Detection of Actinobacillus, actinomycetemcomitans by culture methods and multiplex PCR in subgingival plaque samples of Sudanese aggressive periodontitis patients

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Abstract

Aggressive periodontitis is a destructive periodontal disease which may lead to early loss of teeth. The etiology of the disease is unknown but there are many studies about the implication of gram-negative bacteria Actinobacillus actinomycetemcomitans, Propionibacterium gingivalis, P. intermedia and T. Fusorthia in the disease progression, also there is a genetic factor, (defect in polymorph nuclear leucocytes) that may be involved. This study is done to identify the major causative micro-organism Actinobacillus actinomycetemcomitans, by culture and multiplex PCR.

Method:

Thirty aggressive periodontitis patients were recruited from patients attending department of periodontology, Faculty of dentistry, University of Khartoum for treatment. The age range 18-35 years, both male and female. Sub-gingival plaque samples were obtained from two deepest site per quadrant for each patient for identification of the major causative micro-organisms Actinobacillus actinomycetemcomitans. Identification was done by anaerobic culture in blood agar and sub-cultured using vancomycin. After DNA extraction multiplex PCR technique which is more sensitive was used for confirmation and to identify serotypes of Actinobacillus actinomycetemcomitans if present in the examined samples.

Results:

Actinobacillus actinomycetemcomitans was not identified in all examined sites by culture and this was confirmed with biochemical testes. Using multiplex PCR and after electrophoreses no bands were amplified from all samples. The two methods showed identical results in the identification of Actinobacillus actinomycetemcomitans.

Conclusion: Since A. actinomycetemcomitans was not present in the examined samples using both culture and multiplex PCR, so its role in the pathogenesis of the disease may be questionable.

Key words: Actinobacillus actinomycetemcomitans, Multiplex PCR, Culture.
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] There is familial aggregation of the diseased individuals in addition to racial factors, in the united states the disease is more prevalent among African Americans.[2] 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. Sub gingival flora in aggressive periodontitis is predominated by Gram-negative anaerobic rods, particularly Actinobacillus, actinomycetemcomitans, capnocytophagas species and Eikenella corr.[3] Microbiological laboratory procedures have been involved in diagnoses and therapy control of severe forms of periodontitis.Culture and Nucleic acid based methods (PCR) are used as standard methods in microbiological analyses of subgingival plaque samples, PCR is more sensitive than culture.[4].In culture Aggregatibacter actinomycetemcomitans (formerly Actinobacillus actinomycetemcomitans) is a gram negative coccobacillus or short rod, 0.3 - 0.5 x 0.5 - 1.5 μm, It is facultatively anaerobes does not require X or V factors, it grows best under microaerophilic conditions with added CO2. It grows at 37°C after 24 hours incubation, colonies on blood or chocolate agar firm, adherent, star-shaped. It is oxidase-negative and benzidine-positive, reduced nitrate, produced strong alkaline and acid phosphatases, and fermented fructose, glucose and mannose. Variable fermentation results were obtained with dextran, maltose, manitol and xylose. gas.Haemophilus aphrophilus strain were morphologically and biochemically similar to A. actinomycetemcomitans. selective culture medium are available for identification such as TS BV medium as the reference medium (Tryptone-soy-serum-bacteracinc-vancomycin)and Denta id-1 which lacks both blood and serum for optimal growth of A. actinomycetemcomitans and suppression of oral flora.[5,6,7] Polymerase chain reactions (PCR) including multiplex PCR is used for qualitative 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[8] Actinobacillus actinomycetemcomitans has been implicated as the primary pathogen associated with localized aggressive periodontitis, it was present in high frequency (approximately 90%) in lesions of localized aggressive periodontitis. Sites with evidence of disease progression often showed elevated level of Actinobacillus actinomycetemcomitans and the patients with clinical manifestations of localized aggressive periodontitis have significantly elevated serum antibody titers to actinobacillus actinomycetemcomitans.[9] In some studies actiobacillusactinomycetemcomitans found in healthy and diseased subjects.[10,11,12] 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 is used for genetic identification of using multiplex PCR.[13]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 was identified using loop-mediated isothermal amplification(LAMP) and PCR, JP2, clone of A. actinomycetemcomitans was not detected in the subgingival plaque of either the cases or control. Non-JP2 types of A.actinomycetemcomitans was detected in the subgingival plaque of (70.6%) of the cases and from only (5.9%) control subject. 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.[14] Not all studies support the presence of A. actinomycetemcomitans in aggressive periodontitis patients, in some studies A. actionmycemcomitans was not present in (6.2%) of the samples.[15] In other studies of
subjects with generalized aggressive periodontitis using culture-independent molecular methods DNA, *A. actinomycetemcomitans* was not present, authors suggested that other species, like Selenomonas is the causative micro-organism which is present in their samples. Also in other studies *Actinobacillus actinomycetemcomitans* was the least recovered micro-organisms. Since aggressive periodontitis prevalent among Sudanese school children age (12-16) in Khartoum state and adult, so the aim of the present study was the detection of *A. actinomycetemcomitans* and serotype distribution among Sudanese aggressive periodontitis patients by culture and multiplex PCR which may help in diagnosis and treatment of the disease.

**Material and method:**

**Study design:**

Microbiological hospital based study.

**Sampling:**

Subgingival plaque sample were collected from 30 systemically healthy patients (males and females) with localized and generalized aggressive periodontitis, aged (18-35) years demonstrating pocket depth>5mm. 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. Females were excluded if they were pregnant or taking contraceptives. The present study was done after ethical approval from dental school university of Khartoum. Patients participated in the study gave verbal consent.

**Collection of subgingival plaque samples:**

Plaque samples from each subject were collected from two deepest sites in each quadrant by means of 2 sterile paper points per tooth after isolation using cotton roll. The paper points were introduced into the gingival sulcus for 20 seconds and placed immediately in a pre-reduced transport media (reinforced clostridia media for anaerobic bacteria). Bacterial suspension was vortex, homogenized and divided into two parts one for culture and the other for DNA extraction.

**Isolation of *Actinobacillus actinomycetemcomitans* by culture method:**

Bacterial suspension was vortex and homogenized and incubated anaeropically with cooked meat media for 48 hours. To obtain pure culture, samples were subcultured in blood agar with vancomycin 5Mg/ml (LYKA LABS LIMITED Gujart INDIA) and incubated anaerobically in an anaerobic jar at 37°C using gas generating kits for carbon dioxide (Micromaster laboratories PVT. LTD38/39, KalpataruInd Estate, Thane(W)MAH.INDIA) for 48 hours. The biochemical activities of the purified isolates were studied for identification and confirmation of the presence of *Actinobacillus actinomycetemcomitans*.

**DNA extraction by Guanidine chloride:**

The specimens were vortex and 1-2m µl lyses buffer was added to each sample. 5µl proteinase, K, 1ml guanidine chloride and 300- µl NH4 acetate were added and incubated at 37°C overnight. The samples were then cooled to room temperature and transferred to prechilled chloroform in 30 ml falcon tube, vortex and centrifuge for 5 minutes at 2500 rpm. The upper layer was collected to a new tube and 10 ml of cold absolute ethanol was added, shaken and kept at -20°C 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 was pored off and the pellet dried for 1-2 hours. The pellet was then re-suspended in 100 µl distilled water and stored at -20°C.

LKT primers (LKT2 AND lkt3) were used to identify *Actinobacillus —actinomycetemcommitans* targeting 16SrnRNA(Oligo MACROGEN Seoul,153-781 KOREA) used by Nao Suzuki 2001. as follows:
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.primer(2),1UTaq polymerase, 11µl distilled water and5 µl of template DNA. Negative and positive controls were included in each batch. The positive control consisted of 2 µl of genomic DNA with a concentration of 10.4 actinobacillusactinomycetemcomitans bacteria and the negative control was 2µl 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.

**Result:**
The result of culture was that 5% of the examined sites showed growth of white, translucent smooth non-hemolytic colonies in blood agar, colonies were sticky and adherent to the smooth surface. Gram stain technique and microscopic examination showed gram –ve short rods. The results of biochemical tests were negative because most of isolates did not ferment glucose, were urease positive, and voles proskaur negative. Most of isolates indole -ve, catalase and oxidase positive. Some of isolates hydrolyzed gelatin. The results of Multiplex PCR which is an effective method of detection of A. actinomycetemcomitans and its serotypes were negative. No bands were amplified after electrophoreses from all 30 pooled samples and even in all examined sites Fig (2). The results indicated that A. actinomycetemcomitans is not one of the causative micro-organisms among studied samples and accordingly serotypes were not detected. In the present study both methods showed identical results.

**Discussion:** Sub-gingival microflora consists of different bacterial species that can be detected by many techniques such as culture and multiplex PCR. In the present study primers designed to identify A. actinomycetemcomitans directly from clinical sample containing various micro-organisms was used. Since the result of the present study was negative for the presence of A. actinomycetemcomitans, this is in agreement with previous reports which did not support the association of actinobacillusactinomycetemcomitans with aggressive periodontitis; in some studies bacterial growth was observed in 6.2% of the samples, Mohammad Hossein et al (2004), in another study Aggregatibacter actinomycetemcomitans, was below the limit of detection or may not detected M.Faverir et al (2007) In some studies, actinobacillusactinomycetemcomitans was the least recovered micro-organisms, José Roberto Cortelli (a) (2010), also the result is in agreement with a previous study among Sudanese patients in which A. actinomycetemcomitans JP2 clone genotypes was not detected in the sub gingival plaque of either the cases or the controls. Elamin A et al (2011).

The result is not in agreement with previous studies by Christen LA(1993 ) in which Actiobacillusactinomycetemcomitans was present in 95% of the localized aggressive periodontitis patients and by Tonetti et al.YaMmoto (1999 ),Jose R Cortelli (2005), Wilson Rosalen (2006) ,Meng S, et al (2009),José Roberto (2010), Farshidk Kafilzideh (2010) in which Actiobacillusactinomycetemcomitans was present in healthy and diseased subjects.

**Conclusion:** Aggressive periodontitis patients in the present study were diagnosed according to clinical and radiographic features but the microbiological study for detection of A. actinomycetemcomitans, (which was considered as the main causative micro-organism in previous studies) indicated that the A. actinomycetemcomitans was not present in the examined samples using both culture and multiplex PCR, so its role in the pathogeneses of the disease may be questionable. Both techniques were equally effective in identification of A. actinomycetemcomitans.

**Recommendation:** Since the micro-organism Actinobacillusactinomycetemcomitans was not detected:
1- Investigations are needed for identification of other causative bacteria of aggressive periodontitis such as *P. gingivalis, P.intermedia, capnocytophaga, species* and *spirochetes* which may be more prominent in Sudanese aggressive periodontitis patients.

2- Studies on the role of genetic factors and the role of polymorph leucocytes in the etiology of this destructive disease are needed.

**ACKNOWLEDGEMENT**

I would like to thank Professor Ahmed Hassan Al Fahal Director of Mycetoma Research center and miss Nagwa for their great help in the lab of microbiology. Many thanks for Dr Shami and Miss Nosiba (Departement of Microbiology Faculty of Sciences El Neelain University). I wish to thank Dr Iklass Elkareem (Queen Elthbith University – Belfast) for her advice. Special thanks to Dr Sahar, Mr Abdalla Alser for their generous help in the field of molecular biology (Endemic institute).

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Figure 1 showed white translucent colonies in blood agar.
Fig (2): Agarose gel electrophoresis of PCR product from Subgingival plaque samples of 8 patients. Lane 1 to 8 represent 8 samples (all samples were negative), lane 10 a positive control which showed clear band, lane 11 a negative control.