Identification of periodontal pathogen *actinobacillus actinomycetemcomitans* among Sudanese aggressive periodontitis patient by Multiplex PCR 2012

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ABSTRACT

**Background information:** Aggressive periodontitis is the disease of periodontium which may lead to loss of teeth at a young age. One of the causative micro-organism is *Actinobacillus actinomycetemcomitans* (gram-ve bacteria). **Aim of study:** is the detection of this micro-organism in gingival sulcus of Sudanese aggressive periodontitis patients by Multiplex PCR technique. **Material and method:** Samples were collected by sterile paper points from the deepest sites of periodontal pockets and DNA extraction was done by guanidine. Afterward PCR was carried out using a specific primer for *A. actinomycetemcomitans*. **Result:** No bands were amplified from all 30 pooled samples **Conclusion:** *A. actinomycetemcomitans* is not the main causative micro-organism among Sudanese aggressive periodontitis patients. **Recommendation:** There is a need to investigate other causative bacteria of aggressive periodontitis as *P-gingivalis* which may be more prominent in Sudanese aggressive periodontitis patient.

INTRODUCTION

Aggressive periodontitis is a destructive inflammatory disease; it affects systemically healthy individuals at an age less than 30 years old and also older patients. The progression of the disease is rapid and there is an imbalance between periodontal pathogens, (local factor) and systemic host defense mechanism. Some patients have defects in leukocyte function (systemic factor), (1). Also there is familial aggregation of the diseased individuals in addition to racial factors which was observed in the United States in which the disease is more prevalent among African Americans. (2) There are many studies on the prevalence of
aggressive periodontitis in many countries among different population, in USA prevalence rate is 0.5%. (2). Prevalence rate in Finland and Switzerland among 16 adolescents is 0.1%. (3, 4).

In Sudan Abdalla Saleh et al. 2008 estimated the prevalence and gender distribution of aggressive periodontitis in Sudanese school children age (12-16) in Khartoum state to be 1.7% and 1.2 for male and female respectively. The total prevalence was 1.4% and the disease was found in seven tribes. The group of pure African origins showed high prevalence rates than those of less nigroid. (5)

Amal M. Elamin, et al in 2010 studied ethnic disparities in the prevalence of aggressive periodontitis among high school students in Sudan. A total of 3.4% of the subjects had aggressive periodontitis, Sudanese population consists of an ethnic blend of Africans and Afro-Arabs, significantly higher percentage of the African tribes subjects had attachment loss compared to Afro-Arab tribes (19.8% versus 14.7%), so ethnicity had a significant effect on the prevalence of attachment loss and aggressive periodontitis. The differences in periodontal disease status between these two ethnic groups may be attributed to differences in their socioeconomic status, general and oral health beliefs, habits and other behavioral variable. It was thought that genetic variability may have also contributed to part of the observed differences in the prevalence of the disease (6).

Clinically it is classified into localized aggressive periodontitis and generalized form. The clinical feature of periodontitis is bleeding on probing, pocket formation; inter proximal attachment loss and mobility of the affected teeth. When the periodontal lesions are severe, generalized, rapid bone destruction (vertical loss of alveolar bone) occur within a few weeks or months which may lead to teeth loss.

Epidemiological studies in many parts of the world have demonstrated a strong positive association between bacteria in dental plaque (local factor) and the prevalence and severity of periodontal disease.

Bacterial species in healthy gingiva are Streptocoous, Actinomyces, and Veillonella (7). Microbial examination of sub gingival plaque in periodontitis has a complex flora rich in Gram-negative rods, motile form spirochetes and Black pigmented bacteroides.
Sub gingival flora in aggressive periodontitis is predominated by Gram-negative anaerobic rods, particularly *Actinobacillus, actinomycetemcomitans, capnocytophagas* species and *Eikenella corr*(8)

Microbiological laboratory procedures have been involved in diagnoses and therapy control of severe forms of periodontitis. Culture and Nucleic acid based methods (PCR) have become the standard methods in microbiological analyses of subgingival plaque samples, but PCR is more sensitive than culture (savitt et al 1988) (9)

In study by Christen LA(1993 ) *actiobacillus actinomycetumcomitan* present in 95% of the localized aggressive periodontitis, (10)

Tonetti et al 1999 on study of early onset periodontitis, suggested that *actinobacillus actinomycetumcomitans* has been implicated as the primary pathogen associated with localized aggressive periodontitis, *Actinobacillus actinomycetumcomitans* was present in high frequency (approximately 90%) in lesions of localized aggressive periodontitis. Sites with evidence of disease progression often show elevated level of *actinobacillus actinomycetumcomitans* and the patients with clinical manifestations of localized aggressive periodontitis have significantly elevated serum antibody titers to *actinobacillus actinomycetumcomitans*. (11) Jose R cortelli (2005), Wilson Rosalen (2006) Jose Roberto (2010) Farshidk Kafilzideh(2010) found that *actiobacillus actinomycetumcomitans* present in healthy and diseased subjects.(,12,13,14,15).

The use of Qualitative polymerase chain reactions (PCR) including multiplex PCR is widely spread for microbiological diagnosis of periodontal diseases. The gene encoding the small subunit of 16 ribosomal RNA (16SrDNA) has been frequently used as a target for PCR examination because of its structural characteristics (Ashimoto et al 1996)

Since Actinobacillus, strains are classified into five distinct serotypes: a, b, c, d, and e and serologic specificity is defined by the polysaccharides on the surface of the organisms, five pair of primers from specific DNA sequences for each serotype were developed for genetic methods for identifying serotypes of *A. actinomycetemcomitans* strains, using a multiplex PCR assay with these primers. When the LKT primers were used, the expected
fragments were amplified from all strains of *A. actinomycetemcomitans* but not from other bacteria. These results demonstrated that each set of primers is specific for *A. actinomycetemcomitans* strains and serotypes (17).

Frequency distribution of *A. actinomycetemcomitans* serotypes differs among populations. Serology of oral *A. actinomycetemcomitans* and serotype distribution in humans in the United States, shows that serotypes b is detected more than serotype a and c (18).

In Finland the distribution of serotype b is predominant and serotype c is frequently isolated in periodontally healthy people (19). Japanese patients with Periodontitis, had multiple serotypes which are genetically homogenous in the periodontally diseased patients. (20)

The prevalence of the JP2 clone and non-JP2 genotypes of *A. actinomycetemcomitans* in the subgingival plaque of patients with aggressive periodontitis and controls among Sudanese high-school students were assessed using loop-mediated isothermal amplification (LAMP) and the PCR. The JP2 clone of *A. actinomycetemcomitans* was not detected in the subgingival plaque of either the cases or control. Non-JP2 types of *actinomycetemcomitans* was detected in the subgingival plaque of 12 (70.6%) of the cases and from only one (5.9%) control subject, showing a significantly higher frequency of detection in cases than in controls. The PCR and LAMP methods showed identical results pertaining to the identification of non-JP2 types of *A. actinomycetemcomitans*. The detection of non-JP2 types of *A. actinomycetemcomitans* may be a useful marker of increased risk for development of aggressive periodontitis in young subjects (21).

Not all studies support the presence of *A. actinomycetemcomitans*, Mohammad Hossein et al (2004), in a study for the identification of *A. actinomycetemcomitans* found that the periodonto-pathogenic bacterial growth was not present in 14 of the samples (6.2%).(22) MFaveri et al in study of sub gingival plaque of subjects with generalized aggressive periodontitis using culture-independent molecular methods based on 16S ribosomal DNA, *A. actinomycetemcomitans* was not present, They suggest that other species, like Selenomonas is the causative micro-organism which is present in their sample (2007).
Aim of the study:

is the detection of \textit{A. actinomycetemcomitans} and serotype distribution in Sudanese aggressive periodontitis patient using multiplex PCR.

MATERIALS AND METHODS

Sampling:

After ethical approval from Khartoum dental teaching hospital and informed consent from the patients attending the department of periodontology for treatment, Subgingival plaque sample were collected from 31 systemically healthy patients (male and female) with localized and generalized aggressive periodontitis demonstrating attachment loss >5mm with an age group of (18-35 years) (fig1&2). Subjects were excluded if they had systemic diseases, administration of medication such as antibiotics, steroids or had received periodontal mechanical treatment by scaling and root planning within previous 6 months. And females were excluded if they were pregnant or taking contraceptives.

Plaque samples were collected from two deepest sites in each quadrant by means of two sterile paper points per tooth after isolation using cotton roll (total of 240 sites). The paper points introduced into the gingival sulcus for 20 seconds and placed immediately in pre-reduced transport media (reinforced clostridia media for anaerobic bacteria) and stored in -20C for PCR.

DNA extraction by Guanidine chloride:

The specimens were placed in vortex, 1-2ml lyses buffer was added to each sample. 5ul proteinase, K, 1ml guanidine chloride and 300-ul NH4 acetate were added and incubated at 37 overnight. The samples were then cooled to room temperature and transferd to prechilled chloroform in 30 ml falcon tube, vortex and centrifuge for 5 minutes at 2500rpm. The upper layer was collected to a new tube and 10 ml of cold absolute ethanol was added, shaken and kept at -20C overnight. The solution was then centrifuged at 3000rpm for 15 minutes, the
supernatant was drained and the tube inverted on a tissue paper for 5 minutes. The pellet was then washed with 70% ethanol and centrifuged at 3000rpm for 15 minutes. The supernatant poured off and the pellet dried for 1-2 hours. The pellet was then re-suspended in 100ul distilled water and stored at -20C.

Primers used for PCR were designed to identify *Actinobacillus – actinomycetemcomitans* targeting 16S rRNA as follows:

LKT primers were used (LKT2 AND lkt3) forward: 5-CTAGGT ATT GCG AAA CAA TTT G-3 and reverse: 5-CCT GAA ATT AAG CTG GTA ATC-3.

The PCR mixture consisted of 25 volume of: 2.5 buffer, 1.5 Mg Cl2, 1dNTPs, 1.5 primers (1), 1.5 primer (2), 1UTaq polymerase, 11distaled water and5ul of template DNA.

Negative and positive controls were included in each batch. The positive control consisted of 2ul of genomic DNA with a concentration of 10.4 *actinobacillus actinomycetemcomitans* bacteria and the negative control consisted of 2ul of sterile water, both added to the reaction mixture. After denaturation at 96°C for 2 min, a total of 25 PCR cycles were performed; each cycle consisted of 15 s of denaturation at 94°C, 30 s of annealing at 54°C, and 60 s of extension at 72°C. Amplification products were loaded into 1.8% (wt/vol) agarose gels by electrophoresis, stained with ethidium bromide (0.5 µg/ml), and photographed under UV light. The result is positive in case of presence of clear band after amplification. (5)

**RESULT**

No bands were amplified from all 31 pooled samples and even in about 70 sites fig (3) the result indicates that *A. actinomycetemcomitans* is not detected in these studied samples.
Fig (1): localized aggressive periodontitis in a 23 years old Sudanese female with Loss of lower anterior teeth

![Image of localized aggressive periodontitis](image1)

Fig (2): Generalized aggressive periodontitis in a 25 years old Sudanese female with attachment loss at the upper right lateral incisor and canine

![Image of generalized aggressive periodontitis](image2)
Figure (3): Agrose gel electrophoresis of PCR product from Subgingival plaque samples. Positive control showed clear band but all samples were negative.

DISCUSSION

The result is in agreement with previous reports which did not support the association of *actinobacillus actinomycetum comitans* with aggressive periodontitis; Mohammad Hossein et al (2004), in which no periodontopathogenic bacterial growth was observed in 14 of the samples (6.2%). M. Faveri r et al (2007) , in which *Aggregatibacter actinomycetemcomitans*, was below the limit of detection and may not detected. José Roberto Cortelli (a) 2010 an in which they found that *actinobacillus actinomycetum comitans* was the least recovered micro-organisms, the result is also in agreement with a previous study among Sudanese patients by Elamin A (2011) in which the presence of the *A. actinomycetemcomitans JP2 clone* genotypes of *A. actinomycetemcomitans* was not detected in the sub gingival plaque of either the cases or the controls.


**Conclusion:** The periodontal pathogen *actinobacillus actinomycetumcomitans* is not the causative micro-organism of aggressive periodontitis among Sudanese population.

**Recommendations:** Since the micro-organism actinobacillus *Actinomyctum comitans* was not detected:

1- Investigations should be done to identify other causative bacteria of aggressive periodontitis like *P-gingivalis, P.intermedia, capnocytophga, species* and *spirochetes* which may be more prominent in Sudanese aggressive periodontitis patient.
2- Studies should be conducted on the role of genetic factors and the role of polymorph leucocytes in the etiology of this destructive disease.

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