Molecular Detection of *Aggregatibacter Actinomycetemcomitans* and *Porphyromonas Gingivalis* in Subgingival Plaque of Healthy Young Children

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***Objective**: The aim of the present study was to evaluate the presence of Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans) and Porphyromonas gingivalis (P. gingivalis) in subgingival plaque of healthy young children aged between 3 and 15 years using Polymerase Chain Reaction (PCR) and to compare their presence in children in their primary, mixed and permanent dentitions. **Method**: Subgingival plaque samples were collected from 120 healthy children and were grouped as Group I-Deciduous dentition, Group II- Mixed dentition and Group III- Permanent dentition, and were subjected to PCR assay. **Results**: Binomial test and Proportions test were used for statistical analysis. A. actinomycetemcomitans and P. gingivalis were detected in 5% and 35%, 12.5% and 20%, and 0% and 27.5% samples in group I, II and III respectively **Conclusion**: Both the microorganisms were present in group I and II. In group III only P. gingivalis was present. The results from this study do not necessarily pertain to differences in dentition but possibly to inter-individual differences.*

***Keywords**: Periodontal disease, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Polymerase Chain Reaction.*

INTRODUCTION

Periodontitis is a progressive pathologic condition that has its origin in childhood. Epidemiologically, there is a transmission from childhood gingivitis to adult periodontitis.[1]

Dental plaque is a host-associated biofilm, which is important because of its causal relationship to dental caries and periodontal disease. The tooth associated attached plaque is characterized by gram-positive rods and cocci, including streptococcus species and Actinomyces species which are also responsible for dental caries. The portion of the plaque next to the tissue surfaces is more loosely organized and it has gram-negative rods and cocci, including *P.gingivalis*.[2]

Periodontal diseases are local infections by microorganisms present in the gingival crevice or periodontal pocket. Fewer than 10 to 15 bacterial species are recognized as putative periodontal pathogens[3] of which *Aggregatibacter actinomycetemcomitans* [4] (*A. actinomycetemcomitans*, formerly called as *Actinobacillus actinomycetemcomitans*) and *Porphyromonas gingivalis* (*P. gingivalis*) are the major etiologic agents for periodontitis.[5] These periodontopathic bacteria act as markers for periodontal destruction.[6,7]

Detection of anaerobic bacteria is difficult because of their specific growth requirements like anaerobic environment and certain technical barriers. There have been various methods used for detection of putative pathogens, including direct microscopy, cultivation, enzyme tests, enzyme linked immunosorbent assay (ELISA) and specific DNA probes.[3]

Recently, Polymerase Chain Reaction (PCR) assay was developed for detecting putative organisms. It is quick, relatively simple and has the potential to detect low numbers of bacterial species with detection limits as few as 25–100 cells. Thus it is considered suitable for the detecting periodontal pathogens of subgingival plaque in children where the number of pathogens present is limited.[3]

Various studies have been designed for the purpose of correlating the healthy and diseased periodontal sites with subgingival microbes. Most of these studies used conventional microbiological techniques rather than the newer methodologies.8 Moreover, there are only few studies reported from this country. Keeping these various factors in mind, the present study was designed to detect the

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presence of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in healthy young children from Chennai using the PCR assay.

MATERIALS AND METHOD

The present study was conducted after obtaining clearance from the institutional ethical committee, Sree Balaji Dental College and Hospital, Chennai. Children attending the Department of Paedodontics and Preventive dentistry, Sree Balaji Dental College and Hospital participated in our study.

Children, who fulfilled the following inclusion criteria, were eligible to participate in our study:

- Either sex
- Age between 3-15 years
- Systemically healthy individuals
- Non-carious primary or permanent canine and/ first permanent molars
- Gingival index score ≤ 1
- No history of professional dental cleaning and antibiotic medication for past 3 months

The study was explained to the parents of the participants and written consent was obtained. All participants were examined by a single examiner and 120 participants were selected. They were grouped into three groups: Group I- Deciduous dentition, Group II- Mixed dentition, and Group III- Permanent dentition, each comprising of 40 participants.

Collection of subgingival plaque samples

The samples were collected by the same examiner for all the three groups. Supra-gingival plaque was carefully removed from the buccal surfaces of lower primary canines in group I, and from buccal surfaces of lower primary / permanent canines and first permanent molars, in group II and III with sterile cotton rolls. Then, sub gingival samples were collected from these sites, using site-specific Gracey curettes, and then transferred to tubes containing 500µl of Phosphate Buffered Saline (PBS), and refrigerated until subjected to PCR.

DNA extraction

DNA extraction was done by boiling lysis method using lysis buffer (10 mM Tris- HCl, 1.0mM EDTA, 1.0% Triton X -100, pH 7.8) and added to the PCR reaction mixture. PCR reaction mixture (50µl) for the detecting *16S rRNA* of the two bacterial species contained 5µl of 10X PCR buffer (pH 8.4), 1 U *Taq* DNA polymerase (Bangalore genei, India.), 0.25 mMol/L of each dNTP (Medox Biotech India Private Ltd, India.), 1.5mM $MgCl_2$ (Bangalore genei, India.), 5 µl of the template and 0.5 µM of each species-specific primer (Sigma-Aldrich Private Ltd, India.) as suggested by earlier studies.[10,11] Bacterial sequences submitted to Gen Bank under accession numbers JQ396380 and JQ396381 for *P. gingivalis* and *A. actinomycetemcomitans* were used as positive controls. Sterile Millipore water served as blank control. PCR thermal cycling conditions included an initial denaturation step at 94ºC for 5 min, 35 cycles of denaturation at 94º C for 1minute, primer annealing at 50ºC for 1 minute, extension at 72ºC for 1.5 minutes and final extension step at 72ºC for 7 minutes.

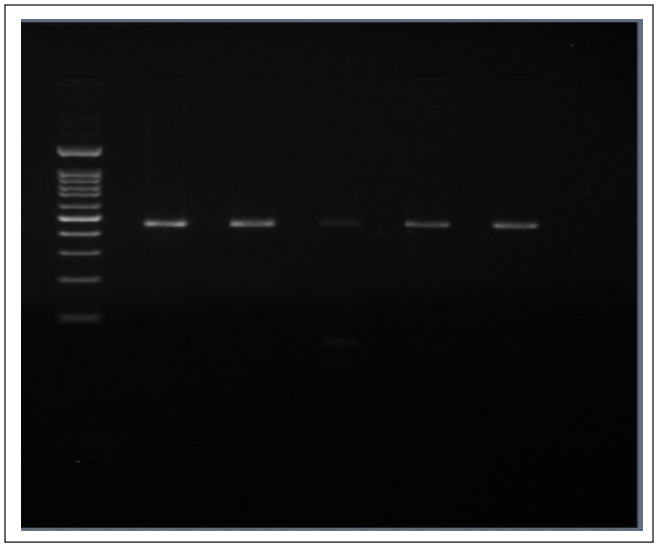

Figure 1. *A. actinomycetemcomitans*
Lane 1-100 bp Ladder, 2- Positive control, 3-6 clinical sample, 7- blank control

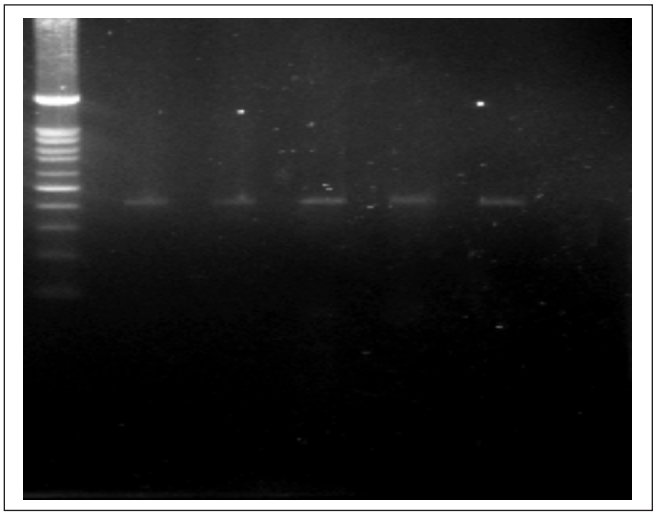

Figure 2. *P. gingivalis*
Lane 1-100 bp Ladder, 4-Positive control, 2, 3-5, 6 clinical samples, 7- blank control

PCR products were fractionated in a 1.5% agarose gel electrophoresis in Tris-Borate EDTA buffer. The gel was stained with 0.5µg/ml ethidium bromide and photographed under BioRad UV gel documentation system. 100 bp ladder (Medox Biotech India Pvt Ltd) served as the molecular weight marker. (Gel photographs for *A. actinomycetemcomitans* and *P. gingivalis* shown in figures 1 and 2)

Statistical Analysis

All statistical tests were done using SPSS software (Version18.0). The distribution of *A. actinomycetemcomitans* and *P. gingivalis* in group I, II, and III were analyzed by Binomial test and p value < 0.05 was considered significant. Proportions test was used to make intergroup comparisons for both the microorganisms (*A. actinomycetemcomitans* and *P. gingivalis*).

Table 1. Mean Age and Standard Deviation of group I, II and III

Group	Group Details	Mean	SD
I	Deciduous Dentition	4.50	0.96
II	Mixed Dentition	7.97	1.00
III	Permanent Dentition	14.50	0.68

RESULTS

The mean age of the participants were 4.55, 7.98 and 14.50 in group I, II and III respectively. (Table 1)

In our study, the prevalence of *A. actinomycetemcomitans* in Group I and II were 5% and 12.5% respectively, whereas, it was absent in group III. The prevalence of *P. gingivalis* was high in group I (35%) followed by group III (27.5%) and II (20%). The binomial test revealed statistically significant prevalence of *A. actinomycetemcomitans* in all the three groups, and *P. gingivalis* in-group II and III only. (Table 2)

Intergroup comparisons were made. A statistically significant difference was present between group II and III for *A. actinomycetemcomitans*, whereas the comparisons were non- significant. (Table 3)

DISCUSSION

The tissue associated dental plaque harbors gram-negative rods and cocci, including *P. gingivalis*.[2] These organisms express a number of potential virulence factors and induce host inflammatory mediators, which eventually leads to connective tissue breakdown and alveolar bone resorption.[2]

A. actinomycetemcomitans was one of the first colonizers on supragingival tooth surfaces in early plaque development in monkeys[12] and in an *in vivo* model in humans.[13] *A. actinomycetemcomitans* is able to colonize in a healthy and clean oral cavity. Cultivable *A. actinomycetemcomitans* occurs in at least 10% of periodontally healthy children with primary dentition[14], whereas *P. gingivalis* is absent or infrequently detected from healthy children.[15-17] These organisms are good markers for destructive periodontal disease and further clinical loss of attachment in a susceptible host. Subgingival sites serve as the ecological niche and natural reservoir for *A. actinomycetemcomitans* and *P. gingivalis*.[2] Hence in this study, the subgingival plaque was collected and presence of *A. actinomycetemcomitans* and *P. gingivalis* were assessed in healthy children aged 3 to 15 years.

Lower canine is one of the least caries-prone teeth, it is fully erupted and remains in the oral cavity for a relatively longer period of time.[5] Mandibular first permanent molar is the first permanent tooth that is commonly fully erupted in children.[9] Hence we selected lower canine (primary/ permanent) and/ first permanent molar for sample collection. Collected samples were subjected to DNA extraction and PCR assay.

PCR is reported to be highly specific and sensitive. Moreover it is quicker, and the results are available within few hours and are less labour-intensive.[18] PCR can be used to detect as few as 50 cells of *A. actinomycetemcomitans* and *P. gingivalis*.[19] The *16S rRNA* PCR method is an accurate, reproducible and highly sensitive test for identification of *A. actinomycetemcomitans* and *P. gingivalis*.[20]

In our study, *A. actinomycetemcomitans* was absent in group III, whereas, the prevalence in Group I and II were 5% and 12.5% respectively. The prevalence of *P. gingivalis* was high in group I (35%) followed by group III (27.5%) and II (20%). The binomial test revealed statistically significant prevalence of *A. actinomycetemcomitans* in all the three groups, and *P. gingivalis* in group II and III only. Intergroup comparisons showed statistically significant difference only between group II and III for *A. actinomycetemcomitans*.

Table 2. Distribution of *A. actinomycetemcomitans* and *P. gingivalis* among three groups.

Group		Present		Absent		Total		Binomial Test
		N	%	N	%	N	%	Sig
Group I	Aa	2	5.0	38	95.0	40	100.0	0.000 (Sig)
	Pg	14	35.0	26	65.0	40	100.0	0.082 (Ns)
Group II	Aa	5	12.5	35	87.5	40	100.0	0.000 (Sig)
	Pg	8	20.0	32	80.0	40	100.0	0.000 (Sig)
Group III	Aa	0	0	40	100.0	40	100.0	0.000 (Sig)
	Pg	11	27.5	29	72.5	40	100.0	0.007 (Sig)

Aa – *Aggregatibacter actinomycetemcomitans*; Pg – *Porphyromonas gingivalis*
Ns – non- significant; Sig – significant; N – No. of patients

Table 3. Intergroup comparison for presence of *A. actinomycetemcomitans* and *P. gingivalis*

Inter Group Comparison	**p- value***	
	A. actinomycetemcomitans	*P. gingivalis*
Group I vs Group II	0.2388 (Ns)	0.1371 (Ns)
Group I *vs* Group III	0.1561 (Ns)	0.4715 (Ns)
Group II *vs* Group III	0.0236 (Sig)	0.4330 (Ns)

Ns – non- significant; Sig – significant

Table 4. Studies regarding prevalence of *A. actinomycetemcomitans*

Studies	Age (in years)	Samples	Methods used	Total subjects	Prevalence
Frisken KW *et al* 1987 [15] *	5-7	Subgingival plaque	Microbiological technique	67	3.3%
Lamell CW *et al* 2010 [20] **	0-18	Samples collected with endodontic paper points	PCR	• At first sampling - 222 • At second sampling (1-3 years later) - 101	48% 51%
Ooshima T *et al* 2003 [5] **	2-15	Saliva and subgingival plaque	PCR	119	10.1%
Kulekci G *et al* 2007 [21] **	6–13	Stimulated saliva	PCR	41	24%
Frisken KW *et al* 1990 [22] *	0-2 ½	Sample swabbed with sterile cotton	Microbiological technique	36	0%
Sakai VT *et al* 2007 [23] *	Mixed dentition	Unstimulated saliva	PCR	First visit (64) Second visit (1year later) – 60	4.7% 1.7%

* – Studies showing low prevalence compared to our study.

** – Studies showing high prevalence compared to our study.

Table 5. Studies regarding prevalence of *P. gingivalis*

Studies	Age (in years)	Samples	Methods used	Total subjects	Prevalence
Frisken KW *et al* 1987 [15] *	5-7	Subgingival plaque	Microbiological technique	67	1.6%
Lamell CW *et al* 2010 [20] **	0-18	Samples collected with endodontic paper points	PCR	• At first sampling - 222 • At second sampling (1-3 years later) - 101	36% 43%
Kimura S *et al* 2002 [24] *	2-13	Plaque	PCR	144	0%
Ardin CA *et al* 2011 [25] *	1-6	Subgingival plaque	PCR	187	20-30%
Nakano K *et al* 2008 [4] *	2-16	Saliva and plaque samples	PCR	26	1.1%
Ooshima T *et al* 2003 [5] *	2-15	Saliva and plaque	PCR	119	1.5%
Kulekci G *et al* 2007 [21] *	6–13	Stimulated saliva	PCR	41	12%
Frisken KW *et al* 1990 [22] *	0-2 ½	Sample swabbed with sterile cotton	Microbiological technique	36c	0%
Sakai VT *et al* 2007 [23] *	Mixed dentition-	Unstimulated saliva	PCR	• First visit (64) • Second visit (1year later – 60)	6.3% 8.3%

* – Studies showing low prevalence compared to our study.

** – Studies showing high prevalence compared to our study

The literature review showed a wide range of prevalence of these two organisms. They are tabulated in table 4 and 5 for *A. actinomycetemcomitans* and *P. gingivalis* respectively.

A. actinomycetemcomitans and *P. gingivalis* are common inhabitants of the oral cavity of children of any age and usually colonize only transiently.20 *P. gingivalis* appears to become more stable in the late teenage, possibly as pockets develop. The reported prevalence of *A. actinomycetemcomitans* and *P. gingivalis* in young subjects varies widely, probably because of differences in site selection, sampling strategies, and the detection methods used. In our study, we did not use Real Time – PCR and quantitate the organisms. Moreover our study did not investigate the virulence factors i.e. the serotypes of *A. actinomycetemcomitans* or fimA genotypes of *P. gingivalis*-positive specimens.

CONCLUSIONS

Though A.actinomycetemcomitans and *P. gingivalis* were detected in subgingival plaque of healthy individuals, their presence does not necessarily mean the existence of active periodontal disease.

The results from this study do not necessarily pertain to differences in dentition but possibly to inter-individual differences. The risk of periodontitis by this putative periodontopathogens can be further substantiated with the screening of the virulence genes.

Future studies on bacterial quantification in a larger sample size of diverse Indian population would be more informative in this regard.

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