

Original Article

Detection of *Aggregatibacter actinomycetemcomitans* leukotoxin and fimbria-associated protein gene genotypes among periodontitis patients and healthy controls: A case-control study

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ABSTRACT

Background: *Aggregatibacter actinomycetemcomitans* has been reported in higher proportions in subgingival microbiota of individuals with aggressive periodontitis (AgP) compared with those with chronic periodontitis (ChP) and healthy controls. The major virulence factors are the ones that help in colonization and evasion of host's defenses. Hence, this study was aimed to assess the prevalence of *A. actinomycetemcomitans* 16S rRNA and its virulent genotypes (leukotoxin [*lktA*] and fimbria-associated protein [*fap*]).

Materials and Methods: In this case-control study We performed periodontal examination and measured probing depth and clinical attachment level (CAL). Subgingival plaque samples from 200 (ChP: $n = 128$ and AgP: $n = 72$) periodontitis patients and 200 healthy controls were screened for the presence of *A. actinomycetemcomitans* 16S rRNA, *lktA*, and *fap* genotypes by polymerase chain reaction. The prevalence of genotypes between periodontitis patients and healthy controls was compared with Pearson's Chi-square test. $P < 0.05$ was considered statistically significant.

Results: Mean pocket probing depth and CAL were high as compared to the healthy controls. The prevalence of *A. actinomycetemcomitans* in ChP ($n = 128$), AgP ($n = 72$), and healthy individuals ($n = 200$) was 32.0%, 61.1%, and 2.5%, respectively. *A. actinomycetemcomitans lktA* genotype prevalence was 71.8% among periodontitis patients, while *A. actinomycetemcomitans fap* genotype showed 31.8% prevalence. The prevalence of these genotypes was insignificant in healthy controls.

Conclusion: The high odds ratio for *A. actinomycetemcomitans* prevalence suggests its strong link to periodontitis. Detection of *A. actinomycetemcomitans lktA* + genotype may be a useful marker for AgP as its prevalence was found to be high in AgP.

Key Words: *Aggregatibacter actinomycetemcomitans*, aggressive periodontitis, chronic periodontitis, polymerase chain

Received: May 2016

Accepted: October 2017

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INTRODUCTION

Aggregatibacter actinomycetemcomitans, formerly *Actinobacillus actinomycetemcomitans*, is a major

etiological agent of aggressive periodontitis (AgP).^[1-3] It also exists in chronic periodontitis (ChP) patients

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How to cite this article: Mahalakshmi K, Krishnan P, Chandrasekaran SC. Detection of *aggregatibacter actinomycetemcomitans* leukotoxin and fimbria-associated protein gene genotypes among periodontitis patients and healthy controls: A case-control study. Dent Res J 2018;15:185-90.

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and periodontally healthy individuals.^[4,5] The prevalence and serotypes of *A. actinomycetemcomitans* strains differ in different periodontal conditions.^[5,6] *A. actinomycetemcomitans* has been shown to possess numerous virulence factors that facilitate its survival in the oral cavity and enable it to evade the host's protective mechanisms. The prime virulence factors are the ones that help in colonization and persistence (adhesins, invasins, bacteriocins, and antibiotic resistance) that interfere with the host's defenses (leukotoxin [*lktA*], lipopolysaccharides, chemotactic inhibitors, cytolethal distending toxin, immunosuppressive proteins, and Fc-binding proteins) and factors that destroy host tissues (cytotoxins, heat shock proteins, collagenase, and bone resorption agents).^[7] Among the various virulence factors reported, the fimbriae of *A. actinomycetemcomitans* plays a major role in colonization of various types of host cells. The fimbria-associated protein (*fap*) gene is strongly expressed in fimbriated *A. actinomycetemcomitans* strains but not in nonfimbriated ones.^[8] Strains possessing fimbriae adhere more efficiently than nonfimbriated variants of *A. actinomycetemcomitans*.^[9] Neutrophils are successful in eradicating the pathogenic bacteria, but *A. actinomycetemcomitans* can produce a unique *lktA* encoded by the *lktA* gene that leads to the destruction of human neutrophils and monocytes.^[10,11]

This bacterial species has been reported in higher proportions in the subgingival microbiota of patients with localized AgP compared with those with ChP or in healthy controls.^[12-15] The main aim of the present study was to determine the prevalence of *16S rRNA* of *A. actinomycetemcomitans* and its virulent genotypes (*A. actinomycetemcomitans lktA* gene and *fap* gene) in AgP, ChP, and healthy individuals.

MATERIALS AND METHODS

Study population

In this case-control study Two hundred periodontitis patients (ChP: $n = 128$ and AgP: $n = 72$) were recruited from the Department of Periodontics and Implantology, Tamil Nadu, Government Dental College and Hospital, Chennai, and Sree Balaji Dental College and Hospital for the study. The age of the study population ranged between 20 and 60 years. The inclusion criteria were more than three teeth with a pocket probing depth (PPD) of ≥ 5 mm and bleeding on probing. Patients who were on periodontal therapy

and dental hygiene procedures in the past 1 year, those who were on antibiotic therapy during the past 6 months, pregnant women, and those with a history of diabetes, other systemic conditions, and smoking were excluded. Two hundred healthy controls with a PPD of ≤ 3 mm and $\leq 3.6\%$ of sites which exhibited gingival bleeding were included as control group. Informed consent was obtained from patients and healthy controls. The present study was reviewed and approved by the human ethics committee of Dr. A.L.M Postgraduate Institute of Basic Medical Sciences, University of Madras, Chennai.

Sample collection and DNA isolation

The PPD and the clinical attachment level (CAL) were recorded. The PPD was measured with a graduated Williams' periodontal probe, and subgingival plaque was collected from three different sites using a sterile Gracey curette after careful removal of supragingival plaque with sterile cotton roll from periodontitis patients and healthy controls. The samples from each patient were pooled into 500 μ l of phosphate buffered saline (PBS, pH 8.0), transported in ice, and stored at -20°C until assay. Subgingival plaque samples in PBS were microcentrifuged at 10,000 rpm for 5 min, supernatant was discarded, and the pellet was resuspended in 150 μ l of lysis broth (10 mmol/L Tris-HCl, 1.0 mmol/L EDTA, 1.0% Triton X-100, pH 8.0).^[16] The lysis broth was further vortexed and kept in water bath at 100°C for 10 min, cooled, and centrifuged at 10,000 rpm for 2 min. The supernatant was stored at -20°C and was used as template for polymerase chain reaction (PCR) assay.

Polymerase chain reaction assays

All the PCR analyses were performed in duplicate. Positive and negative controls were incorporated in all batches of the samples which were examined. The positive controls consisted of DNA extracted from clinical samples (subgingival plaque), which was previously tested and was found to be positive as was confirmed by PCR and gene sequencing (*16S rRNA* gene of *A. actinomycetemcomitans* GenBank accession no: HQ188689 and *A. actinomycetemcomitans lktA* gene GenBank accession no: HQ269830). Sterile millipore water was included as a negative control. The PCR reactions were run in a thermal cycler (Eppendorf Mastercycler Gradient; Eppendorf AG, Hamburg, Germany). The PCR mixture (50 μ l) for detection of *16SrRNA* gene of *A. actinomycetemcomitans* contained 5 μ l of 10X PCR buffer

(20 mM/L Tris-HCl, 50 mM/L KCl, pH 8.4), 1.25 U Taq DNA polymerase (Bangalore Genei Pvt., Ltd., India), 0.25 mmol/L of each dNTP (Medox Biotech India Pvt., Ltd.), 1.5 mM MgCl₂ (Sigma-Aldrich Pvt., Ltd., India), 0.5 μM of each primer (Sigma-Aldrich Pvt., Ltd., India) [Table 1] and 5 μl of the template. PCR thermocycling conditions included an initial denaturation at 95°C for 2 min, followed by 36 cycles at 94°C for 30 s, at 55°C for 1 min, and at 72°C for 2 min, and a final extension step at 72°C for 10 min.

Polymerase chain reaction protocol for the detection of fimbria-associated protein and leukotoxin genes of *Aggregatibacter actinomycetemcomitans*

To detect *fap* and *lktA* genes of *A. actinomycetemcomitans*, PCR amplification was performed in a volume of 50 μl containing 5 μl of template, 5 μl PCR buffer (20 mM/L Tris-HCl, 50 mM/L KCl, pH 8.4), and 1 U Taq polymerase (Bangalore Genei Pvt., Ltd., India), 0.25 mmol/L of each dNTP, 2.5 mM/L MgCl₂, 50 pmol primers [Table 1] for *A. actinomycetemcomitans fap* gene, and 25 pmol primers for *A. actinomycetemcomitans lktA* gene. Thermal cycling conditions included an initial denaturation at 94°C for 5 min, followed by 36 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, and a final extension step at 72°C for 7 min.

Gel electrophoresis

The PCR products were fractionated in a 1.5% agarose gel electrophoresis in Tris-borate EDTA buffer. The gel was stained with 0.5 μg/ml ethidium bromide and photographed under a Bio-Rad UV gel documentation system. 100 bp ladder (Medox Biotech India Pvt., Ltd.) was served as a molecular weight marker [Figures 1-3].

Statistical analysis

The mean clinical measurements such as PPD and CAL were computed for each patient, and they

Table 1: Primers used for the study

Gene	Primers	Amplicon size (bp)
16S rDNA	Forward-5'-AAACCCATCTCTGAGTTCTTCTTC-3' Reverse-5'-ATGCCAACTTGACGTTAAAT-3' (Slots et al., 1995)	443
<i>lktA</i>	5'TCGCGAATCAGCTCGCCG-3' 5' GCTTTGCAAGCTCCTCAC-3' (Watanabe and Frommel, 1996)	285
<i>fap</i>	5'-ATTAAATACTTTAACTACTAAAGC-3' 5'-GCACTGTAACTGTACTAGC-3' (Ishihara et al., 1997)	210

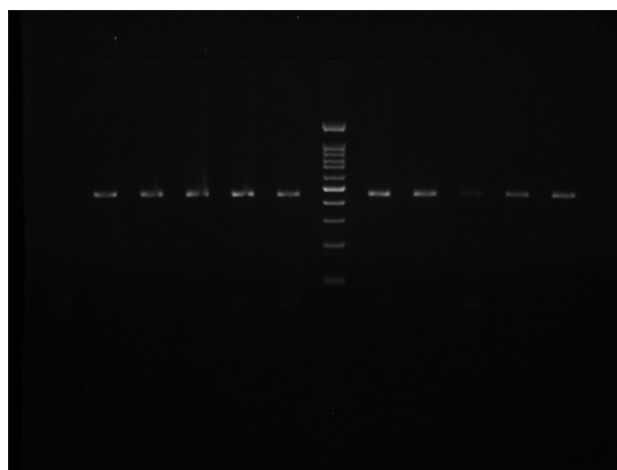


Figure 1: Gel picture of *Aggregatibacter actinomycetemcomitans* 16S rRNA (443 bp): 8 - Positive control, 1-6 and 9-12 - Clinical sample, 7-100 bp ladder, 13 - Blank control.

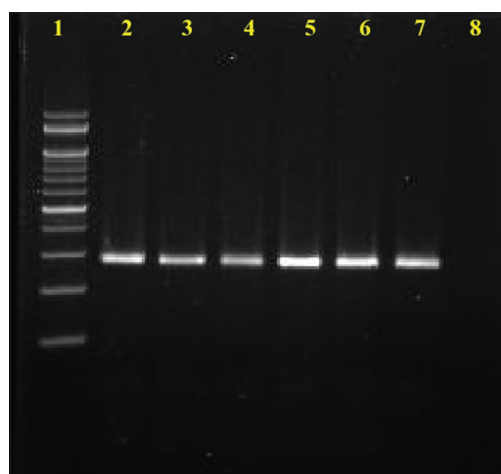


Figure 2: Gel picture of *Aggregatibacter actinomycetemcomitans lktA* gene (285 bp): 1-100 bp ladder, 2-6 - Clinical samples, 7 - Positive control, 8 - Blank control.

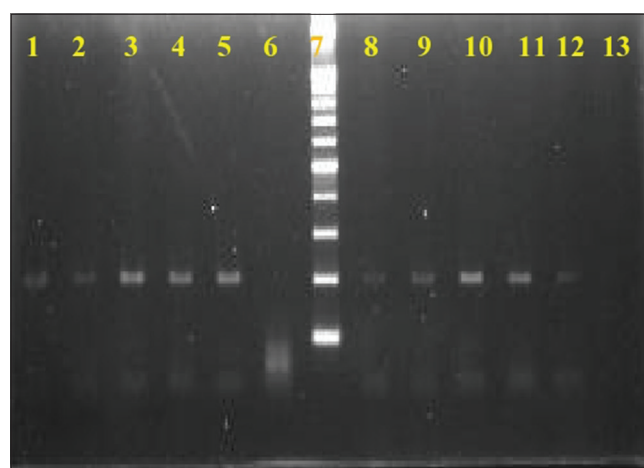


Figure 3: Gel picture of *Aggregatibacter actinomycetemcomitans fap* gene (210 bp): 7th lane - 100 bp ladder, 1-6 and 8-12 lanes - Clinical samples, 13th lane - Blank control.

were then averaged across the patients within the groups. Any difference of $P < 0.05$ was considered as statistically significant. The prevalence of the genotypes between periodontitis patients and healthy controls was compared with Pearson's Chi-square test. A $P < 0.05$ was considered as statistically significant. Odds ratio was calculated using Fisher's exact test.

RESULTS

A significant difference was observed for mean PPD and CAL ($P = 0.0001$) between the study population and control group [Table 2]. The prevalence in ChP ($n = 128$), AgP ($n = 72$), and healthy individuals ($n = 200$) was 32.0%, 61.1%, and 2.5% for *A. actinomycetemcomitans 16S rRNA*, respectively. The prevalence of *A. actinomycetemcomitans lktA* gene and *fap* gene among periodontitis (ChP and AgP) patients was 71.8% and 31.8%, respectively. Two out of five *A. actinomycetemcomitans*-positive healthy controls showed the presence of *lktA* gene. Table 3 shows *A. actinomycetemcomitans lktA* gene and *fap* gene prevalence in different groups. The odds of detecting *A. actinomycetemcomitans 16S rRNA* was 25.82 times high in individuals with periodontitis as compared to those in healthy controls. A significant prevalence was observed for *lktA* genotype among ChP and AgP patients compared to *fap* genotype. The presence of both *lktA* and *fap* genes was observed in 17 (23.6%) AgP and 6 (4.7%) ChP patients, respectively ($P > 0.05$). *A. actinomycetemcomitans*

lktA + fap + genotype were present in periodontitis patients with pocket depth ≥ 7 mm.

DISCUSSION

In agreement with the previous studies,^[2,14,17-21] the results of the present study support the notion that *A. actinomycetemcomitans* is a predominant pathogen in the etiology of AgP. The occurrence of *A. actinomycetemcomitans* in AgP and ChP is associated with severity of the disease in regard to PPD and CAL. *A. actinomycetemcomitans* was most frequently detected in sites with pocket depth ≥ 5 mm and clinical attachment loss ≥ 7 mm. The prevalence of *A. actinomycetemcomitans* (61.1%) among AgP in the present study is statistically significant ($P = 0.0001$).

In contrast to the results of the present study by PCR (61.1% in AgP, 32.0% in ChP, and 2.5% in healthy controls), Yang *et al.*^[5] have reported a higher prevalence of 84.3%, 60.4%, and 64.0% in AgP, ChP, and healthy periodontal individuals, respectively, by indirect immunofluorescence assay using serotype-specific polyclonal antisera to *A. actinomycetemcomitans*. The prevalence of *A. actinomycetemcomitans* among ChP is consistent with few earlier studies.^[22-24] The present study shows a very low prevalence of *A. actinomycetemcomitans* in healthy controls compared to Kumar *et al.*, 2003,^[25] who have reported 21% and 30% in ChP and healthy individuals, respectively. On the contrary, Junior *et al.*, 2006^[26] have reported a very high prevalence (76.7%) in healthy controls. The present study reports a high prevalence of *A. actinomycetemcomitans* among periodontitis patients compared to few other studies.^[27-32] On the contrary, a number of studies have reported a higher prevalence than the present study in AgP.^[16,20,26,33]

One of the most intriguing properties of *A. actinomycetemcomitans lktA* is its ability to selectively kill human neutrophils and monocytes. Here, we report a very high prevalence (81.8%) of *A. actinomycetemcomitans lktA +* genotype among AgP patients exhibiting a very good statistical significance in comparison with healthy controls ($P < 0.033$). The prevalence of *A. actinomycetemcomitans lktA +* genotype (61.0%) among ChP patients was low compared to the finding of Wu *et al.*, who have reported 75.4% in the Chinese population.^[16] Junior *et al.* have observed leukotoxic genotype only in patients with generalized aggressive disease, while we have shown a significant presence in ChP as well.^[26]

Table 2: Clinical parameters (mean \pm standard deviation) of periodontitis patients and healthy controls

	Periodontitis	Health	P
Age [‡]	34.26 \pm 10.23 [†]	26.09 \pm 7.70 [†]	<0.0001*
Gender (males/females) [#]	108/92	115/85	0.5
Mean PPD (mm) [‡]	7.52 \pm 1.57 [†]	2.00 \pm 0.07 [†]	<0.0001*
Mean CAL (mm) [‡]	9.67 \pm 1.70 [†]	3.00 \pm 0.00 [†]	<0.0001*

[#]Refers to Chi-square test; [‡]Mann-Whitney test; [†]SD; *Significant P value. SD: Standard deviation; PPD: Pocket probing depth; CAL: Clinical attachment level

Table 3: Prevalence of *lktA* and *fap* genotypes

Virulence gene of <i>Aggregatibacter actinomycetemcomitans</i> screened	ChP (n=41), n (%)	AgP (n=44), n (%)	Health (n=5), n (%)
<i>lktA</i>	25 (61.00)	36 (81.80)	2 (40.00)
<i>fap</i>	9 (22.00)	18 (40.90)	1 (20.00)

n: Number of *Aggregatibacter actinomycetemcomitans 16S rRNA*-positive samples; ChP: Chronic periodontitis; AgP: Aggressive periodontitis

A. actinomycetemcomitans fap + genotype showed a prevalence of 22.0% in ChP patients which is relatively low when compared to the findings of Wu *et al.*, 2007, who have reported 50% in ChP patients. Conversely, *A. actinomycetemcomitans fap* + genotype were detected among 40.9% of AgP patients. Only one (20.0%) healthy controls showed the presence of *A. actinomycetemcomitans fap* + genotype. Statistical significance was not observed for *fap* genotype ($P = 0.581$).

As predicted, none of the *A. actinomycetemcomitans 16S rRNA*-negative samples was positive for either *lktA* or *fap*. AgP is a disease of adolescents that can have profound cosmetic, functional, and psychological effects with the highest prevalence of *A. actinomycetemcomitans*. Hence, microbiological analyses to identify *A. actinomycetemcomitans* in subgingival plaque samples are important for screening, treatment planning, treatment evaluation, and monitoring of periodontal diseases.

CONCLUSION

The high odds ratio for *A. actinomycetemcomitans* suggests a strong association between them and periodontitis. Detection of *A. actinomycetemcomitans lkt* + genotype may be a useful marker for AgP as its prevalence was found to be very high in AgP group in the present study.

Acknowledgments

We thank Dr. K. H. Panishankar, Professor, Department of Periodontics and Implantology, Tamil Nadu, Government Dental College and Hospital, Chennai, for help rendered in collection of clinical samples.

Financial support and sponsorship

This work was supported by the funds which were generated through the university–industry collaborative research program of the University of Madras.

Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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