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Characterization and serotype distribution of *Aggregatibacter actinomycetemcomitans*: Relationship of serotypes to herpesvirus and periodontal status in Indian subjects

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ABSTRACT

Background: The virulence of *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) in any individual depends on the type of strain of this bacterium. To our knowledge, there have been no studies reported in Indian subjects about *A. actinomycetemcomitans* serotype occurrence, co-existence with herpes virus and the possible influence of such co-existence on periodontal pathology.
Methods: Subjects for this study were a subset of a larger study to identify the prevalence of *A. actinomycetemcomitans* in chronic periodontitis. A total of 63 subjects (12 periodontally healthy and 51 with chronic periodontitis) who were positive for *A. actinomycetemcomitans* were serotyped for strain-level identification. The presence of Human Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) was tested in subgingival plaque samples by polymerase chain reaction.
Results: All five serotypes a to e were detected. Of the samples analyzed 38.09% harbored a single serotype, 36.5% had two serotypes, 6.3% demonstrated three and 4.7% demonstrated four serotypes. None of the samples showed presence of JP2 strain. Serotypes b, c, and e were most frequently identified in these individuals (46.03%, 36.5% and 38.09% respectively). Presence of serotypes b and c and absence of serotype d was associated with increased PD and CAL. Among 63 samples analyzed, 11 samples had CMV, four samples had EBV and nine samples had both these viruses. The PD and CAL were significantly higher ($p = 0.04$) when a combination of CMV and one of the serotypes was present indicating a pathological role of the coexistence.
Conclusion: Multiple serotypes are associated with chronic periodontitis in Indians, however, JP2 strains are not detectable in this cohort. Presence of multiple serotypes and a combination of any serotype with herpesvirus is associated with greater severity of the disease.

1. Introduction

Periodontitis is a multi-factorial disease of which microbial infection is considered to be the most important. Usually, the periodontal infections are poly-microbial in nature, with the most commonly studied include bacteria belonging to the red and orange complexes and herpes viruses [1]. *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) is a resident oral microbiota and an opportunistic pathogen. The virulence of this organism in any individual depends on the type of strain of this bacterium [2]. Certain strains of *A. actinomycetemcomitans* are highly leukotoxic [3], and serotype b (also known as JP2 clone) is one which exhibits properties of a true exogenous pathogen [2], and produces high amounts of leukotoxin [4].

Periodontal disease has been correlated to co-colonization with herpesvirus and red complex bacteria [5]. The herpes viruses, especially Epstein-Barr virus (EBV) and Human Cytomegalovirus (CMV), have been detected at destructive periodontitis sites and sites with active periodontal disease [6]. The higher prevalence of these viruses in combination with the anaerobic bacteria and the host immune factors might aggravate the progress of the disease

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[7]. It has been considered that the herpesviruses infect the structural and host defense cells of the periodontium and might decrease the capacity of periodontal tissues to resist bacterial insults [8,9]. The confrontation between the virus related anti-host response and the host defense leads to release of pro-inflammatory cytokines which have the ability to activate osteoclasts [8,10] and weaken the host defense against putative periodontal pathogens like *Porphyromonas. gingivalis* and *A. actinomycetemcomitans* [11].

The distribution of *A. actinomycetemcomitans* serotypes may vary with different geographical location, ethnicity and periodontal status of the study population [12–16]. To our knowledge there have been no studies reported in Indian subjects about *A. actinomycetemcomitans* serotypes, co-existence with herpes virus and the possible influence of such co-existence on periodontal pathology. Hence in this study we aim i) to examine the occurrence of *A. actinomycetemcomitans* serotype a-e in subjects positive for *A. actinomycetemcomitans* with and without periodontitis ii) to assess the association between serotypes and periodontal status and iii) to examine whether there is an association between serotypes and herpesvirus (CMV, EBV). The findings of this study will help in understanding the *A. actinomycetemcomitans* serotype distribution in this part of the subcontinent, prognosticate clinical outcomes and append the knowledge of the global distribution of this organism.

2. Materials and methods

2.1. Study population

Subjects for this study were a subset of a larger study to identify the prevalence of *A. actinomycetemcomitans* in chronic periodontitis [17]. Subjects were recruited for this study from the outpatient department of Maratha Mandal's NGH Institute of Dental Sciences and Research Centre, Belagavi, Karnataka, India. After a clinical examination and selection of the subjects for the study, a signed consent was obtained. The study was approved by the Institutional Ethics Committee. Sample size estimation was done based on a comparison of proportions of *A. actinomycetemcomitans* in healthy and diseased volunteers. Samples from 63 subjects who were positive for *A. actinomycetemcomitans* from the larger investigation were used for serotyping in this study [17].

The study subjects were segregated into healthy and chronic periodontitis based on American Association of Periodontology guidelines [18]. For the healthy group, the inclusion criteria required subjects with healthy periodontium with the probing depth (PD) of $\leq$3 mm in all the sites. For the chronic periodontitis group, subjects with at least four sites in different teeth presenting both PD of $\geq$5 mm and clinical attachment loss (CAL) of $\geq$3 mm were recruited. Exclusion criteria included diabetes, medical conditions requiring prophylactic antibiotic coverage, HIV infection, current pregnancy, current orthodontic treatment, professional dental cleaning or antibiotic therapy in past 3 months and lower than 20 teeth in oral cavity. For this study, we recruited subjects who were lifelong nonsmokers.

2.2. Clinical examination

A complete periodontal examination, excluding third molars, was conducted to register PD, CAL, gingival index (GI) [19], and plaque index (PI) [20] in six periodontal sites per tooth by one trained and calibrated examiner using a manual periodontal probe (UNC-15).

2.3. Sampling and processing of subgingival bacterial plaque

To determine the colonization pattern of *A. actinomycetemcomitans* strains, a pooled subgingival sample was collected. The site that was deepest in each quadrant was selected. A total of six sites were isolated with sterile cotton rolls, and the supragingival plaque was removed with sterile cotton pellets. The subgingival sample was collected using a Universal curette (2R/2L, 4R-4L: Hu-Freidy, USA) and was placed in the plastic vial with reduced transport fluid. The plaque samples were diluted 10 fold (10^{-3}) and were immediately plated onto TSBV (trypticase soy, serum, bacitracin, vancomycin) agar plates and Dentaid plates [21,22]. The plates were incubated at 37° C in an environment with 5% CO2 and 95% N2 (microaerophilic) for the growth of *A. actinomycetemcomitans*. From each plate data was recorded as the count of colony forming units/ml (CFU/ml). The colonies were subcultured to obtain a pure culture, which was then collected and DNA extraction was done.

2.4. Isolation of DNA

For the DNA isolation, the colonies were collected in a vial containing Tris-EDTA (TE) buffer. DNA extraction was done using the protocol described below. In brief, the bacterial cell from culture or the plaque samples were vortexed with TE buffer. Following this, the cells were treated with lysis buffers and proteinases K and incubated for 2 h at 60 °C. Finally, the enzyme was inactivated by boiling for 10 min. The vial centrifuged and the supernatant containing the isolated genomic DNA is aliquoted in a plastic vial and stored at −80 °C until further use [23].

2.5. Identification of A. actinomycetemcomitans serotypes by DNA-PCR analysis

Only those samples which were culture positive for the detection of *A. actinomycetemcomitans* were selected for serotyping. To detect the five serotypes a to e, the primers used for DNA amplification were designed in accordance with Suzuki et al. [24](Table 1). The expected amplicon sizes for serotypes a, b, c, d, and e were 428-bp, 298-bp, 559-bp, 690-bp, and 211-bp, respectively. The PCR was carried out in 20 μl final volume, and the conditions included initial denaturing at 94 °C for 5 min, followed by a total of 30 cycles of denaturation at 94 °C for 45 s; annealing at 50° C for 45 s, and extension at 72 °C for 1 min. The final extension was carried out at 72° C for 5 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide (0.5 μg/ml), and photographed under UV light by Gel doc system. PCR fragments were detected by comparing the band position with the 100-bp DNA ladder.

Table 1
Primer sequences used in serotyping of *A.actinomycetemcomitans* strains.

Name	Sequence	Product size
SA-F	5′-GCAATGATGTATTGTCTTCTTTTGGA-3′	428
SA-R	5′-CTTCAGTTGAATGGGGATTGACTAAAAC-3′	
SB-F	5′-CGGAAATGGAATGCTTGC-3′	298
SB-R	5′-CTGAGGAAGCCTAGCAAT-3′	
SC-F	5′-AATGACTGCTGTCGGAGT-3′	559
SC-R	5′-CGCTGAAGGTAATGTCAG-3′	
SD-F	5′-TTACCAGGTGTCTAGTCGGA-3′	690
SD-R	5′-GGCTCCTGACAACATTGGAT-3′	
SE-F	5′-CGTAAGCAGAAGAATAGTAAACGT-3′	211
SE-R	5′-AATAACGATGGCACATCAGACTTT-3′	