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Evaluation of Dental Caries Experience and Salivary IgA, IgG and IgM In Children Between 3-6 Years of Age Using Two Immunoassays (SRID, RE)

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Abstract

Host resistance to dental caries is a complex phenomenon. The present study evaluated the role of Salivary Immunoglobulins IgA, IgG and IgM in children aged 3-6 years using two Immunoassays Single Radial immunodiffusion (SRID) and Rocket Electrophoresis (RE). 90 children selected were divided into 3 groups Non-Rampant Caries (NRC), Rampant Caries (RC) and Caries free (CF). Stimulated saliva was collected and cultured for streptococcus mutans under anaerobic conditions. The colony forming units (CFU/cc) and Immunoglobulins were quantified by SRID described by Mancini et al (1965). A standard curve was constructed by plotting d^2 against concentrations of standard solution and unknown were read off. RE peak heights of standard and the test samples were read off. Statistical analysis of IgA, IgG and IgM, age, deft scores and CFU/cc of saliva was done using unpaired student's t test and Karl's Pearson's coefficient of correlation was obtained. Results and Analysis depicted significantly higher concentration of IgA against CF group by SRID in RC. No significant difference in the IgG concentration in all 3 groups. The values of IgM were found to be zero in all the ninety subjects. Mean values of IgA, IgG and IgM in the 3 groups showed fair concordance by both SRID and RE. A significant positive correlation between IgG and deft scores by SRID in RC group and positive correlation between IgG and deft scores by RE in NRC group. The

present study suggests that the antibodies in whole saliva have a role in caries control and secretory immunity deserves attention in the development of vaccination against dental caries.

Keywords: SRID, RE, NRC. RC, CF. IgA, IgG and IgM

Introduction

Host resistance to dental caries is a complex phenomenon since the recognition of caries as an infectious disease by several species of cariogenic bacteria. The mouth is colonized by a variety of microorganisms and health of mouth is dependent on the integrity of the mucosa which does not allow microorganisms to penetrate.

There is substantial evidence that Streptococcus mutans plays an important role in caries development in humans. Number of cariogenic bacteria, volume and microbial composition of the plaque in which they are present is an important factor. The factors which are responsible for maintaining oral health are the integrity of the mucosa, saliva, gingival crevicular fluid and their humoral and cellular immune components. A remarkable feature of the tooth surface is that it is influenced by both local and systematic immune mechanisms [1],[2].

The line of division between the two immune mechanisms occurs near the gingival margin and it is perhaps the only site in the body where the interface can be found between the secretory and systematic immune mechanisms.

The salivary dominant found in the blood is largely dependent on the function of secretory IgA [3], the gingival domain is controlled by host, the immune component found in the blood. However, reliable and reproducible measurements of salivary immunoglobulins are extremely difficult to achieve.

The present study was conducted with the aim and objective:

1.To determine the concentrations of immunoglobulins IgA[4],[5],[6],[7], IgG[8],[9],[10] and IgM levels[11] in saliva in 3 groups aged 3-6 years, in Non-Rampant Caries (NRC), Rampant Caries (RC) and Caries free (CF) children.

2.To determine any possible correlation between the dental caries experience and the salivary immunoglobulins levels in children having Non-Rampant caries, Rampant caries, no caries patterns in the above age groups [12],[13].

3.To evaluate the selective ability of two different immunoassays viz, Single Radial Immunodiffusion technique (SRID)[14],[15] and Rocket Electrophoresis (RE)[16].

Materials and Method

This study population comprised of 90 children with 28 females and 62 males between range of 3 to 6 years from schools:

1. Phoenix School, Nehru Nagar, Belgaum, Karnataka

2. Anganwadi, S.S. High School, Nehru Nagar, Belgaum, Karnataka

After obtaining informed consent from the parents the children were clinically screened and on the basis of their caries experience they were divided into:

Group I: Non-Rampant caries (NRC) (criteria deft > equal to 1)

Group II: Rampant caries (RC) (criteria two or more labial or lingual lesions on maxillary or mandibular incisors: **Winter, Hamilton and James (1966)** [17]

Group III: Acting as Control Group- Caries Free (CF) (criteria deft = 0)

Dental examination of each child was done using sterile mouth mirrors and explorers under day light and deft scores were calculated for each child as described by **Gruebell (1944)** [18]

Saliva Sampling Procedure

Each child was asked to chew two pieces of thermocol for 2 minutes so as to stimulate the salivary flow [19] and approximately 2-3 cc of the specimen of whole saliva was collected in the wide mouthed cylinders (picture 3). Saliva

was then transferred to sterilized vials which were stored at -20 degrees Centigrade for the immunoassay by Single Radial Immunodiffusion and Rocket Electrophoresis.

Culture and Quantitation of Streptococcus mutans [20],[16],[22] (picture1)

0.5 cc of whole saliva samples of each subject was transferred to 4,5 cc of modified Stuart's transport medium from Hi Media lab Pvt. ltd., Mumbai (picture 4), 0.01cc of this from each sample was taken on loop and plated on mitis salivarius agar (Hi Media lab Pvt. ltd., Mumbai) containing 20% sucrose and bacitracin. The plates were incubated in anaerobic jar from Dyna Micro Laboratories, Thane (picture 6) for 48 hours at 37 degrees Centigrade in which anaerobic catalyst Dyanox charge:P:2, saturated sodium carbonate solution (10 cc) and 1:4 sulphuric acid (40cc).

Quantitation of Immunoglobulins

Armamentarium (picture 9)

Single Radial immunodiffusion [23] (KUMARURATNE et al 1992)

This technique was first described by **Mancini et al** in 1965[24]

Principal: Here the protein antigen diffuses radially from a point application into antibody containing gel, a circular precipitate being formed at the point of equivalence. With constant antibody concentration and constant gel thickness, the area encompassed by the precipitin ring is proportionate to the concentration of antigen (Cag)

Procedure (picture 10)**:**

a) Preparation of gel

The volume of the buffered 1% agarose needed to produce 1,5 mm thick gel (1.5 cc) was taken. Pipette the requisite volume into a universal container and place in the water bath. The agar -antiserum mixture was poured evenly on the clean glass plate, allowed to set and equilibrated at 4°C.

b) Origin wells: The template was located over the gel and origin wells were cut. These wells were evenly spaced and followed a regular pattern to facilitate identification (Picture 2)

c) Sample loading: measured volumes of test and standard solutions were applied to the origin wells by micropipette. 2 microliters were used to load the wells.

d) Diffusion: The loaded gels were placed in a moist chamber at room temperature until the completion of diffusion.

e) Quantitation: The gels were placed in 1% tannic acid for 5 minutes to enhance the precipitin rings. The ring diameter was measured to nearest 0.1mm and diameter (d^2) obtained against antigen concentration and provided diffusion and this will be a straight line which can be expressed by the equation:

$d^2 + k\,(Cag) + S_0$

Rocket electrophoresis (Yemul and Huley 1980) [16] (picture 12)

Principal (picture 11)**:** The protein under estimation migrates to the antibody containing gel under the influence of the electric field, antigen antibody complexes are formed which aggregate to form visible precipitate at the point of equivalence. Standard antisera IgA, IgG, and IgM (raised in the Department of Microbiology, J.N. Medical College, Belgaum) and protein reference set from Orion Diagnostica, Espoo, Finland (picture 5).

Procedure

a) **Preparation of gel:** 60 mg of agar + 60 mg agarose + 12 cc of barbitone buffer were used to make 1% gel solution. Take 4 .8 cc of gel in the test tube kept in water bath at 56 °C.

b) **Antisera:** Raised against IgA, IgG and IgM 60 micro litres of the antisera was added to the tube containing 4.8 cc of gel solution (picture 7).

c) **Origin wells:** Place the gel over the template (picture

8) and 1.2 mm wells are cut 5mm apart.

d) Sample loading: The prepared slide was placed in Electrophoresis Chamber in contact with agarose. Water cooling was started and a low voltage of 1 v/cm was applied across the plate. Two micro litres volume of standards and test samples were applied to the origin wells by micropipette.

e) Electrophoresis: The voltage is increased to 10-12 v/cm and electrophoresis was allowed to proceed to completion.

f) Processing (picture 13)**:** After completion the slide was removed from the tank and placed in moist chamber and kept in refrigerator overnight. It was removed and placed in saline (4-5 hours). A filter paper was placed on the plate and kept in incubator overnight. Slide was then stained (15 minutes).

g) Quantitation: Peak heights were measured to the nearest half mm; measurements being taken from the tip of the peak to the anodal border of the origin wells.

h) Sensitivity and precision: Rocket electrophoresis is of sufficient sensitivity as to allow diagnosis of complete absence of a protein and a minimum of 10 mm peak is required for accurate quantitation, the effective lower limit of sensitivity of the method is in order of 1 mg/l

The concentration of intact secretory IgA molecule was calculated by multiplying by factor of 3.25 as introduced by Brandtzaeg (1970) [25],[11],[26].

Statistical analysis

The variables IgA, IgG and IgM, age, deft score and CFU/cc of saliva the value of mean, standard deviation, variance, standard error and range were compared by unpaired student's t test (Table VIII)**.**

The values of Karl Pearson's coefficient were obtained to establish relationship between the antibodies and dental caries (Table IX).

Results And Discussion (Graph I and Graph II)

1. The effects of salivary IgA on oral streptococci which are involved in the caries process have been evaluated extensively. In the present study the Caries Free (CF) children are compared to caries susceptible (NRC and RC) who experience caries before age of 4 years.

2. When any infection occurs in the human body, the body's immune system gets activated and gives rise to increased production of antibodies. As dental caries is known to be an infective disease it is logical to assume that antibody titres would increase in children with caries. A small difference in concentration of Ig A levels between CF and NRC groups reflects the chronicity of dental caries (Challacombe and Lehner 1976) [10].

3. In the present study higher the titres of salivary IgA in subjects with Rampant Caries experience reflects either there is cumulative antigenic stimulation or repeated attacks of caries. Also, memory of secretory immune system is shorter than that in serum and frequent exposure to antibodies is required to maintain antibody titres in saliva and other secretions.

This concept has been used and extended to employ that secretory IgA levels may be used as markers of incipient caries infection.

4. The RC group showed significantly higher concentration of IgA as compared with CF group by SRID technique.

5. There was no significant difference in the IgG concentration in all 3 groups NRC, RC, and CF

6. The values of IgM was found to be zero in all the ninety subjects.

7. The mean values of IgA, IgG and IgM in the 3 groups- NRC, RC, CF showed fair concordance by both SRID and RE techniques.

8. There was a significant positive correlation between IgG and deft scores by SRID technique in RC group and

positive correlation between IgG and deft scores by RE technique in NRC group.

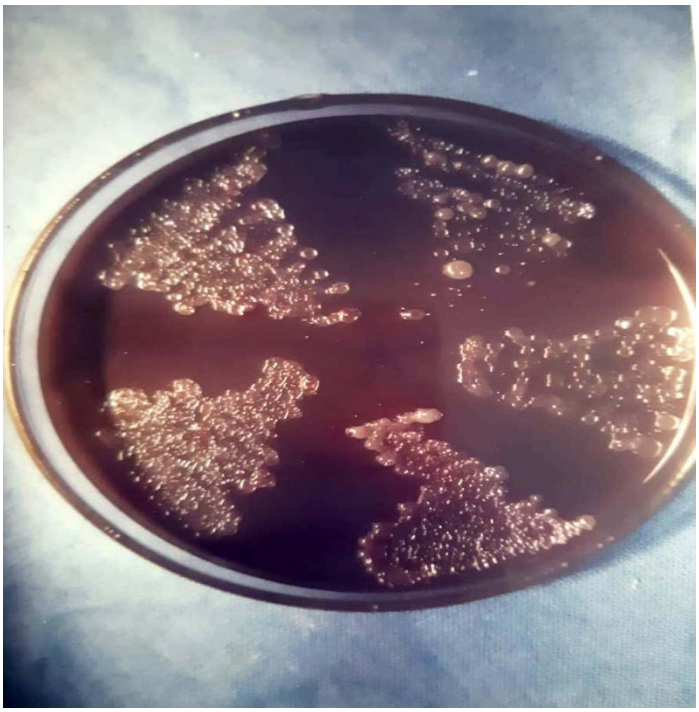


Figure 1: Colony Forming Units of Streptococcus mutans on mitis Salivarius bacitracin agar.

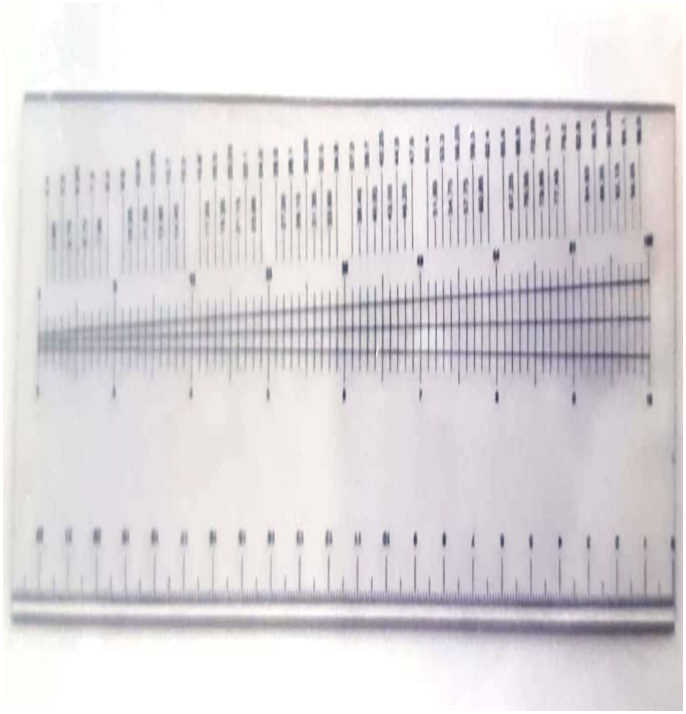


Figure 2: Tripartigen ruler

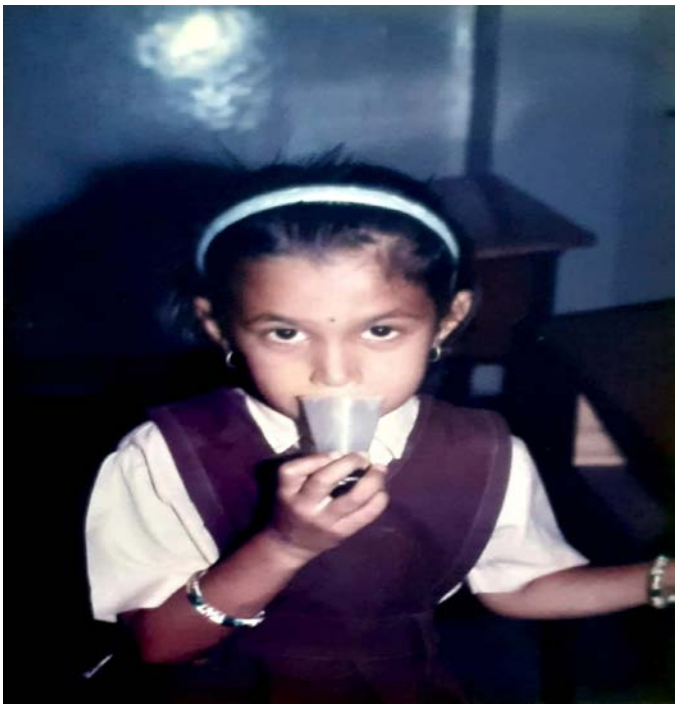


Figure 3: Collection of Saliva

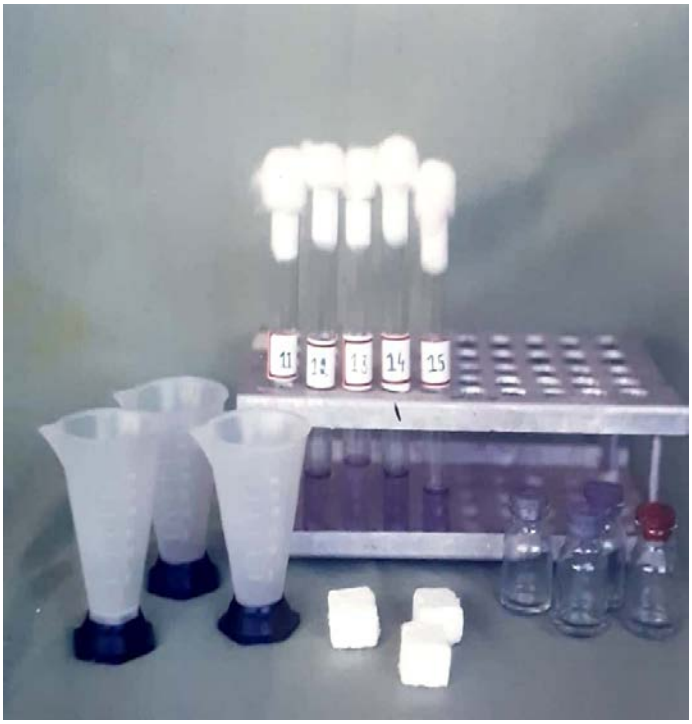


Figure 4: Wide mouth cylinder, Modified Start's transport media, vials, thermocol

Figure 5: Control Serum

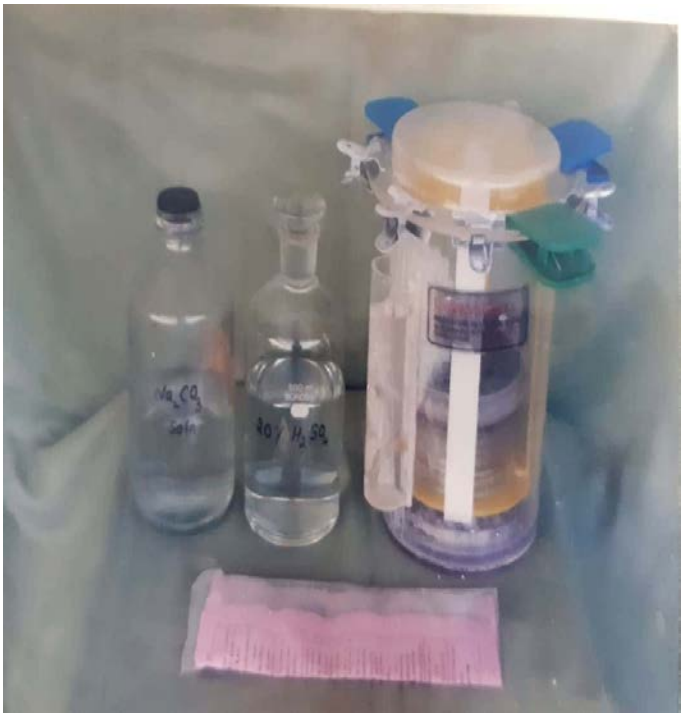


Figure 6: 20% Sulphuric acid, saturated sodium carbonate solution, anaerobic jar, Petri plates, Dyanox charge P:2.

Figure 7: Antisera

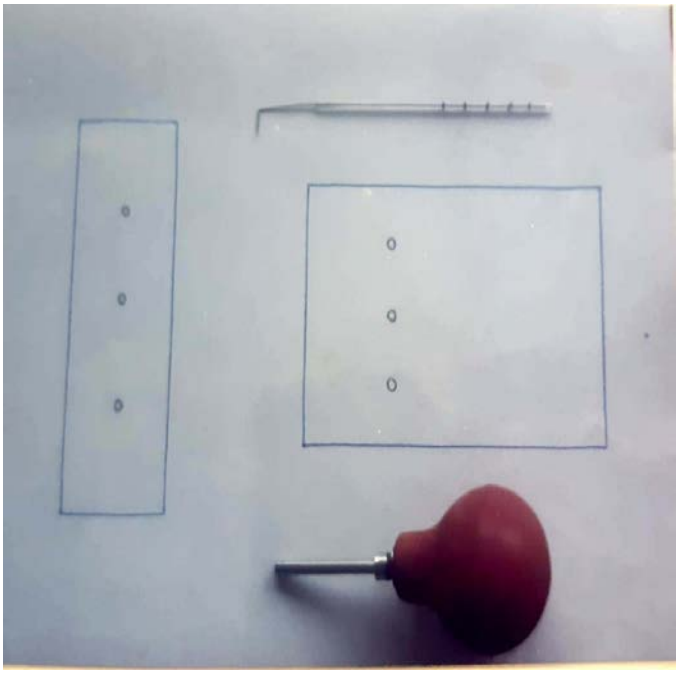


Figure 8: Template, Punch Dispenser for both techniques (SRID, RE)

Figure 9: Sodium hypophosphate, antiserum, potassium hypophosphate, micropipette tips, agarose

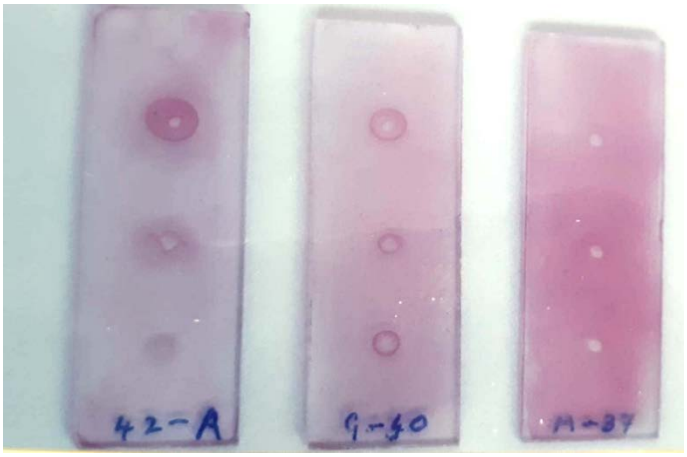


Figure 10: SRID Technique used for estimation of salivary IgA, IgG, IgM

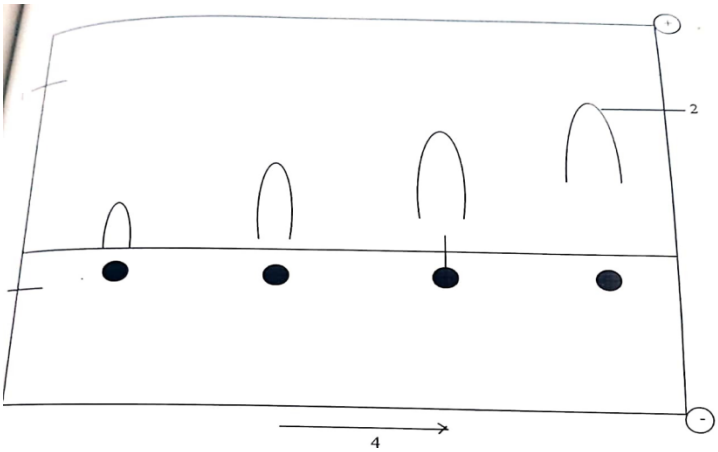


Figure 11: Rocket Electrophoresis: Antigen is driven into gel containing antibody. 1. Antibody in agarose gel 2. Precipitin arcs 3. Antigen wells 4.Increasing antigen concentration

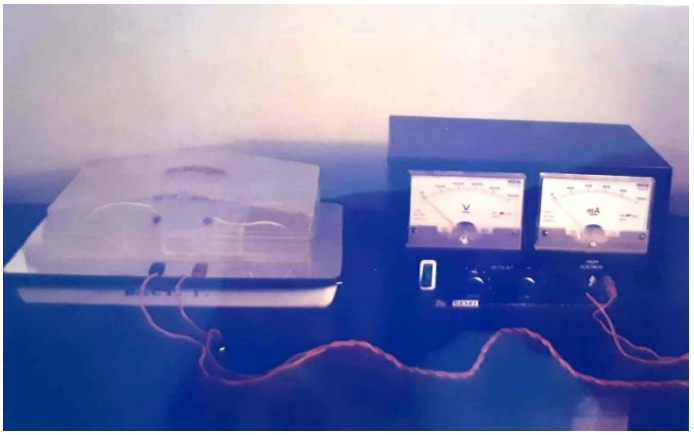


Figure 12: Electrophoresis apparatus with tank.

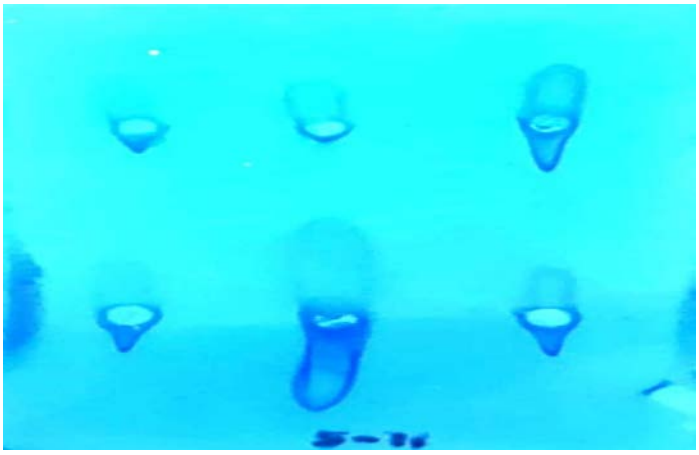
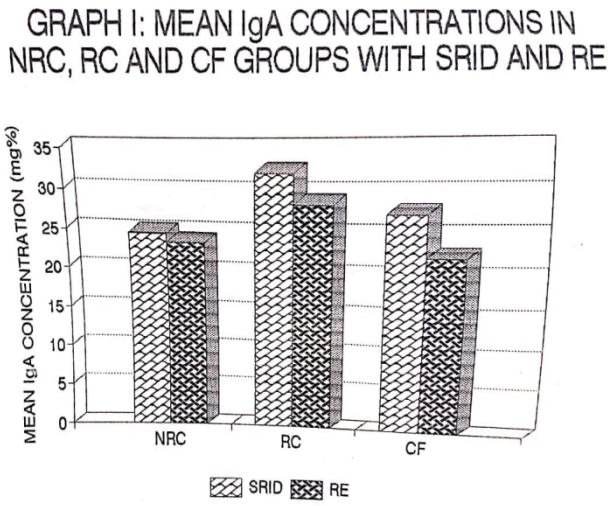
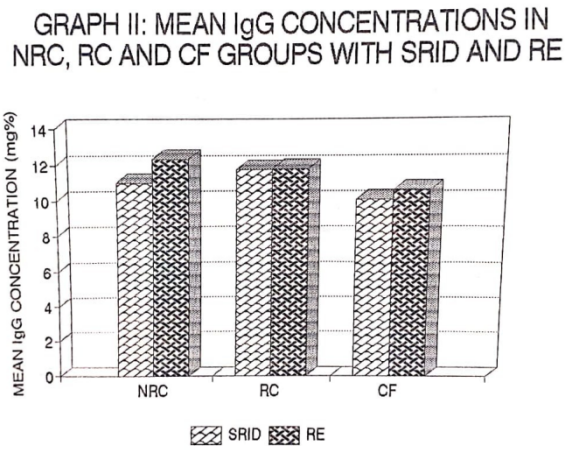


Figure 13: Rocket Electrophoresis used for estimation of salivary IgA, IgG, IgM



Graph 1: Mean IgA Concentrations in NRC, RC and CF groups with SRID and RE.



Graph 2: Mean IgG Concentrations in NRC, RC and CF groups with SRID and RE

Table I

Distribution of sample according to sex

Group	Females	Males	Total
Non-Rampant caries	12	19	31
Rampant caries	8	22	30
Caries free	8	21	29

Table 1: Distribution of sample according to sex

Table II

Mean values of Age, deft score and CFU per cc of saliva

Group	Age	deft	CFU/cc
Non-Rampant caries	4.60	3.87	22.16×10^4
Rampant caries	4.42	7.86	18.03×10^4
Caries free	4.47	0.00	18.09×10^4

Table 2: Mean value of age, deft score and CFU per cc of saliva

Table III

Distribution of immunoglobulin levels of IgA, IgG and IgM in saliva of children with NRC group by SRID and RE techniques

		IgA (mg%)	IgG (mg%)	IgM (mg%)
SRID	Mean	24.78±2.86	11.03±0.71	0.00
	S.D.	15.95	3.95	0.00
	Variance	246.22	15.16	0.00
	Range	6.50-91.00	3.50-21.00	0.00
RE	Mean	23.63±2.55	12.32±0.85	0.00
	S.D.	14.21	4.78	0.00
	Variance	195.46	21.92	0.00
	Range	7.47-78.65	4.00-24.50	0.00

The concentration of IgM was found to be zero in all the subjects.

Table 3: Distribution of immunoglobulins levels of IgA, IgG, IgM in saliva of children with NRC group by SRID and RE technique

Table IV

Distribution of immunoglobulin levels of IgA, IgG and IgM in saliva of children with RC group by SRID and RE techniques

		IgA (mg%)	IgG (mg%)	IgM (mg%)
SRID	Mean	32.77±2.174	11.73±0.671	0.00
	S.D.	17.38	3.677	0.00
	Variance	292.17	13.07	0.00
	Range	3.25-104.00	5.00-20.00	0.00
RE	Mean	28.50±2.308	11.76±0.830	0.00
	S.D.	12.64	4.54	0.00
	Variance	154.51	19.97	0.00
	Range	9.10-58.50	4.00-20.50	0.00

The concentration of IgM was found to be zero in all the subjects.

Table 4: Distribution of immunoglobulins levels of IgA, IgG, IgM in saliva of children with RC group by SRID and RE technique

Table V

Distribution of immunoglobulin levels of IgA, IgG and IgM in saliva of children with CF group by SRID and RE techniques

		IgA (mg%)	IgG (mg%)	IgM (mg%)
SRID				
	Mean	21.48±1.79	10.07±0.68	0.00
	S.D.	9.62	3.64	0.00
	Variance	89.40	12.81	0.00
	Range	6.50-45.50	4.00-19.00	0.00
RE				
	Mean	22.25±2.74	10.60±1.065	0.00
	S.D.	14.77	5.73	0.00
	Variance	210.65	31.76	0.00
	Range	6.50-71.50	3.00-19.00	0.00

The concentration of IgM was found to be zero in all the subjects.

Table 5: Distribution of immunoglobulins levels of IgA, IgG, IgM in saliva of children with CF group by SRID and RE technique.

Table VI

Comparison of antibody titers between (NRC-RC), (RC-CF), (NRC-CF) by SRID and RE techniques using student's t test

	Between	t Values		
		IgA	IgG	IgM
SRID	NRC-RC	1.8706	0.7870	-
	RC-CF	3.0704**	1.7314	-
	NRC-CF	0.9620	0.9723	-
RE	NRC-RC	1.4128	0.4684	-
	RC-CF	1.7484	0.8648	-
	NRC-CF	0.3686	1.2690	-

** - Significant at p<0.01
All the other t values are not statistically significant
Since IgM values were zero t values could not be calculated.

Table 6: Comparison of Antibody titres between (NRC-RC), (RC-CF), (NRC-CF) by SRID and RE using student's t test

Table VII

Comparison of CFU per cc of saliva between (NRC-RC), (RC-CF), (NRC-CF) using student's t test

Between	t Value
NRC-RC	1.0056
RC-CF	0.0180
NRC-CF	0.9273

The t values are not statistically significant

Table 7: Comparison of CFU per cc of saliva between (NRC-RC), (RC-CF), (NRC-CF) by SRID and RE using student's t test.

Table VIII

Comparison of two techniques (SRID, RE) for immunoglobulins IgA, IgG, IgM using student's t test in repsective groups (NRC, RC, CF)

	IgA	IgG	IgM
Non-Rampant caries	0.3005	1.1600	-
Rampant caries	1.0876	0.0343	-
Caries free	0.2845	0.4154	-

The t values are not statistically significant

Since IgM values were zero t values could not be calculated.

Table 8: Comparison of two techniques (SRID, RE) for immunoglobulins IgA, IgG, IgM using student's t test in respective groups (NRC, RC, CF)

Table IX

Correlation coefficients between immunoglobulins IgA, IgG, IgM, deft score and CFU per cc of saliva using SRID and RE techniques in the three groups (NRC, RC, CF)

		NRC	RC	CF
SRID				
	IgA - deft	-0.0649	0.0287	-
	IgG - deft	0.2780	0.4505**	-
	IgM - deft	-	-	-
	IgA - CFU/cc	0.1911	-0.0456	-
	IgG - CFU/cc	-0.1454	0.0381	-
	IgM - CFU/cc	-	-	-
RE				
	IgA - deft	-0.0469	-0.0351	-
	IgG - deft	0.3707*	0.2956	-
	IgM - deft	-	-	-
	IgA - CFU/cc	0.1855	-0.2633	-
	IgG - CFU/cc	-0.0845	-0.1636	-
	IgM - CFU/cc	-	-	-
	deft - CFU/cc	0.0541	0.1119	-

** - Significant at p<0.02
* - Significant at p<0.05
Since the deft score in caries free was zero the correlation coefficient could not be calculated.

Table 9: Correlation coefficients (Karl Pearson) between IgA, IgG, IgM, deft score and CFU/cc of saliva using SRID and RE techniques NRC. RC, CF groups.

Conclusion

The present study helps us to corroborate the role of antibodies in whole saliva and the measurements of secretory immunity deserves attention in the development of vaccination against dental caries [27],[28].

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