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Salivary Immunoglobulin Gene Expression in Patients with Caries

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Abstract

BACKGROUND: Immunoglobulins mediate the host's humoral immune response are expressed in saliva.

AIM: To quantify the *FcαR*, *FcγRIIB*, and *FcαμR* gene expression in the saliva of Mexican patients with caries in mixed and permanent dentition.

SUBJECTS AND METHODS: This was a comparative cross-sectional study. mRNA was isolated from 200 µL of saliva following the RNA III Tissue Fresh-frozen protocol of the MagNA Pure LC Instrument 2.0 (Roche Diagnostics GmbH, Nederland BV) and the *FcαR*, *FcαμR* and *FcγRIIB* were quantified through TaqMan Assays.

RESULTS: One hundred individuals, 50 with mixed dentition and 50 with permanent dentition, were included in the study. Statistically, it was found a significant difference ($p = 0.025$) in the IgG (*FcγRIIB*) expression between the studied groups.

CONCLUSION: Although we confirmed the existence of *FcαR*, *FcγRIIB* and *FcαμR* gene expression in saliva, only a significant difference in the expression of *FcγRIIB* between the mixed dentition and permanent dentition was found.

Introduction

Caries is an infectious and multifactorial disease characterised by the progressive dissolution of the hard tissues of the tooth, due to demineralization of the mineral portion and the subsequent disintegration of the organic part, produced by the acid fermentation of the carbohydrate metabolism employed by oral microorganisms. It is the most prevalent oral health problem in the world, affecting 95–99% of the population [1,2].

Through a variety of studies in Mexico over the past two decades in different age groups, it has been reported that caries has prevalence of 48–95% in preschoolers [3–6], of 42–88% in children 12 years of age [7–10], of 53.4% in teenagers [11] and of 74.4% in young adults <26 years [12].

Paul Keyes [13] established that the aetiology of dental caries was due to a scheme consisting of three agents (host, organism and diet) that of necessity must interact. These are known as basic or primary factors [14]. Subsequently, "time" was added as a fourth etiologic factor required to produce caries [15].

Among the etiological factors related to the host, it is important to consider the saliva, as it possesses a relevant role in the formation of caries [16,17]. Additionally, it has been found that the IgA, IgG, and IgM antibodies [1,17] that mediate the host's humoral immune response are expressed in saliva and are involved in the development of caries. This is because functionally intact immunoglobulins in the oral cavity have the ability to bind to specific antigens of oral bacteria, thereby blocking certain bacterial surfaces that are important for bacterial adhesion to

oral surfaces. The innate immune response is also involved in anticaries activities by cellular immunity, represented by macrophages as well as by the activity of enzymes [18].

Furthermore, it has been observed that molecules IgA and IgG possess binding activity against orally isolated Streptococcus α-hemolytic and reduce the incidence of new carious lesions, but do not necessarily eliminate the disease [19]. Specifically, secretory IgA aids in the following: maintaining the integrity of the oral surfaces by limiting microbial adhesion to epithelial surfaces and the hydroxyapatite of tooth enamel [20]; reducing the reduction in plaque formation by controlling streptococcal glucosyltransferase [21]; neutralizing toxins, enzymes, and viruses [22, 23]; or acting in synergy with other antibacterial factors such as lysozyme[24, 25], lactoferrin [26–28], salivary peroxidase [29, 30], and mucins [27, 31]. It can also prevent penetration of antigens into the oral mucosa [32].

IgG and IgM arise from gingival fluid circulation (or crevicular); thus, plaque in the cervical region of the tooth is subjected to the influence of these antibodies, as well as to complementary factors and various components of cellular immunity, such as lymphocytes, and of innate immunity, such as macrophages and PolyMorphoNuclear (PMN) neutrophils from the gingival sulcus [33, 34].

The components of innate immunity (human myeloid cells, natural killer cells) and B cells, in addition, have a variety of receptors that allow them the interaction with monomeric or aggregated immunoglobulins, immune complexes and opsonized particles (coated with antibody). These receptors bind to the Fc portion of immunoglobulins (FcR) and endow these cells with the ability to interact with IgA, IgG and IgM [35].

Fcα/μR is a transmembrane protein that weakly binds IgA, but that binds IgM with higher affinity [36–38]. FcαRI (CD89) is the sole receptor specific for IgA, and it presents a high affinity for the antibody; it is responsible for activating IgA-dependent cellular responses such as respiratory burst, degranulation, and phagocytosis by granulocytes, monocytes, and macrophages [37–39]. FcγRIIB (CD32) binds very weakly to monomeric IgG, but demonstrates substantially increased affinity with the associated IgG (immune or antibody-coated target cells); thus, cells having FcγRII can join antibody-coated target elements in the presence of elevated serum levels of monomeric IgG. Activation of these immune pathways leads to negative regulation of cell-response capacity, purposefully attenuating activating pathways to avoid an excessive activation state of cells of the innate or adaptive immune system [35, 37, 40]. Considering the presence of caries in different age groups, thus in different types of teething, this study aimed to determine the existence of IgA (*FcαR*), IgG (*FcγRIIB*), and IgM (*FcαμR*) gene expression in the saliva of patients with mixed or permanent dentition and caries.

Materials and Methods

Study design

This was a prospective, comparative cross-sectional study developed between March 2013 and February 2015 at the Centro de Investigaciones y Estudios Avanzados en Odontología (CIEAO) "Dr Keisaburo Miyata", the Autonomous University of the State of Mexico (UAEMex), Toluca, Mexico. The sampling process was deterministic.

Participants

Subjects complying with the following conditions were included in the study: the presence of caries in at least one tooth; free of orthodontic, orthopaedic, or post orthodontic treatments and without any pharmacotherapy. Subjects with chronic or acute diseases were excluded from the study.

Population characterization

We employed a data-collection instrument containing the following sections: demographics (age, gender, marital status, education, and occupation); hygienic habits (frequency of brushing, brushing time, and auxiliary hygienic tool), and food (number of meals per day, number of nutritive and non-nutritive foods with sugar content and food ingestion 1 h before bedtime).

Caries diagnosis

The intraoral examination was performed by a trained dentist using No.-3 flat mirrors and sterile fine-tipped probes with natural light and a biological barriers technique. For caries diagnosis, World Health Organization (WHO) criteria were utilised [41]. The number of decayed, sealed, or extracted teeth was recorded, indicating whether it was a temporary or permanent dentition, to determine the Decayed, Missing, Filled (DMF) index and the def (d: decayed tooth, e: decayed tooth indicated for extraction, f: filled tooth) index. The values obtained are added together and divided by the number of subjects examined for each index. The scale of severity of dental caries according to WHO is as follows: 0–1.1 = very low; 1.2–2.6 = low; moderate, 2.7–4.4; high, 4.5–6.5, and very high, ≥ 6.6.

In addition to the previously mentioned

indexes, the laser fluorescence device DIAGNOdent Pen (KaVo, Biberach, Germany) was employed to make the diagnosis of caries for each tooth. The reference values were those established by the manufacturer (0–13 = no caries; 14–20 = caries in enamel, and 21–99 = dentin caries) [42].

Saliva sample

A sample of 1.5 mL of spontaneous saliva was collected from each subject. The volunteers were instructed to refrain from eating, drinking, smoking, or performing oral hygiene procedures for at least one h before sampling. To obtain saliva, the subjects were instructed to expectorate non-stimulated mixed saliva into a flat plastic cone, transferring this later into a 1.7-mL microcentrifuge tube (Costar, CA, USA) using a sterile Beale 1654 spatula (Arain, Sialkot, Pakistan). Samples were stored at 4–8°C during collection and were subsequently stored at –70°C until their processing.

RNA extraction

Messenger RNA (mRNA) was isolated from 200 µL of saliva following the RNA III Tissue Fresh-frozen protocol of MagNA Pure LC Instrument 2.0 (Roche Diagnostics GmbH, Nederland BV) employing MagNA Pure Lc RNA Isolation Kit III (Tissue) (Roche Applied Science, Mannheim, Germany). Fifty µL of salivary mRNA aliquots were obtained. Quantification of mRNA concentration and purity was carried out by spectrophotometry (NanoPhotometer Ver. 2.0; Implen GmbH, Schatzbogen, Germany).

cDNA

Salivary mRNA transcription to complementary DNA (cDNA) was performed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Ten µL of Buffer Mix and 1 µL of Enzyme Mix were used, adding 9 µL of saliva-RNA sterile water at a concentration of 28.8 ng for a total volume of 20 µL. The reaction was performed in a Life Express Thermocycler (Bioer, Hangzhou, China), with the following cycles: 60 min at 37°C; 5 min at 95°C, and 10 min at 4°C.

Real Time-Polymerase Chain Reaction (RT-qPCR)

TaqMan Assays (Applied Biosystems, CA, USA) for *FcαR* (CD89), Hs00370197_m1, *FcαµR* (CD351), Hs01049679_m1, *FcγRIIB* (CD32), Hs00269610_m1, and the constitutive gene 18S, Hs99999901_s1, were utilized in this step. cDNA samples were processed in a Fast MicroAmp® reaction plate (Applied Biosystems). We used 10 µL of TaqMan Universal Master Mix II, No UNG (Applied Biosystems) and 1 µL of TaqMan Assay, specific for the indicated genes, adding 9 µL of salival cDNA-sterile water, with a concentration of 28.8 ng, for a final volume of 20 µL. A negative control for each sample was established. The samples were processed in 7500 Fast Real-Time PCR Systems (Applied Biosystems). Cycling parameters of the RT-qPCR for all genes were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min.

Analysis of relative gene expression was through the $2^{-\Delta\Delta CT}$ method (43) and 7500 Fast Real-Time PCR Software Systems, which provided the threshold cycle (CT) (C1T, X, C2T, X). Two options were considered to determine the calibrator sample for the method: a) *FcαR*, *FcγRIIB*, and *FcαµR* gene expression of a caries-free subject in each study group (with a value of 1), and b) *FcαR*, *FcγRIIB*, and *FcαµR* gene expression obtained from Raji cDNA human male (Part No. 4352575, Applied Biosystems).

Statistical analysis

Results were expressed employing descriptive statistics. The Student t-test was performed to compare the Relative Units (RU) of gene expression between the following two groups analysed: 1) mixed dentition, and 2) permanent dentition. Multiple Linear Regression was used to know the association between caries and the gene expression of the immunoglobulins (FcαR, FcγRIIB, and FcαµR). All tests were performed with SPSS ver. 19.0 statistical software (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA).

Ethics

Informed consent was obtained and, in the case of children, the consent of their parents was mandatory. The study was reviewed and approved by the Ethics Committee of the Faculty of Dentistry, UAEMex (code: MCIOO-0113) and followed the General Health Research Law of Mexico and the Declaration of Helsinki (Fortaleza, Brazil).

Results

General Characteristics

One hundred individuals, including 50 with mixed dentition (mean age, 9.5 years) and 50 with permanent dentition (mean age, 22.9 years) were included in the study. While in the first group, all study participants were students, in the second group, this percentage decreased to 72%. In both groups, the predominant gender was feminine, with 60 females

and 58% males, respectively. Information on the selected population and dental hygiene are depicted in Table 1.

Table 1: General characteristics of the studied population

Variable	Category	Mixed dentition (N = 50) Frequency (%)	Permanent dentition (N = 50) Frequency (%)
Gender	Male	20 (40)	21 (42)
	Female	30 (60)	29 (58)
Marital status	Single	50 (100)	46 (92)
	Married	0 (0)	4 (8)
Scholarly	Primary school	50 (100)	0 (0)
	High school	0 (0)	2 (4)
	Preparatory	0 (0)	4 (8)
	University	0 (0)	43 (86)
	Postgraduate	0 (0)	1 (2)
Occupation	Worker	0 (0)	9 (18)
	Merchant	0 (0)	1 (2)
	Student	50 (100)	36 (72)
	Other	0 (0)	4 (8)
Time for the toothbrush	Before meals	1 (2)	1 (2)
	After meals	49 (98)	49 (98)
Frequency of toothbrush per day	1	11 (22)	0 (0)
	2	29 (58)	30 (60)
	3	10 (20)	16 (32)
	> 3	0 (0)	4 (8)
Use of hygienic tool	Yes	10 (20)	42 (84)
	No	40 (80)	8 (16)
Type of hygienic tool	Mouthwash	4 (8)	29 (58)
	Toothpick	1 (2)	2 (4)
	Dental floss	5 (0)	11 (22)
	None	40 (80)	8 (16)
Frequency of dental brush change	Every month	9 (18)	7 (14)
	Every 2 months	10 (20)	13 (26)
	Every 3 months	19 (38)	23 (46)
	After 3 months or more	12 (24)	7 (14)

In the group of mixed dentition, the number of meals per day was 2.7 on average, with a consumption frequency per day of nutritive and non-nutritive foods of 1.61 and 1.19, respectively, both with sugar content. Similarly, in the permanent-dentition group, the number of meals per day was 2.7 on average, with a consumption frequency per day of nutritive and non-nutritive foods of 1.46 and 1.73, respectively, both with sugar content. Statistically significant differences between the study groups were detected regarding age (p ≤ 0.001) and consumption of unhealthy foods containing sugar (p = 0.022).

Dental Caries

DMF and def indexes in the mixed-dentition group were 2.88 and 3.14, respectively, resulting in a DMF-def index of 6.02; this group reached the high level of caries severity. Regarding the group of permanent dentition, the DMF index was 8.24, at the very high level of the caries severity scale.

Results with the DIAGNOdent Pen (KaVo) were as follows: in the group of mixed dentition, overall average demineralization fluorescent units were 12.91: 21.51 in the upper right quadrant, 12.34 in the upper left quadrant, 13.01 in the lower right quadrant, and 13.82 in the lower left quadrant. In the group of permanent teeth, units in the same quadrants were 6.32, 10.59, 11.07, and 10.89; with an average of 9.69. Statistically, there were significant differences in the right upper quadrant (p ≤ 0.001), left lower quadrant (p = 0.034), and in mean overall demineralization (p ≤ 0.001) between the studied groups (Table 2).

Table 2: Demineralization fluorescence units

Caries localization	Mixed dentition	Permanent dentition	p
Upper right quadrant	12.52	6.32	≤ 0.000
Upper left quadrant	12.34	10.59	0.322
Lower right quadrant	13.01	11.07	0.203
Lower left quadrant	13.82	10.89	0.034
General	12.91	9.69	≤ 0.001

Gene Expression

All samples revealed IgA gene expression (*FcαR*), while IgG (*FcγRIIB*) and IgM (*Fcα/μR*) were expressed only in some. Statistically, a significant difference (p = 0.025) was found in IgG (*FcγRIIB*) expression between the groups studied (Table 3).

Table 3: Gene expression*

Inmunoglobulins	Group	Positive (frequency)	Negative (frequency)	Mean ± SD (range)	p
FcαR	A	50	0	1.914 ± 6.451 (0.00-41.99)	
	B	50	0	4.152 ± 9.537 (0.00-36.73)	
FcγRIIB	A	29	21	2.936 ± 6.109 (0.00-26.52)	0.025
	B	26	24	13.22 ± 15.593	
Fcα/μR	A	2	48	0.050 ± 0.0141 (0.04-0.06)	
	B	1	49	21.89 (22-22)	

SD: standard deviation. *: relative units. A: Mixed dentition, B: Permanent dentition.

Multiple Linear Regression showed no association between caries and gene expression of immunoglobulins, either in the mixed or in the permanent dentition (Table 4).

Table 4: Multiple lineal regressions for caries and *FcαR*, *FcγRIIB* and *Fcα/μR* gene expression

Type of dentition	Variables	B	Coefficient	p
Mixed	Demineralization	13.676		≤0.001
R^2= 0.191	*FcαR*	-0.378	[-0.178]	0.358
	FcγRIIB	-0.178	[-0.157]	0.419
	Fcα/μR	161.079	[0.306]	0.104
Permanent	Demineralization	10.171		≤0.001
R^2= 0.037	*FcαR*	-0.016	[-0.040]	0.785
	FcγRIIB	-0.067	[-0.269]	0.185
	Fcα/μR	.	.	.

Discussion

The first analysis is that in addition to the expected difference in age, the fact that auxiliary hygiene is more frequent during permanent dentition than during mixed dentition suggests a deficiency in the oral hygiene of the former group.

Evidence shows that saliva comprises a useful body-fluid tool for the development of molecular diagnostics because it contains components found in serum, and its collection possesses the advantage of being cost-effective, safe, easy, and non-invasive [44]. Also, salivary proteins serve as biomarkers for the study of several diseases, for example, autoimmune disorders [45], cancer [46], cardiovascular diseases

[47], metabolic syndrome [48], and for viral [49] and bacterial infections [50].

In the field of dentistry, saliva has already been employed to study oral pathologies such as periodontal diseases [51] and to evaluate the risk for caries [52]; however, in the present work, saliva has been utilized as the medium for quantitative measurement of the expression of immunoglobulin Fc fractions A (*FcαR*), G (*FcγRIIB*), and M (*FcαμR*). In this line, it is clear from this study that quantification of *FcαR*, *FcγRIIB*, and *FcαμR* gene expression confirms their existence in saliva. In fact, because *FcαR* was expressed in all subjects in both groups, this is suggestive of IgA being secreted by local plasma cells [38].

It is noteworthy that several studies have linked immunoglobulin concentrations in saliva and the presence of caries. For example, it has been found that levels of salivary IgA and IgG concentrations are significantly higher in children with caries in early childhood, compared to the levels of children without caries [53]. This phenomenon has been explained partially by that the high concentration of salivary immunoglobulins may be associated with increased antigen [54] or specific chemokine content [55], leading to high antibody production. Even more so, another study found a significant positive relationship between the concentration of salivary IgA and the presence of early childhood caries, but without correlation between salivary IgA and the Decayed, Missing, or Filled Teeth (DMFT) index [56]. Moreover, another group reported that salivary IgA levels in children with rampant caries were significantly lower compared with the levels of caries-resistant children [57]. According to this informarton and our data, we propose some mechanisms that explain the increase in IgA when a child has caries (Figure 1).

FcγRIIB expression occurred in approximately one-half of the subjects in both groups. This could correspond to the crevicular fluid containing PMN neutrophils, which express low-affinity receptors for the Fc domain of IgG [58] and represent the first line of defence in the gingival sulcus [59]. However, the difference in *FcγRIIB* gene expression between the studied groups was statistically significant, higher in the permanent dentition; in this respect, it could be mentioned that the presence of PMN neutrophils in saliva has been reported extensively [60] and that these immune cells are involved in the main cells responsible for the progression of periodontal disease [61, 62]. This latter point suggests that the expression of *FcγRIIB* could be related to the presence of gingival or periodontal disease, both in mixed and in permanent dentition. However, it is noteworthy that the information refers to the periodontal disease, in that there are, to our knowledge, no reports on *FcγRIIB* expression in dental caries.

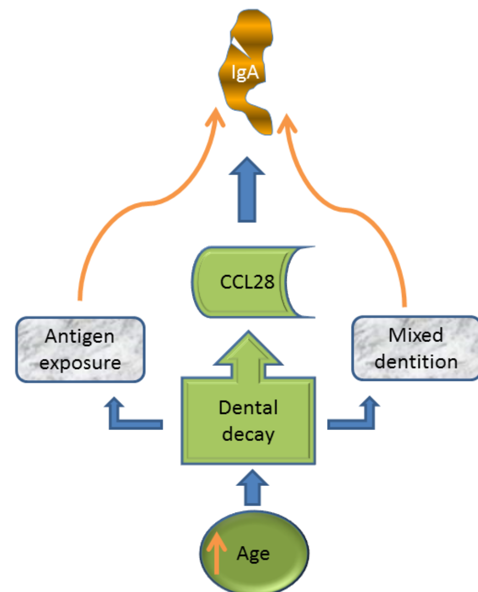

Figure 1: Factors that stimulate the IgA production CCL28: Chemokine (C-C motif) ligand 28

FcαμR exhibited minimal expression, possibly due to IgM affinity to multiple antigens [63-65]; this is the first Ig produced during infection, acting as an early defence mechanism against systemic and mucosal pathogens [52]. Taking this information into account, we can infer that the majority of study subjects had no infections.

About the supposed association of *FcαR*, *FcγRIIB*, and *FcαμR* gene expression and caries, multiple linear regression demonstrated a lack of significant association in the studied groups; this cannot be compared with other studies, due to the lack of more papers.

Given the previously mentioned material, it is important to note that this study would be complemented by quantifying the concentration of immunoglobulins; however, no such procedure was performed, which comprises a limitation of our study. Likewise, observations regarding the expression of *FcγRIIB* entertains the limitation that identification of PMN neutrophils was not conducted, and neither the diagnosis of periodontal disease nor habits such as smoking [57] or alcohol consumption [30], considered important in the PMN Neutrophil count in saliva.

It is noteworthy that as calibrator sample for the $2^{-\Delta\Delta CT}$ method, *FcαR*, *FcγRIIB*, and *FcαμR* gene expression in subjects without caries was chosen from each group. This was due to that the relative gene expression units obtained from Raji cDNA, although proportional, were higher compared with those obtained from caries-free subjects.

Although reaction efficiency for *FcαR*, *FcγRIIB*, *FcαμR*, and *18s* was optimal, it has been shown that the use of a single reference gene is susceptible to error in interpreting the results of real-

time PCR [66]; therefore, this concept was another limitation of our study.

The importance of this study lies in verifying the existence of *FcαR*, *FcγRIIB*, and *FcαμR* gene expression in saliva, data that, to our knowledge, is missing in the scientific literature. Furthermore, the use of molecular biology techniques permits us to expand knowledge in the dental area, because this study could drive future research regarding *FcγRIIB* expression and its relationship to periodontal disease, FcαμR expression in oral infections, as well as direct research concerning the association of humoral immunity and caries.

In conclusion, although we confirmed the existence of *FcαR*, *FcγRIIB*, and *FcαμR* gene expression in saliva, a significant difference was only found in *FcγRIIB* expression between mixed dentition and permanent dentition. A limitation of this finding is the small sample and the lack of more complex analysis such as proteomics, but the clinical relevance of this study is that the type and quantity of immunoglobulins in saliva can be implicated in the development of caries in mixed and permanent dentition.

The use of saliva as an associated factor in the development of caries is a suitable route for the study of elements related to the oral environment. Clearly, future research in this area must include FcαR, FcγRIIB, and FcαμR protein quantification and measurement of immunoglobulins in saliva, to improve knowledge on humoral immunity and its relationship with dental caries in different teething.

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