Clin Oral Invest (2016) 20:1809–1818
DOI 10.1007/s00784-015-1653-7

ORIGINAL ARTICLE

Characterization of *A. actinomycetemcomitans* strains in subgingival samples from periodontitis subjects in Morocco

M. Mínguez[1] · O. K. Ennibi[2] · X. Pousa[1] · L. Lakhdar[2] · L. Abdellaoui[2] · M. Sánchez[3] · M. Sanz[1,4] · D. Herrera[1,4]

Received: 15 March 2015 / Accepted: 9 November 2015 / Published online: 23 November 2015

Abstract
Objective *Aggregatibacter actinomycetemcomitans*, specially its highly leucotoxic strain (JP2 clone), represents an etiological factor for the onset and progression of aggressive types of periodontitis. The aims of this investigation were to investigate the most relevant periodontal pathogens in the subgingival microbiota of periodontitis patients from Morocco and to describe the clinical and microbiological characteristics of subjects positive for *A. actinomycetemcomitans*, including serotype, leukotoxin gene, and operon of the cytolethal distending toxin (*cdt*) distribution.
Material and methods In consecutive Moroccan subjects diagnosed of periodontitis, subgingival samples were taken and processed by culture. From the positive samples for *A. actinomycetemcomitans*, one to three isolates were subcultured and characterized by means of polymerase chain reaction (PCR), assessing their specific serotype distribution, the variation in the sequences of the leukotoxin gene, and the operon of the *cdt*.
Results Twenty-one (35.6 %) out of 59 periodontitis patients harbored *A. actinomycetemcomitans*. These patients demonstrated statistically significant deeper pockets ($p = 0.035$) and higher proportions of *P. micra* ($p = 0.045$) than did the negative group. The 39 studied isolates were serotype "b"; in 16 out of 17 patients, there was mono-colonization with this serotype. Five isolates, from two patients, presented the 530-bp deletion in the leukotoxin's promoter region. Thirty-two isolates (78 % of the strains) were *cdt*-positive.
Conclusion *A. actinomycetemcomitans* was frequently found (35.6 %) in our sample. All strains were serotype "b," and most (78 %) were also *cdt*-positive. The JP2 strain type was only detected in 12.2 % of the strains.
Clinical relevance *A. actinomycetemcomitans* can be frequently found in Morocco. This fact can influence the therapeutic approach of this type of patients.

Keywords *Aggregatibacter actinomycetemcomitans* · Subgingival microbiota · Periodontitis · Morocco · Polymerase chain reaction

✉ D. Herrera
davidher@odon.ucm.es

[1] Section of Periodontology, Faculty of Odontology, University Complutense, Plaza Ramón y Cajal S/N, 28040 Madrid, Spain

[2] Clinical Department of Periodontology, Dental School, Mohammed V Souissi University, Rabat, Morocco

[3] Laboratory of Research, Faculty of Odontology, University Complutense, Madrid, Spain

[4] ETEP (Etiology and Therapy of Periodontal Diseases) Research Group, University Complutense, Madrid, Spain

Introduction

Periodontitis is a chronic inflammatory disease caused by bacteria present in the subgingival biofilm. This disease is characterized by loss of the connective tissue attachment and the supporting alveolar bone and if untreated can eventually progress to tooth loss. This biofilm consists of a complex structure of bacterial communities adhered to the tooth surface in a well-protected subgingival environment conferring the bacteria-specific resistance properties, pathogenic synergism, and reduced susceptibility to antibiotics that allows the overgrowth of pathogenic bacterial species [1].

From the more than 700 bacterial species detected in the biofilm, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* have been considered the most important pathogens, since they

have shown a strong level of evidence in their association with periodontitis. [2] *A. actinomycetemcomitans* has been specifically implicated in the etiology of the aggressive forms of periodontitis, especially in populations of North and West African origin, where these aggressive forms of periodontitis are highly prevalent [3, 4].

This pathogen has evidenced a high degree of variability, both in terms of geographical prevalence and in the presence of virulence factors (serotype distribution and toxins, mainly leukotoxin and cytolethal distending toxin, Cdt), which has resulted in different patterns of disease association [3, 5]. *A. actinomycetemcomitans* has been characterized in six different clonal lineages representing the different serotypes (from "a" to "f") and one untyped strain (serotype "g") [6]. The distribution pattern of the serotypes has been associated with different diseases and with different patterns of colonization depending on the geographical region and patient's ethnicity [7].The most frequently detected serotypes are "a," "b," "c," and "e," while the serotypes "d" and "f" are rarely detected [8, 9].

One of the most studied virulence factors of *A. actinomycetemcomitans* is the secretion of a potent leukotoxin, which specifically destroys human polymorphonuclear leukocytes [10]. While most strains express relatively low levels of this leukotoxin, there are variations among the different isolates, and one specific hyperleukotoxic phenotype has been isolated and characterized by a 530-base pair deletion in the leukotoxin gene operon (*lxt*) encoding the toxin [10, 11]. The vast majority of strains of this highly leukotoxic clone, also known as the JP2 clone, have been isolated from subjects of African origin. In a population-based longitudinal study of 700 adolescents in Morocco, there was a direct etiological relationship between the presence of JP2 strains of *A. actinomycetemcomitans* and the initiation or progression of aggressive periodontitis [12]. This pathogen also produces a Cdt, which triggers DNA damage, mainly by its CdtB, the subunit that exhibits both type I deoxyribonuclease-like and phosphatase activities. Some specific classes of eukaryotic cells and cell lines, mainly oral epithelial cells and T lymphocytes, are susceptible to this toxin, resulting in the arrest of their cell cycle. Different studies have shown that not all *A. actinomycetemcomitans* strains present this cytotoxic activity [13].

In spite of this demonstrated strong association with the presence of *A. actinomycetemcomitans* and periodontitis in Morocco, there is only one study that has characterized the subgingival microbiota of a Moroccan population and has shown a clear association between disease severity and the most virulent clones (JP2-positive) of this pathogen [14]. There are, however, no studies assessing the microbiota and disease severity of patients negative for *A. actinomycetemcomitans* or the presence of other virulence factors in patients positive for *A. actinomycetemcomitans*. This investigation, therefore, was aimed, firstly, to investigate the most relevant periodontal pathogens in the subgingival microbiota of periodontitis patients in Morocco, comparing those with and without *A. actinomycetemcomitans*, and, secondly, to assess in the isolated *A. actinomycetemcomitans* strains, the presence of virulence factors, the serotype distribution, the operon of the *cdt*, and the variation in the sequences of the genes that codify the leukotoxin.

Material and methods

Patients

Microbiological subgingival samples were collected from a convenience sample of consecutive patients diagnosed of periodontitis and seeking therapy at the Dental School in the Mohammed V, Souissi University, in Rabat. All included patients were Moroccan. All samples were taken from patients as part of their periodontal diagnostic process, and they were informed about the benefits of the microbiological diagnosis, an overview of the microbiological procedures, and that, eventually, some of the cultured colonies could be isolated for further processes, including definitive identification. Verbal informed consent was obtained from all individual participants included in the study. The study protocol was approved by The Biomedical Research Ethical Committee at the University Mohammed V Soussi of the Faculty of Medicine and Pharmacy of Rabat (reference number 400/2010).

Every selected patient satisfied the following criteria:

- 1. Being systemically healthy, without any acute pathology.
- 2. Having at least 16 teeth, at least three on every quadrant, to make possible the proposed microbiological sampling strategy.
- 3. Having at least one site with probing pocket depth (PPD) > 4 mm in each quadrant.

Subjects were excluded if pregnant or in lactation or when on any medication relevant for the study, such as intake of systemic antibiotics within the last 3 months.

These patients were clinically examined after taking a brief medical and smoking history. Using the criteria of the American Academy of Periodontology classification, each patient was diagnosed as chronic or aggressive periodontitis [15]. Since the criteria of rapid loss of attachment and the bone destruction were difficult to evaluate due to the lack of previous records in the present population, an estimation of rapid progression of the disease was made taking into account the actual destruction and the age of the patient. A separate group of patients was classified as "refractory" periodontitis,

including patients previously treated and demonstrating relapsing periodontitis, as defined in the inclusion criteria.

Four diseased sites were selected for microbiological sampling. In these selected sites, the presence of dental plaque (PlI), bleeding on probing (BOP), suppuration, PPD, and recession was recorded by two trained investigators (XP and OE).

Microbiological sampling

Subgingival microbiological samples were taken from the deepest pocket in each quadrant [16]. At these sites, and once supragingival plaque was carefully removed with one sterile gauze to avoid bleeding and isolated with sterile cotton rolls and gentle air-drying, two consecutive sterile paper points (medium size, Maillefer, Ballaigues, Switzerland) were inserted as deep as possible in the pocket and left in place for 10 s. The paper points were then transferred to a vial containing 1.5 mL of reduced transport fluid (RTF) and pooled with all the other paper points from the same patient [17]. Samples were transported for processing to the Laboratory of Microbiology at the Faculty of Odontology, University Complutense, Madrid, Spain, within 24 h from the time of sampling.

Culture, isolation, and storage

Samples were homogenized by vortexing and were serially diluted in phosphate-buffered saline (PBS). Aliquots of 100 μL were plated in two different media: blood agar medium (No. 2 of Oxoid; Oxoid Ltd, Basingstoke, England), with 5 % horse blood and haemin (5 mg/L) and menadione (1 mg/L), and Dentaid-1 medium [18].

The blood agar plates were studied after 7 and 14 days of anaerobic incubation (80 % N_2; 10 % H_2; 10 % CO_2 at 37 °C) and the specific pathogens *P. gingivalis*, *Prevotella intermedia/nigrescens*, *T. forsythia*, *Parvimonas micra*, *Capnocytophaga* spp., *Eikenella corrodens*, and *Fusobacterium nucleatum* were identified based on the morphology of the colony. Colonies of each bacterial species were counted as the total number of colonies in a representative plate (between 30 to 300 colonies). The Dentaid-1 plates, used for selective isolation and growth of *A. actinomycetemcomitans*, were incubated at 37 °C in air with 5 % CO_2 and after 3–5 days were carefully examined for the identification of *A. actinomycetemcomitans*, based on its typical colony morphology, a positive catalase reaction, and a set of specific enzymes (RapID NH system Remel Inc., USA). From each patient positive for *A. actinomycetemcomitans*, and in order to evaluate the intra-individual variability, one to three strains were adequately isolated (up to 10 passes per strain) and stored at −80° until further use.

Isolation of genomic DNA

Cells were harvested by centrifugation and re-suspension in 1 mL of Tris–HCl 10 mM, ethylenediaminetetraacetic acid (EDTA) 0.8 mM (ph 8.0), and lysozyme (final concentration 5.0 mg/mL). After incubation at 37 °C for 30 min, proteinase K was added to a final concentration of 2 mg/μL, 1 mg/μl, and 1 % respectively. The mixture was incubated at 37 °C for 30 min. The DNA was then extracted with equal volumes of phenol (saturated with 10 mM Tris–HCl, pH 8.0) and phenol–chloroform–isoamyl alcohol (25:24:1). Bulk nucleic acids were precipitated from the solution using ethanol followed by centrifugation (12,000 rpm) for 10 min. The DNA precipitate was re-suspended in 50 μL of sterile distilled water. The result of the DNA extraction was assessed by electrophoresis. A 5-μL aliquot of the extracted DNA was electrophoresed through a 1.0 % agarose gel, in a Tris-acetate-EDTA (TAE) buffer [19]. The gel was stained with ethidium bromide (10 μg/ml) and visualized under UV illumination, to confirm the identity of the species that were previously morphologically identified by culture.

Serotyping of strains

Serotypes “a” to “f” were determined by polymerase chain reaction (PCR) technique, based on specific sequences from the gene clusters responsible for the distinct serotypes described by Kaplan and coworkers, listed in Table 1 [20].

The PCR reaction was performed in a 25-μL final volume containing 2.5 μL of 10× PCR buffer, 1.0 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM deoxynucleoside triphosphates, 0.5 U Taq DNA polymerase (Invitrogen Corporation, CA, USA), 1.0 μL of 25 μM primers, and 2 μl of genomic DNA. A 15-μL aliquot of each PCR was electrophoresed through a 1.0 % agarose gel in 1× TAE buffer; the PCR products were visualized by staining with ethidium bromide (10 mg/mL) and visualized under UV illumination. The PCR assays were performed with an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc). After the initial step of denaturation at 96 °C for 3 min, a total of 35 PCR cycles were performed; each cycle consisted of 30 s of denaturation at 95 °C, 1 min of annealing at 55 °C and 72 °C for 2 min, and a final step of extension at 72 °C for 10 min. Positive and negative controls were included.

Detection of putative leukotoxin overproducers

The deletion of 530 bp in the promoter region of the leukotoxin gene was determined in every isolate by means of PCR. The PCR primers and conditions for detecting the JP2 strain were those described by Haubek [21]. The PCR reaction was performed in a 25-μL final volume containing 2.5 μL of 10× PCR buffer, 1.0 μL of 25 mM $MgCl_2$, 0.5 μL of

Table 1 Primers described by Kaplan et al. (2002) selected for the PCR serotyping of *A. actinomycetemcomitans*

Primer	Sequence (5′–3′)	Serotype	PCR product size(bp)
P11	TCTCCACCATTTTTGAGTGG	b	333
P12	GAAACCACTTCTATTTCTCC	c	268
P13	CCTTTATCAATCCAGACAGC	f	232
P15	TGGGTCATGGGAGGTACTCC	a	293
P16	GCTAGGAACAAAGCAGCATC		
P17	TGGAACGGGTATGGGAACGG	d	411
P18	GGATGCTCATCTAGCCATGC		
P19	ATTCCAGCCTTTTGGTTCTC	e	311
P20	TGGTCTGCGTTGTAGGTTGG		

bp base pair

10 mM deoxynucleoside triphosphates, 0.5 U Taq DNA polymerase (Invitrogen Corporation, CA, USA), 1.0 μL of 25 μM primers, and 2 μL of genomic DNA.

The PCR primer upstream from the deletion had the sequence 5′-CAGATCAAAACCTGATAACAGTATT-3′ and the primer downstream from the deletion had the sequence 5′TTTCTCCATATTCCCTCCTTCTGT-3′.The PCR temperature profile included an initial step of denaturation at 94 °C, a total of 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, and a final step of extension at 72 °C for 2 min. The PCR fragment of 504 bp indicated deletion of 530 bp corresponding to the JP2 strain whereas a PCR fragment of 1034 bp indicated no deletion in the *A. actinomycetemcomitans* leukotoxin operon. Positive and negative controls (both previously tested in our laboratory) were included in the PCR assays. The molecular weights of the PCR products were determined by visualization and comparison with standard molecular weight markers using agarose gel electrophoresis.

cdt detection

The presence of the operon that codifies for the Cdt was also determined by means of PCR using the same procedure previously described for the leukotoxin but using the primers that amplify the complete operon CdtA1 and CdtC2, which are listed in Table 2. The total molecular weight of the amplified segment was of 2016 bp when the complete operon was present. The PCR products were also determined by visualization in 1.0 % agarose gel.

Statistical analysis

Patients were divided into positive of negative depending whether *A. actinomycetemcomitans* was detected. The clinical and microbiological findings were compared between these two groups by means of unpaired *t* test. Variables were first evaluated for normal distribution by assessing the skewness and kurtosis of the distribution, and microbiological variables (proportions of microbiota and bacterial counts) were previously log-transformed. Frequencies of detection were compared using the chi-square test. The microbiological findings were also compared depending on the clinical diagnosis: chronic, aggressive, or refractory periodontitis patients.

In *A. actinomycetemcomitans*-positive patients, descriptive statistics, including frequency distribution, were used to characterize the samples in terms of the different serotypes and the variation of the sequences of the genes that codify the leukotoxin and the operon of the *cdt*. Contingency tables were constructed, and comparisons were performed using the chi-square test.

Results

A total of 59 periodontitis patients were included in the study and provided subgingival microbiological samples. Their mean age was 27.6 years (range 12–55), and the percentage of females was 66.1 %. Most patients were non-smokers (Table 3).

Complete demographic and clinical data were available from 45 patients; correlations between clinical and microbiological variables were done only for those patients with complete data available, while 59 samples were only considered for the assessment of microbiological profiles.

From the 45 patients with complete clinical and demographic data, 12 were diagnosed of chronic periodontitis (20.34 %), 32 (54.24 %) of aggressive periodontitis, and one (1.7 %) as refractory periodontitis. The clinical data of this population is depicted in Table 4, with a mean PPD of 6.2 mm, a mean recession of 0.85 mm, and mean percentages of BOP, plaque index (PlI), and suppuration of 82, 78, and 21 %, respectively.

Tables 5, 6, and 7 show the microbiological results. The mean bacterial total count (expressed as total colony forming units [CFU], per milliliter) for the 59 samples was 1.6×10^7. Twenty-one of them (35.6 %) were positive for *A. actinomycetemcomitans*, with a mean count of 6.5×10^3

Table 2 Primers used for the detection of the presence of the *cdt* operon of *A. actinomycetemcomitans* (designed in this study)

Primer	Sequence (5′–3′)	Gen	PCR product size (bp)
$CdtA_1$	ATGAAGAAGTTTTTACCTGGTC	CdtA	669
$CdtA_2$	TAATTAAGGGGTCAAGCTTCT		
$CdtB_1$	TGCAATCCCATTTCGAATTA	CdtB	851
$CdtB_2$	TTAGCGATCACGAACAAAAC		
$CdtC_1$	CTTTAGGTACATGTATTGAA	CdtC	560
$CdtC_2$	TTAGCTACCCTGATTTC		

and a mean proportion of the total microbiota of 0.1 %. The mean detection of the other pathogens was higher, including *P. gingivalis* (84.7 %), *F. nucleatum* (98.3 %), and *P. intermedia* (94.9 %), representing the mean proportions of these species between the 10 and the 20 % of the total bacteria counts in both the *A. actinomycetemcomitans*-positive and *A. actinomycetemcomitans*-negative patient group, except for *F. nucleatum* in the first group, where the proportion was 2.2 % (Table 6). The frequency of detection of these species was high in both groups, where the values were 90.48 and 81.58 % for *P. gingivalis* and 100 and 97.37 % for *F. nucleatum*, while the frequency values for *P. intermedia* were 85.71 and 100 %, respectively (Table 7).

Characterization of *A. actinomycetemcomitans*-positive patients

The 21 patients positive for *A. actinomycetemcomitans* had a mean age of 26.6 years (ranging between 13 and 46). Their mean PPD was 6.96 mm and the recession 0.76 mm. The mean values for BOP, PlI, and suppuration were 79 %, 75 %, and 20 %, respectively (Table 4).

The mean total bacterial count was 1.8×10^7, the mean count of *A. actinomycetemcomitans* was 1.8×10^4, and the mean proportion was 0.3 % (Tables 5 and 6). Patients positive for *A. actinomycetemcomitans* presented frequently *F. nucleatum* (100 %) or *P. gingivalis* (90.4 %) (Table 7).

The comparison between the 38 *A. actinomycetemcomitans*--negative and the 21 *A. actinomycetemcomitans*-positive patients revealed statistically significant differences in terms of higher mean counts of *P. micra* ($p = 0.045$) and deeper mean PPD ($p = 0.035$), in the *A. actinomycetemcomitans*-positive group. No other statistically significant differences were detected, and very similar levels of BOP, PlI, and SUP were observed. In addition, more mean recession was measured in the *A. actinomycetemcomitans*-negative group.

From the 21 *A. actinomycetemcomitans*-positive patients, 41 strains were characterized in 17 patients: except one patient, all provided 2 or 3 strains. Most of the patients (88.2 %) had the leukotoxin gene (ltx 1034), while only two patients (11.8 %) had the JP2 clone (Table 8).These two patients were women suffering from aggressive periodontitis. Samples from one of the patients were positive for *P. gingivalis*, *T. forsythia*, *P. intermedia*, and *F. nucleatum*, and others were positive for *P. micra*. The bacterial total counts ranged between 10^6 and 10^7 and the mean PPD between 7.75 and 8 mm, deeper than the mean PPD for the *A. actinomycetemcomitans*-positive patients group (6.96 mm).

Of the patients, 64.7 % were characterized as *cdt* gene-positive. All patients, except one, were characterized as "b" serotype. This particular patient was characterized by "a" and "e" serotypes (Table 8).

From the 41 strains, only five (12.2 %) were defined as JP2 clone, while 36 were characterized as ltx 1034. *cdt*-positive gene predominated (78 %) over *cdt*-negative gene (22 %). All the strains, except two, were serotype "b" (95.1 %), while these two were "a" and "e" (Table 8).

Regarding the genetic distribution of the *A. actinomycetemcomitans* strains, every JP2 clone strain ($n = 5$) was serotype b and *cdt*-positive; 27 out of 36 (75 %) of the ltx 1034 strains were *cdt*-positive; and only two strains were serotype "a" and "e," both *cdt*-negative and ltx 1034 (Table 9).

Discussion

In this population of Moroccan patients with periodontitis, the presence of *A. actinomycetemcomitans* was a relatively frequent finding (35.6 %), and the clinical condition of those harboring *A. actinomycetemcomitans* was significantly worse (deeper pockets, $p = 0.035$) and with a more pathogenic microbiota (higher proportions of *P. micra*, $p = 0.045$) when compared with those patients negative for *A. actinomycetemcomitans*. The recovered strains of this pathogen were very homogeneous, belonging almost exclusively to serotype "b," with the genes responsible for the codification of leukotoxin found in every strain (and five, 12.2 %, belonging to the JP2 strain type) and the operon that codifies the *cdt* detected in 78 % of the strains.

The reported figure of prevalence of *A. actinomycetemcomitans* (35.6 %) should be interpreted with caution, since the aim and the design of this study were not to assess prevalence. However, in this population in Morocco with 61.9 % cases of aggressive periodontitis and 14.29 % of chronic periodontitis, the prevalence may be considered as

Table 3 Demographic data, periodontal status, and patient characteristics for the whole sample and for each compared group (according to the detection of *A. actinomycetemcomitans* [*Aa*])

PERIODONTITIS	All	*Aa*-positive	*Aa*-negative
n	59	21	38
Chronic	12	3	9
Aggressive	32	13	19
Refractory	1	1	0
Not available	14	4	10
% Chronic	20.34 %	14.29 %	23.68 %
% Aggressive	54.24 %	61.90 %	50.00 %
% Refractory	1.69 %	4.76 %	0.00 %
% Not available	23.73 %	19.05 %	26.32 %
SMOKING	all	*Aa*-positive	*Aa*-negative
n	59	21	38
Non-smoker	45	18	27
Smoker	1	0	1
Not available	13	3	10
Non-smoker	76.27 %	85.71 %	71.05 %
Smoker	1.69 %	0.00 %	2.63 %
Not available	22.03 %	14.29 %	26.32 %
GENDER	all	*Aa*-positive	*Aa*-negative
n	59	21	38
Male	6	0	6
Female	39	18	21
Not available	14	3	11
% Male	10.17 %	0.00 %	15.79 %
% Female	66.10 %	85.71 %	55.26 %
% Not available	23.73 %	14.29 %	28.95 %
AGE	All	*Aa*-positive	*Aa*-negative
Mean	27.6	26.6	28.2
Standard deviation	9.2	8.3	9.8
Maximum	55	46	55
Minimum	12	13	12
n	45	17	28

Table 4 Clinical characteristics, expressed as mean and standard deviation (SD) of patients in each group

Clinical data	All			*Aa*-positive			*Aa*-negative		
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
PPD	45	6.20	1.90	17	6.96	2.08	28	5.74	1.65
REC	45	0.85	1.02	17	0.76	0.85	28	0.90	1.12
BOP	45	0.82	0.29	17	0.79	0.28	28	0.84	0.29
Pl	45	0.78	0.34	17	0.75	0.36	28	0.79	0.33
SUP	40	0.21	0.20	14	0.20	0.20	26	0.21	0.20

Aa A. Actinomycetemcomitans, *PPD* probing pocket depth (in mm), *REC* recession (in mm), *CAL* clinical attachment level (in mm), *BOP* bleeding on probing (in percentage), *Pll* plaque index (in percentage), *SUP* suppuration (in percentage)

relatively low, when comparing with other studies assessing similar populations. In a cross-sectional study, Haubek and coworkers, using microbial culturing, found 60.4 % of *A. actinomycetemcomitans* in a group of Moroccan adolescents [22]. The use of PCR-based microbial diagnosis has provided very heterogeneous results with frequencies of detection of 13 and 37.5 % in severe chronic and aggressive periodontitis patients, respectively [14]. Similarly, in an aggressive periodontitis patient group younger than 35 years, the reported prevalence was 83 % [22]. There may be different explanations for these variable findings, including the use of different methods of detection, diversity in demographic characteristics (especially age), and differences in periodontal status, although all studies coincide that *A. actinomycetemcomitans* was a key factor in the onset and progression, especially in aggressive periodontitis [2, 23]. The fact that in our sample the mean age is 27.6 years, including 20.34 % of the patients suffering from chronic periodontitis, is due, on one hand, to the convenience nature of the sample that consisted of consecutive patients seeking treatment at that dental school and, on the other hand, to the high prevalence of periodontitis in young people in Morocco, as it has been described by several authors [14, 22].

In other geographical locations, the reported presence of *A. actinomycetemcomitans* in periodontitis patients has shown wide variability, mainly depending on the used microbial technologies [24]. In a previous study from our group, using the same technology, the prevalence of *A. actinomycetemcomitans* in a Spanish population was 5.7 % [25]. In Ghana, the prevalence of *A. actinomycetemcomitans* in aggressive periodontitis adolescents was 54.4 %, while in Sudan, also in aggressive periodontitis, the prevalence was 70.3 % [26, 27]. Whether these important differences are due to the methodologies used, the different geographical location, or the fact that they represent a specific microbiological profile directly related with the onset of progression of periodontitis remains unclear. It is important to take into account that the presence of *A. actinomycetemcomitans* is not just determined by genetics but also by several factors influencing the appropriate environment for the growth of *A. actinomycetemcomitans* [20].

The fact that deeper PPD with a more complex microbiota (higher counts of *P. micra)* was reported in those patients positive for *A. actinomycetemcomitans* is congruent with results from other studies associating the presence of *A. actinomycetemcomitans* with a greater extension and severity of periodontitis or even directly related with the specific diagnosis of aggressive periodontitis [26, 27].

This study has also shown that almost every isolate of *A. actinomycetemcomitans* corresponded to serotype "b." Only one patient harbored two different serotypes, namely "a" and "e." Finding a patient harboring more than one serotype is not so unusual, as reported in other investigations assessing patients of different ethnic origins [28, 29]. Also in Morocco,

Table 5 Microbiological data: bacterial counts (transformed to log10 cfu) in the compared groups, expressed as mean and standard deviation (SD) of the colony-forming units/milliliter

Bacterial counts	All			*Aa*-positive			*Aa*-negative		
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
Total	59	16,902,734	15,651,442	21	18,604,771	13,651,066	38	15,962,135	16,754,697
A.a	59	6584	15,158	21	18,499	20,854	38	0	0
P. gingivalis	59	3,458,713	8,181,444	21	3,943,280	5,545,568	38	3,190,926	9,385,969
P. intermedia	59	3,317,138	6,731,681	21	4,900,469	9,389,824	38	2,442,139	4,600,780
T. forsythia	59	340,515	1,009,729	21	657,171	1,594,209	38	165,521	368,877
P. micra	59	106,047	364,589	21	233,514	587,216	38	35,605	87,526
C. rectus	59	21,277	61,810	21	3143	14,402	38	31,298	74,745
F. nucleatum	59	321,051	371,254	21	266,514	273,351	38	351,189	415,989
Eubacterium	59	671	5155	21	0	0	38	1042	6424
Capnocytop.	59	6063	18,753	21	8234	23,117	38	4863	16,071
E. corrodens	59	36,020	88,262	21	45,257	121,882	38	30,916	64,083

A. a Aggregatibacter actinomycetemcomitans, *P. gingivalis Porphyromonas gingivalis*, *P. intermedia Prevotella intermedia*, *T. forsythia Tannerella forsythia*, *P. micra Parvimonas micra*, *C. rectus Campylobacter rectus*, *F. nucleatum Fusobacterium nucleatum*, *Eubacterium Eubacterium* spp., *Capnocytop Capnocytophaga* spp., *E. corrodens Eikenella corrodens*

a study by Haubek et al. reported that most of aggressive periodontitis patients (77 %) were positive for serotype "a," 11 % for serotype "c," and 5.5 % for serotype "d," which is in contrast with our results, although in their study a serotype-specific antiserum was used, instead of molecular techniques [22].

Using the same methodology, our group evaluated a population of periodontitis patients in Spain, and subjects positive for *A. actinomycetemcomitans* were infected by multiple different serotypes, including "a," "b," or "c" [25]. These results are clearly in contrast with the pattern distribution of the results of the present study, in a Moroccan periodontitis population, characterized by mono-colonization and "b" being the clearly predominant serotype. There is convincing evidence of differences in serotype distribution related to geography and/or ethnic groups; however, whether there is an association between the serotype distribution and the clinical periodontal status is still unknown. In USA, serotype "b" strains were frequently isolated from patients with localized juvenile periodontitis, while another study in the same geographical location found a clear predominance of serotype "c" [3, 30, 31]. Similarly, serotype "c" was found as the predominant serotype in diseased sites in some Asian countries and in some African populations as Ghanaian [29, 32–34].

The virulence of *A. actinomycetemcomitans* strains has also been attributed to the presence of the leukotoxin gene and

Table 6 Microbiological data: proportions of total microbiota, expressed as mean and standard deviation (SD)

Proportions	All			*Aa*-positive			*Aa*-negative		
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
A. actinomyc.	59	0.1 %	0.4 %	21	0.3 %	0.6 %	38	0.0 %	0.0 %
P. gingivalis	59	15.3 %	17.7 %	21	17.8 %	18.0 %	38	13.9 %	17.6 %
P. intermedia	59	12.7 %	17.2 %	21	15.7 %	22.5 %	38	11.0 %	13.5 %
T. forsythia	59	2.6 %	7.5 %	21	2.5 %	5.4 %	38	2.7 %	8.5 %
P. micra	59	0.8 %	2.1 %	21	1.2 %	2.5 %	38	0.5 %	1.7 %
C. rectus	59	0.2 %	0.8 %	21	0.0 %	0.2 %	38	0.3 %	0.9 %
F. nucleatum	59	3.8 %	8.8 %	21	2.2 %	3.2 %	38	4.7 %	10.6 %
Eubacterium	59	0.0 %	0.1 %	21	0.0 %	0.0 %	38	0.0 %	0.2 %
Capnocytophaga	59	0.1 %	0.3 %	21	0.1 %	0.3 %	38	0.1 %	0.2 %
E. corrodens	59	0.3 %	1.0 %	21	0.4 %	1.4 %	38	0.3 %	0.6 %

Aa Aggregatibacter actinomycetemcomitans, *A. actinomyc Aggregatibacter actinomycetemcomitans*, *P. gingivalis Porphyromonas gingivalis*, *P. intermedia Prevotella intermedia*, *T. forsythia Tannerella forsythia*, *P. micra Parvimonas micra*, *C. rectus Campylobacter rectus*, *F. nucleatum Fusobacterium nucleatum*, *Eubacterium Eubacterium* spp., *Capnocytophaga Capnocytophaga* spp., *E. corrodens Eikenella corrodens*

Table 7 Microbiological data: number of positive samples (n-positive) and frequency of detection of different species

Frequency	All			*Aa*-positive			*Aa*-negative		
	n	n-positive	Frequency	*n*	n-positive	Frequency	*n*	n-positive	Frequency
A. actinomyc.	59	21	35.59 %	21	21	100.00 %	38	0	0.00 %
P. gingivalis	59	50	84.75 %	21	19	90.48 %	38	31	81.58 %
P. intermedia	59	56	94.92 %	21	18	85.71 %	38	38	100.00 %
T. forsythia	59	24	40.68 %	21	9	42.86 %	38	15	39.47 %
P. micra	59	15	25.42 %	21	7	33.33 %	38	8	21.05 %
C. rectus	59	10	16.95 %	21	1	4.76 %	38	9	23.68 %
F. nucleatum	59	58	98.31 %	21	21	100.00 %	38	37	97.37 %
Eubacterium	59	1	1.69 %	21	0	0.00 %	38	1	2.63 %
Capnocytophaga	59	8	13.56 %	21	4	19.05 %	38	4	10.53 %
E. corrodens	59	18	30.51 %	21	6	28.57 %	38	12	31.58 %

Aa Aggregatibacter actinomycetemcomitans, *A. actinomyc Aggregatibacter actinomycetemcomitans*, *P. gingivalis Porphyromonas gingivalis*, *P. intermedia Prevotella intermedia*, *T. forsythia Tannerella forsythia*, *P. micra Parvimonas micra*, *C. rectus Campylobacter rectus*, *F. nucleatum Fusobacterium nucleatum*, *E. corrodens Eikenella corrodens*

mainly to the presence of the serotype "b" JP2 clone. In the present study, this clone was detected in five isolates from two patients (11.8 %). In a large school children cohort, this highly leucotoxic strain was detected in 14.5 % of the cases, using the same primers as in this study [22]. Ennibi and coworkers found a prevalence of 77 % of the JP2 clone among a group of aggressive periodontitis, young adult patients in Morocco, using a different set of primers, while in Ghana, the prevalence of the JP2 clone was 8.8 % [26, 35, 36]. The prevalence reported in the present study was lower than expected, since it is well known that this clone affects more likely to subjects of African origin [36–38]. One explanation to this discrepancy may be the use of culture as the first detection method, which due to its inherent low sensitivity may have underestimated the prevalence of *A. actinomycetemcomitans* and consequently the prevalence of high leukotoxic strains, in comparison with the other studies using PCR as the first detection method.

Table 8 Patient and strain based analysis: leukotoxin, CDT, and serotype distribution

Strains	Patients	Percent		
1	1	5.9		
2	8	47.05		
3	8	47.05		
Total	17			
	Patients		Strains	
Leukotoxin	*n*	%	*n*	%
ltx 1034	15	88.2	36	87.8
JP2	2	11.8	5	12.2
Both	0	0.00	0	0.00
Total	17		41	
CDT	*n*	%	*n*	%
CDT +	11	64.7	32	78
CDT -	3	17.6	9	22
Both	3	17.6	0	0.00
Total	17		41	
Serotype	*n*	%	*n*	%
b	16	94.1	39	95.1
a & e	1	5.9	2	4.9
Other	0	0.00	0	0.00
Total	17		41	

The *cdt* gene was detected in 32 out of the 41 strains of periodontitis patients (78 %). The reported prevalence of this toxin in *A. actinomycetemcomitans* is highly variable. In a PCR-based disease progression study, a similar prevalence was described in a Ghanaian adolescent population (79 %), although in this study there was no relation between the *cdt* genotype and the disease progression nor with the Cdt activity [34]. Ahmed and coworkers found 43 of 50 strains from periodontitis patients containing all three *cdt* genes and expressing Cdt activity [39]. In another PCR-based study, however, only 13 out of 106 diseased sites were positive for these genes, while Fabris et al. reported its presence in 39 out of the 40 patients studied [40, 41].

Table 9 Strain based analysis: genetic distribution of the *A. actinomycetemcomitans* samples

Serotype	Leukotoxin				Total
	JP2		ltx 1034		
	cdt+	*cdt*−	*cdt*+	*cdt*−	
a and e	0	0	0	2	2
b	5	0	27	7	39
Total	5	0	27	9	41

It is important to acknowledge the limitations of the present investigation. First, we did not use uniform criteria in the clinical diagnosis. In addition, in one patient, only one strain could be studied, which made it impossible to assess the presence of different serotypes in this subject. Moreover, the primer for the serotype "e" described by van der Reijden was not used (it was described after our samples were analyzed); therefore, it is possible that these so called non-typable strains could belong to that serotype [42]. The small size of this convenience sample and the lack of clinical and demographic data of 14 subjects (who were clinically assessed and diagnosed as periodontitis, but the data files were lost) should be recognized as other limitations of this investigation. The convenience nature of the sample is a common limitation found in other studies with a similar methodology, as Ennibi and coworkers in Morocco, or Sakellari and coworkers in Greece, also recruited patients from the Periodontology Department of the University in order to study the microbiological profiles of a particular group of the population [35, 38].

In conclusion, this research has evidenced that the presence of *A. actinomycetemcomitans* was a relatively frequent finding (35.6 %) in patients with periodontitis in Morocco. The isolated strains were mostly from serotype "b." Most of these strains were positive for the operon that codifies the *cdt* (78 %) and for the genes responsible for the codification of leukotoxin (87.8 %), although only five (12.2 %) of the isolates belonged to the JP2 highly leukotoxic strain.

Acknowledgments We thank Arancha Llama-Palacios, María José Marín, Itziar González and Ana O'Connor from the Laboratory of Dental Research, University Complutense, Madrid, for their technical assistance.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Listgarten MA (1976) Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study. J Periodontol 47:1–18
2. Consensus report (1996) Periodontal diseases: pathogenesis and microbial factors. Ann Periodontol 1:926–932
3. Asikainen S, Chen C, Slots J (1995) *Actinobacillus actinomycetemcomitans* genotypes in relation to serotypes and periodontal status. Oral Microbiol Immunol 10:65–68
4. Poulsen K, Theilade E, Lally ET, Demuth DR, Kilian M (1994) Population structure of *Actinobacillus actinomycetemcomitans*: a framework for studies of disease-associated properties. Microbiology 140:2049–2060
5. Paju S, Carlson P, Jousimies-Somer H, Asikainen S (2000) Heterogeneity of *Actinobacillus actinomycetemcomitans* strains in various human infections and relationships between serotype, genotype, and antimicrobial susceptibility. J Clin Microbiol 38:79–84
6. Kanasi E, Dogan B, Karched M, Thay B, Oscarsson J, Asikainen S (2010) Lack of serotype antigen in *A. actinomycetemcomitans*. J Dent Res 89:292–296
7. Cortelli JR, Aquino DR, Cortelli SC, Roman-Torres CV, Franco GC, Gomez RS et al (2012) *Aggregatibacter actinomycetemcomitans* serotypes infections and periodontal conditions: a two-way assessment. Eur J Clin Microbiol Infect Dis 31: 1311–1318
8. Holtta P, Alaluusua S, Saarela M, Asikainen S (1994) Isolation frequency and serotype distribution of *mutans streptococci* and *Actinobacillus actinomycetemcomitans*, and clinical periodontal status in Finnish and Vietnamese children. Scand J Dent Res 102: 113–119
9. Celenligil H, Ebersole JL (1998) Analysis of serum antibody responses to periodontopathogens in early-onset periodontitis patients from different geographical locations. J Clin Periodontol 25:994–1002
10. Baehni PC, Tsai CC, McArthur WP, Hammond BF, Shenker BJ, Taichman NS (1981) Leukotoxic activity in different strains of the bacterium *Actinobacillus actinomycetemcomitans* isolated from juvenile periodontitis in man. Arch Oral Biol 26:671–676
11. Zambon JJ, Christersson LA, Slots J (1983) *Actinobacillus actinomycetemcomitans* in human periodontal disease. Prevalence in patient groups and distribution of biotypes and serotypes within families. J Periodontol 54:707–711
12. Haubek D, Ennibi OK, Poulsen K, Vaeth M, Poulsen S, Kilian M (2008) Risk of aggressive periodontitis in adolescent carriers of the JP2 clone of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* in Morocco: a prospective longitudinal cohort study. Lancet 371:237–242
13. Yamano R, Ohara M, Nishikubo S, Fujiwara T, Kawamoto T, Ueno Y et al (2003) Prevalence of cytolethal distending toxin production in periodontopathogenic bacteria. J Clin Microbiol 41:1391–1398
14. Benrachadi L, Bouziane A, Azziman Z, Bouziane-Ouartini F, Ennibi O (2012) Screening for periodontopathogenic bacteria in severe chronic periodontitis in a Moroccan population. Med Mal Infect 42:599–602
15. Armitage GC (1999) Development of a classification system for periodontal diseases and conditions. Ann Periodontol 4:1–6
16. Mombelli A, McNabb H, Lang NP (1991) Black-pigmenting gram-negative bacteria in periodontal disease. II Screening strategies for detection of P gingivalis. J Periodontal Res 2:308–313
17. Syed SA, Loesche WJ (1972) Survival of human dental plaque flora in various transport media. Appl Microbiol 24:638–644
18. Alsina M, Olle E, Frias J (2001) Improved, low-cost selective culture medium for *Actinobacillus actinomycetemcomitans*. J Clin Microbiol 39:509–513
19. Yorgancioglu A, Turktas H, Kalayci O, Yardim N, Buzgan T, Kocabas A et al (2009) The WHO global alliance against chronic respiratory diseases in Turkey (GARD Turkey). Tuberk Toraks 57: 439–452
20. Kaplan JB, Schreiner HC, Furgang D, Fine DH (2002) Population structure and genetic diversity of *Actinobacillus actinomycetemcomitans* strains isolated from localized juvenile periodontitis patients. J Clin Microbiol 40:1181–1187
21. Haubek D, Dirienzo JM, Tinoco EM, Westergaard J, Lopez NJ, Chung CP et al (1997) Racial tropism of a highly toxic clone of *Actinobacillus actinomycetemcomitans* associated with juvenile periodontitis. J Clin Microbiol 35:3037–3042
22. Haubek D, Ennibi OK, Poulsen K, Poulsen S, Benzarti N, Kilian M (2001) Early-onset periodontitis in Morocco is associated with the highly leukotoxic clone of *Actinobacillus actinomycetemcomitans*. J Dent Res 80:1580–1583

23. Van der Velden U, Abbas F, Armand S, Loos BG, Timmerman MF, Van der Weijden GA et al (2006) Java project on periodontal diseases. The natural development of periodontitis: risk factors, risk predictors and risk determinants. J Clin Periodontol 33:540–548
24. Stingu CS, Jentsch H, Eick S, Schaumann R, Knofler G, Rodloff A (2012) Microbial profile of patients with periodontitis compared with healthy subjects. Quintessence Int 43:23–31
25. Minguez M, Pousa X, Herrera D, Blasi A, Sanchez MC, Leon R et al (2014) Characterization and serotype distribution of *Aggregatibacter actinomycetemcomitans* isolated from a population of periodontitis patients in Spain. Arch Oral Biol 59:1359–1367
26. Aberg CH, Kwamin F, Claesson R, Johansson A, Haubek D (2012) Presence of JP2 and Non-JP2 Genotypes of *Aggregatibacter actinomycetemcomitans* and attachment loss in adolescents in Ghana. J Periodontol 83:1520–1528
27. Elamin A, Albandar JM, Poulsen K, Ali RW, Bakken V (2011) Prevalence of *Aggregatibacter actinomycetemcomitans* in Sudanese patients with aggressive periodontitis: a case–control study. J Periodontal Res 46:285–291
28. Aberg CH, Sjodin B, Lakio L, Pussinen PJ, Johansson A, Claesson R (2009) Presence of *Aggregatibacter actinomycetemcomitans* in young individuals: a 16-year clinical and microbiological follow-up study. J Clin Periodontol 36:815–822
29. Yoshida Y, Suzuki N, Nakano Y, Shibuya K, Ogawa Y, Koga T (2003) Distribution of *Actinobacillus actinomycetemcomitans* serotypes and Porphyromonas gingivalis in Japanese adults. Oral Microbiol Immunol 18:135–139
30. Chen C, Wang T, Chen W (2010) Occurrence of *Aggregatibacter actinomycetemcomitans* serotypes in subgingival plaque from United States subjects. Mol Oral Microbiol 25:207–214
31. Zambon JJ, Slots J, Genco RJ (1983) Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. Infect Immun 41:19–27
32. Chung HJ, Chung CP, Son SH, Nisengard RJ (1989) *Actinobacillus actinomycetemcomitans* serotypes and leukotoxicity in Korean localized juvenile periodontitis. J Periodontol 60:506–511
33. Mombelli A, Gmur R, Lang NP, Corbert E, Frey J (1999) *Actinobacillus actinomycetemcomitans* in Chinese adults. Serotype distribution and analysis of the leukotoxin gene promoter locus. J Clin Periodontol 26:505–510
34. Hoglund Aberg C, Antonoglou G, Haubek D, Kwamin F, Claesson R, Johansson A (2013) Cytolethal distending toxin in isolates of *Aggregatibacter actinomycetemcomitans* from Ghanaian adolescents and association with serotype and disease progression. PLoS One 8:65781
35. Ennibi OK, Benrachadi L, Bouziane A, Haubek D, Poulsen K (2012) The highly leukotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans* in localized and generalized forms of aggressive periodontitis. Acta Odontol Scand 70:318–322
36. Poulsen K, Ennibi OK, Haubek D (2003) Improved PCR for detection of the highly leukotoxic JP2 clone of *Actinobacillus actinomycetemcomitans* in subgingival plaque samples. J Clin Microbiol 41:4829–4832
37. Haubek D, Poulsen K, Asikainen S, Kilian M (1995) Evidence for absence in northern Europe of especially virulent clonal types of *Actinobacillus actinomycetemcomitans*. J Clin Microbiol 33:395–401
38. Sakellari D, Katsikari A, Slini T, Ioannidis I, Konstantinidis A, Arsenakis M (2011) Prevalence and distribution of *Aggregatibacter actinomycetemcomitans* serotypes and the JP2 clone in a Greek population. J Clin Periodontol 38:108–114
39. Ahmed HJ, Svensson LA, Cope LD, Latimer JL, Hansen EJ, Ahlman K et al (2001) Prevalence of cdtABC genes encoding cytolethal distending toxin among *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* strains. J Med Microbiol 50:860–864
40. Tan KS, Woo CH, Ong G, Song KP (2001) Prevalence of *Actinobacillus actinomycetemcomitans* in an ethnic adult Chinese population. J Clin Periodontol 28:886–890
41. Fabris AS, DiRienzo JM, Wikstrom M, Mayer MP (2002) Detection of cytolethal distending toxin activity and cdt genes in *Actinobacillus actinomycetemcomitans* isolates from geographically diverse populations. Oral Microbiol Immunol 17:231–238
42. van der Reijden WA, Brunner J, Bosch-Tijhof CJ, van Trappen S, Rijnsburger MC, de Graaff MP et al (2010) Phylogenetic variation of *Aggregatibacter actinomycetemcomitans* serotype e reveals an aberrant distinct evolutionary stable lineage. Infect Genet Evol 10:1124–1131