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Prevalence of *Aggregatibacter actinomycetemcomitans* in Sudanese patients with aggressive periodontitis: a case–control study

A. Elamin[1], J. M. Albandar[2], K. Poulsen[3], R. W. Ali[4], V. Bakken[1]

[1]Section for Microbiology and Immunology, The Gade Institute, University of Bergen, Bergen, Norway, [2]Periodontal Diagnostics Research Laboratory, Department of Periodontology and Oral Implantology, Temple University School of Dentistry, Philadelphia, PA, USA, [3]Department of Medical Microbiology and Immunology, Aarhus University, Aarhus, Denmark and [4]Department of Periodontology, University of Science and Technology, Omdurman, Sudan

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Background and Objective: Aggregatibacter actinomycetemcomitans is considered a possible etiological agent for aggressive periodontitis. The aim of this study was to determine 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.

Material and Methods: In a previous study we examined a large representative sample of students attending high schools in Khartoum, Sudan. In this population, 17 patients with aggressive periodontitis and 17 controls (14–19 years of age) consented to participate in the present study. The subjects underwent a clinical periodontal examination, and subgingival dental plaque samples were collected using paper points. The presence of the *A. actinomycetemcomitans* JP2 clone and non-JP2 genotypes were assessed using loop-mediated isothermal amplification (LAMP) and the PCR.

Results: The JP2 clone of *A. actinomycetemcomitans* was not detected in the subgingival plaque of either the cases or the controls. Non-JP2 types of *A. actinomycetemcomitans* were detected in the subgingival plaque of 12 (70.6%) patients with aggressive periodontitis and from only one (5.9%) control subject, showing a significantly higher frequency of detection in cases than in controls ($p = 0.0001$). The odds ratio for the detection of *A. actinomycetemcomitans* in the subgingival plaque of the patients with aggressive periodontitis was 38.4 (95% confidence interval: 4.0–373.0; $p = 0.002$). The PCR and LAMP methods showed identical results pertaining to the identification of non-JP2 types of *A. actinomycetemcomitans*.

Conclusions: The JP2 clone of *A. actinomycetemcomitans* was not detected in the subgingival plaque of high school subjects in Sudan. 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.

Amal Elamin BDS, Section for Microbiology and Immunology, The Gade Institute, The Laboratory Building, Haukeland University Hospital, N-5021 Bergen, Norway
Tel: +47 92428911
Fax: +47 55974689
e-mail: Amal.elamin@gades.uib.no

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Aggregatibacter actinomycetemcomitans (formerly *Actinobacillus actinomycetemcomitans*) is a gram-negative facultative anaerobe that has been implicated in the pathogenesis of aggressive periodontitis (1,2). This bacterium is classified into seven serotypes: a, b, c, d, e, f and g (3–5). Serotype b strains exert a markedly high periodontal pathogenicity, possibly related to leukotoxic activity (6,7). A particularly virulent clone of *A. actinomycetemcomitans* serotype b, termed the JP2 clone after the first isolate of this type (8), is strongly associated with aggressive periodontitis (9–11). The JP2 clone of *A. actinomycetemcomitans* has a characteristic 530-nucleotide deletion in the promoter region of the leukotoxin operon, resulting in enhanced production of leukotoxin, and is therefore termed highly leukotoxic, in contrast to non-JP2 genotypes, which are minimally leukotoxic (12). Lack of a relevant animal model precludes definitive proof for the significance of elevated leukotoxin activity in the pathogenesis of aggressive periodontitis caused by the JP2 clone.

The JP2 clone of *A. actinomycetemcomitans* serotype b, as well as other genotypes representing different serotypes of *A. actinomycetemcomitans*, have been implicated in the initiation of periodontitis in children and adolescents (13–15). The prevalence of various serotypes of *A. actinomycetemcomitans*, and their association with periodontal disease, seem to vary among geographically and ethnically distinct populations (16–26). Notably, the JP2 clone shows a pronounced ethnical tropism, as it has been isolated almost exclusively from aggressive periodontitis adolescents of West and Northwest African descendants, including both Africans and Arabs (27,28).

It has been suggested that aggressive periodontitis may occur as a result of different etiological scenarios. In one instance, primarily found in adolescents of North- and West-African descendants, the highly leukotoxic JP2 clone was found to act as an exogenous pathogen. In another scenario, diverse commensal *A. actinomycetemcomitans* genotypes may play a role of opportunistic pathogens, if any role at all (27). In the latter scenario, other bacteria and/or the genetic constitution of the host (e.g. aberrant immune functions) may also contribute to the etiology of the disease. Conceivably, aggressive periodontitis depends on a number of risk factors, which together contribute to lowering the threshold for initiation of the disease (29).

The prevalence of aggressive periodontitis is high among African populations, with estimates ranging from 0.5 to 5% (30). In Sudan, the prevalence of aggressive periodontitis among school children is 3.4% (31). The high prevalence of this disease in African populations may be attributed to colonization of these patients by highly leukotoxic strains of *A. actinomycetemcomitans*. The Sudanese population is comprised of diverse African and Afro-Arab ethnic groups that may have increased susceptibility for colonization with certain bacterial strains (3).

The aim of the present study was to assess the presence of the JP2 clone and non-JP2 genotypes of *A. actinomycetemcomitans* in the subgingival plaque of aggressive periodontitis patients and healthy controls in Sudan, an East Saharan African nation.

Material and methods

Subjects

We have recently conducted a large survey of aggressive periodontitis in Sudanese students attending grades 9 to 11 (age 13–19 years) in high schools in the Khartoum metropolitan area, Sudan. The target population of the survey included more than 150,000 students from 744 high schools. Of these, we selected 1200 students from 38 high schools using a multi-stage, stratified sampling design, and these students were interviewed and examined clinically at their schools by one examiner (A. Elamin). A detailed description of the study sample and methods has been published elsewhere (31).

Aggressive periodontitis was defined as subjects having at least four teeth with interproximal sites showing ≥ 4 mm of attachment loss, or at least three teeth with interproximal sites showing ≥ 5 mm of attachment loss. A total of 41 patients with aggressive periodontitis were identified in this student population, a disease prevalence rate of 3.4%. Of these, 17 subjects (the cases) agreed to participate in the present study, a response rate of 41.5%. An interview with the subjects showed that the most frequent reason for nonresponse was a cultural belief that the study could lead to transmission of dangerous diseases. Dental fear was the second major reason for declining to participate. Seventeen subjects with no periodontal attachment loss (the controls) were randomly selected from the same classes after matching to cases on gender and ethnicity. Hence, a total of 34 subjects were included in the present study. Only consenting subjects, for whom parental consent had also been given, were included. Exclusion criteria included subjects who had used systemic antibiotics within the previous 3 mo, a history of periodontal treatment or having an acute infection. The subjects included 15 male and 19 female participants, and ranged in age between 14 and 19 years.

Ethical consideration

The protocol was reviewed by ethical committees at the following institutions in Sudan: the University of Science and Technology (UST), Omdurman; the Ministry of Health; and the Ministry of Education. Written consent was obtained from each of the participants and from their parents/guardians, as well as from school principals. Study participants were informed of the study objectives and the importance of the findings.

Plaque samples

Four subgingival plaque samples per subject were collected from the deepest periodontal pocket; one sample from each quadrant. The sites were isolated with cotton rolls, the supragingival deposits were carefully removed with sterile periodontal curettes and

Table 1. Primers used for detection of *Aggregatibacter actinomycetemcomitans* by loop-mediated isothermal amplification (LAMP)

Target	Primer	Sequence
Primers for detection of 16S ribosomal RNA genes of *A. actinomycetemcomitans*[a]	FIP	5′-CCCCACGCTTTCGCACATCATACCGAAGGCGAAGGCAG-3′
	BIP	5′-AGATACCCTGGTAGTCCACGCTTTCGGGCACCAGGGCTAAAC-3′
	F3	5′-TGCGTAGAGATGTGGAGGAA-3′
	B3	5′-GGCGGTCGATTTATCACGT-3′
	LB	5′-AAACGGTGTCGATTTGGGGAT-3′
Primers for detection of JP2 clone of *A. actinomycetemcomitans*[b]	F3	5′-CCGTTTTATTCAGTTCCCAAG-3′
	B3	5′- TCTCCATATTAAATCTCCTTGTT-3′
	FIB	5′-ACGATTTGTGCTAATCTTACCGTTACTCTATGAATACTGGAAACTTGT-3′
	BIP	5′-TCTCGGCGAAAAAACTATTTGGAATTTGAATAAGATAACCAAACCAC-3′
	LF	5′-ACTAACCTTTTGTACAAAATTCTGA-3′
	LB	CCAAAGTACTTTTAATGATGGCA-3′

[a]Primers for detection of 16S ribosomal RNA genes of *A. actinomycetemcomitans*, as described by Miyagawa *et al.* (36).
[b]Primers for detection of the JP2 clone of *A. actinomycetemcomitans*, as described by Seki *et al.* (35).

then two sterile paper points (#35; Zipperer Co., Munich, Germany) were placed in the periodontal pocket and kept there for 10 s. The paper points from each participant were placed in one vial containing 2 mL of Viability Medium Göteborg Anaerobically prepared III transport medium (Sahlgrenska Academy, University of Gothenburg, Sweden) (32,33). Samples were transported to the laboratory of the Section for Microbiology and Immunology, The Gade Institute (University of Bergen, Bergen, Norway), and were stored at −80°C for subsequent DNA isolation.

Bacterial strains and culture conditions

A. actinomycetemcomitans strains JP2 (CCUG 56172) and Y4 (ATCC 43718) were used as positive controls in the PCR and in loop-mediated isothermal amplification (LAMP). The two strains were cultured on fastidious anaerobic blood agar plates (Lab M, Bury, UK), which were incubated anaerobically (5% CO_2, 10% H_2 and 85% N_2) using the Anoxomat system (MART Microbiology BV, Lichtenvoorde, the Netherlands) at 37°C for 4–5 d.

DNA extraction

DNA templates for PCR and LAMP obtained from subgingival plaque samples were extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Duren, Germany). Briefly, the samples were thawed at room temperature, paper points were transferred to tubes containing 180 μL of buffer T1 (lysis buffer) from the NucleoSpin Tissue kit, and, after homogenization by vortexing for 60 s, the DNA was prepared according to the protocol. DNA from the two cultured strains was purified using the E.Z.N.A. Bacterial DNA kit (Omega Bio-Tek, Norcross, GA, USA). The quantity and quality of DNA were determined by measuring the absorbances of the samples at 260 and 280 nm and by electrophoresis on a 1% agarose gel.

PCR

The primers *ltx3* and *ltx4* were used to amplify the promoter region of the leukotoxin gene operon, as previously described (34). These primers amplify a 686-bp product characteristic of the highly leukotoxic JP2 clone and a 1216-bp amplicon from other genotypes of *A. actinomycetemcomitans*. PCR was performed in a volume of 25 μL using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Bucks, UK) containing 23 μL of sample and 10 pmol each of two primers. PCR was performed using a Gene Amp PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). The thermocycling program was as follows: denaturation for 5 min at 94°C and 30 cycles of 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 8 min. The purity and size of the amplification products were analyzed by electrophoresis on a 1% agarose gel.

LAMP

The primers used in the LAMP reactions are listed in Table 1. One set of primers used for LAMP specifically detects the highly leukotoxic JP2 clone, and the second set targets sequences of the 16S ribosomal RNA genes of *A. actinomycetemcomitans* and specifically amplifies DNA fragments from all strains of the species, as shown previously (35,36). LAMP reactions were performed in a 25-μL reaction mixture containing 1.6 μM each of the inner primers FIP and BIP, 0.2 μM of outer primers F3 and B3, 0.4 μM of loop primers LF and LB, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs, Ipswich, MA, USA), 1.4 mM each of the four deoxynucleoside triphosphates, 0.8 M betaine (Sigma, St Louis, MO, USA), 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween-20 and template DNA in a volume of up to 5 μL, as previously described (35). The mixture was incubated at 63°C for 60 min and then heated at 80°C for 2 min to terminate the reaction. The reactions were analyzed by visual inspection and by agarose-gel electrophoresis. There was complete agreement between the two methods.

Data analysis

Ethnicity was categorized into Afro-Arab and African tribes. The Mantel–Haenszel chi-square test and the Fisher's exact test were used for the

comparison of frequencies between the groups, and the Student's *t*-test was used for comparison of means. Data analysis was performed using the statistical package SAS (v9.2; SAS Institute Inc., Cary, NC, USA). The odds ratio and the 95% confidence interval were calculated, and the significance was tested using the Wald test.

Results

Seventeen subjects with aggressive periodontitis and 17 healthy controls, who were previously identified in a large survey of aggressive periodontitis among high school students in Sudan, were included in this study. The cases and controls had similar demographics, with no significant differences in mean age, gender or ethnicity (Table 2).

A PCR method was used that detects *A. actinomycetemcomitans* and distinguishes between the JP2 clone and other genotypes (Fig. 1). In addition, two LAMP methods – one that detects all *A. actinomycetemcomitans* strains and one that specifically detects the JP2 clone – were applied (Figs 2 and 3). The results of the different methods showed complete concordance. The JP2 clone of *A. actinomycetemcomitans* was not detected in the subgingival plaque of either the cases or the controls. Non-JP2 types of *A. actinomycetemcomitans* were detected in 12 (70.6%) patients with aggressive periodontitis but in only one (5.9%) control subject (Table 3), showing a significantly higher frequency of detection in cases than in controls ($p = 0.0001$). The odds ratio of the detection of *A. actinomycetemcomitans* in the subgingival plaque of the subjects with aggressive periodontitis was 38.4 (95% confidence interval: 4.0–373.0; $p = 0.002$). The detection frequencies were higher in male than in female study participants, and in Afro-Arabs than in African tribes, although the differences were not statistically significant (Table 3).

Discussion

In this study, subgingival plaque samples were collected from subjects with aggressive periodontitis and healthy

Table 2. Demographics of the cases and controls

	Aggressive periodontitis ($n = 17$)	Control ($n = 17$)	Total	*p*-value
Age: mean (SD)	15.4 (1.7)	15.7 (1.7)		0.6
Gender				
Male: *n* (%)	8 (53.3)	7 (46.7)	15	0.7
Female: *n* (%)	9 (47.4)	10 (52.6)	19	
Ethnicity[a]				
Afro-Arab: *n* (%)	12 (48.0)	13 (52.0)	25	0.7
African: *n* (%)	4 (50.0)	4 (50.0)	8	

[a]One subject was excluded from the table because the ethnicity was unknown.
SD, standard deviation.

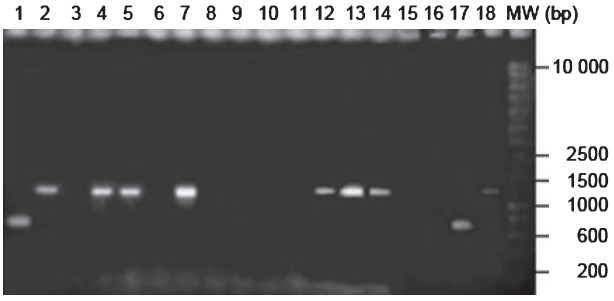

Fig. 1. Detection of *Aggregatibacter actinomycetemcomitans*, with and without the 530-bp deletion, from subgingival plaque samples by PCR using primers *ltx3* and *ltx4*. Lanes 1 and 17, control 1: CCUG 56172 (=JP2); lanes 2 and 18, control 2: ATCC 43718 (=Y4); lane 3, negative control (water); lanes 9 and 16 are empty; lanes 4, 5, 7, 12, 13 and 14 are clinical samples positive for non-JP2 types of *A. actinomycetemcomitans* in both PCR and loop-mediated isothermal amplification (LAMP); and lanes 6, 8, 10, 11 and 15 are clinical samples negative for *A. actinomycetemcomitans* in both PCR and LAMP. The PCR products were electrophoretically detected on a 1% agarose gel and stained by ethidium bromide. A DNA ladder, with molecular weight (MW) in base pairs, is indicated to the right (Hyperladder I; Bioline, London, UK).

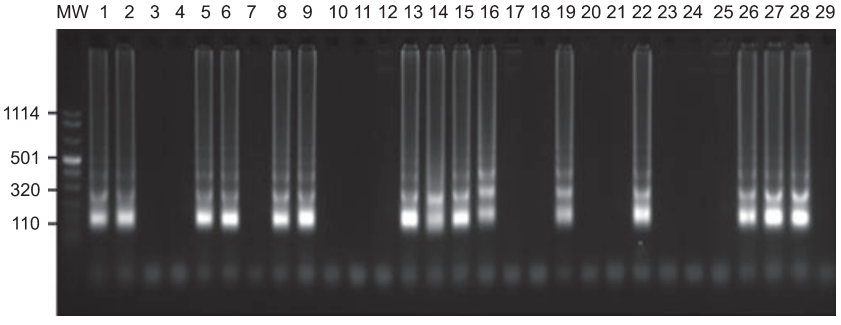

Fig. 2. Detection of non-JP2 types of *Aggregatibacter actinomycetemcomitans* in subgingival plaque samples using loop-mediated isothermal amplification (LAMP). The DNA template was prepared from the plaque samples and subjected to analysis by both PCR and LAMP. For electrophoresis, 5 μL of the reaction mixture was loaded onto a 3% agarose gel and stained by ethidium bromide. Lane 1, positive control strain ATCC 43718 (=Y4); lane 29, negative control *Porphyromonas gingivalis* ATCC 33277; lane 3, water; lanes 2, 5, 6, 8, 9, 13, 14, 15, 16, 19, 22, 26, 27 and 28 are clinical samples that are positive for the non-JP2 types of *A. actinomycetemcomitans* in both LAMP and PCR; and lanes 4, 7, 10, 11, 12, 17, 18, 20, 21, 23, 24 and 25 are clinical samples that are negative for *A. actinomycetemcomitans* in both LAMP and PCR. A DNA ladder, with molecular weight (MW) in base pairs, is indicated to the left (Roche Diagnostics, Mannheim, Germany).

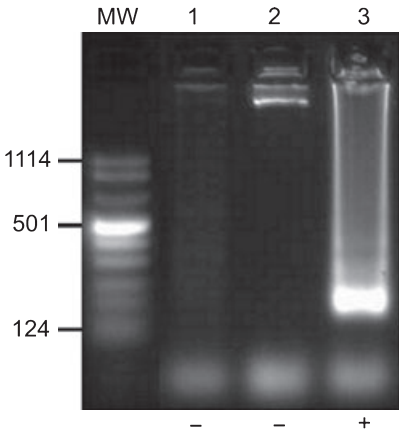

Fig. 3. Testing the specificity of loop-mediated isothermal amplification (LAMP) primers that specifically detect the JP2 clone of *Aggregatibacter actinomycetemcomitans*. LAMP products were separated by electrophoresis on a 3% agarose gel and stained by ethidium bromide. Lane 1, water; lane 2, negative control strain ATCC 43718 (=Y4); lane 3, positive control strain CCUG 56172 (=JP2). A DNA ladder, with molecular weight (MW) in base pairs, is indicated to the left (Roche Diagnostics, Mannheim, Germany).

controls in Sudan, transported to Europe, stored at −80°C and analyzed by PCR and LAMP techniques for the presence of *A. actinomycetemcomitans*, including specific detection of the JP2 clone (34–36). The methods were previously shown to be highly specific and sensitive, and, in this study, the PCR and LAMP results were in complete agreement. The samples were stored in frozen medium before further analysis and this may have reduced the sensitivity of the detection method as a result of the lysis of bacteria. On the other hand, DNA was extracted from the samples before performing PCR and LAMP, and this may have improved the detection limit compared with previous studies where the samples were boiled (34,35). The agreement between the results of the PCR and LAMP methods indicates that the detection limit was adequate in this study.

The JP2 clone of *A. actinomycetemcomitans* was not detected in either the cases or controls in this Sudanese population, suggesting that the JP2 clone does not show a general dissemination pattern among various African populations. Absence of the JP2 clone in East Africa is in agreement with a previous study where this particular clone was not detected among clinical isolates from 12 subjects from Kenya, Tanzania and Somalia (28). Population genetic analyses indicated that the JP2 clone of *A. actinomycetemcomitans* originated in the Mediterranean part of Africa perhaps 2400 years ago and has subsequently spread to West Africa and further, to the American continents during the Transatlantic slave trade (37). In a recent study on the genetic diversity of African human populations, it was shown that the indigenous population in Saharan Africa are genetically quite distinct from those in Western and Central Africa, indicating that a reported historic migration from West Africa to the East-Saharan area has been very limited (38). Thus, absence of the JP2 clone in East Africa, including Sudan, is in agreement with the proposed scenario of origin and spread of the clone. However, African populations are genetically very diverse (38), and genetic differences may result in differences in susceptibility for colonization by certain microorganisms.

In this cross-sectional study, non-JP2 types of *A. actinomycetemcomitans* were detected predominantly (70.6%) from the subgingival plaque of patients with aggressive periodontitis, whereas only one subject (5.9%) without periodontitis also tested positive for the microorganism. Although the sample size was small, the odds ratio for demonstrating the presence of non-JP2 types of *A. actinomycetemcomitans* in the subgingival plaque of the patients with aggressive periodontitis was 38.4 (95% confidence interval: 4.0–373.0), suggesting a strong association between subgingival colonization above the detection limit with non-JP2 types of the bacterium and the diagnosis of aggressive periodontitis in this Sudanese population. These results are in contrast to studies performed in a Moroccan population, which showed only a weak or no association between the presence of non-JP2 types of *A. actinomycetemcomitans* and aggressive periodontitis among adolescents (15,39). This difference may suggest that in Sudan other pathogenic strains of *A. actinomycetemcomitans* are present, or that differences in human host factors exist between the two populations. However, in this study, detection of the bacterium was based on non-culture techniques and therefore further studies are needed to test these hypotheses.

Current advances in periodontal molecular technology are increasingly adding to our understanding of periodontal infections. Accordingly, it is currently believed that periodontal destruction is caused by a number of periodontopathic microorganisms, rather than by one microorganism solely, and is mediated by inflammatory and immunological host responses (40,41). A substantial number of studies have incriminated bacteria as key putative pathogens, namely

Table 3. Detection frequencies of the non-JP2 types of *Aggregatibacter actinomycetemcomitans*, by disease groups, gender and ethnicity

	Non-JP2 types of *A. actinomycetemcomitans*		
	n (%)	Total	*p*-value
Disease group			
Aggressive periodontitis	12 (70.6)	17	0.0001
Control	1 (5.9)	17	
Gender			
Male	7 (46.7)	15	0.37
Female	6 (31.6)	19	
Ethnicity[a]			
Afro-Arab	10 (40.0)	25	0.26
African	2 (25.0)	8	

[a]One subject was excluded from the table because the ethnicity was unknown.

A. actinomycetemcomitans, Porphyromonas gingivalis and *Tannerella forsythia* (41–43). Recently, more diverse putative microorganisms are considered to play a role in aggressive periodontitis, including pathogens in the *Archaea* domain and herpes viruses (44,45). However, the precise identity of the casual pathogens is still a challenging task that is confused by the complexity of the oral biofilms and population differences with regard to ethnic, environmental and genetic make-up of the host (46,47). A putative exception from the mixed-plaque hypothesis is infection with the highly leukotoxic JP2 clone of *A. actinomycetemcomitans* (15,39).

In conclusion, the observed association between the presence of *A. actinomycetemcomitans* and aggressive periodontitis implies that *A. actinomycetemcomitans* may be useful as a marker of increased risk for developing aggressive periodontitis in this young Sudanese population.

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