Characterization and serotype distribution of *Aggregatibacter actinomycetemcomitans* isolated from a population of periodontitis patients in Spain.

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Introduction

The etiology of periodontal diseases has been associated with microorganisms colonizing the subgingival biofilm.[1] Among these, *Aggregatibacter actinomycetemcomitans* has received particular attention and it is regarded a major pathogen, being a key factor in the etiology of early onset and refractory forms of periodontitis. [1–4] This notion is derived primarily from association studies, conducted in North America and Europe, which significantly correlated the presence and amount of these microorganisms with the presence and severity of disease and also linked a worse than expected treatment outcome with presence of high levels of *A. actinomycetemcomitans* after therapy. In prospective studies, its presence has also been identified as a risk factor for the onset of periodontitis.[5] On the other hand, *A. actinomycetemcomitans* has also been frequently detected in subjects without clinical evidence of periodontal disease, what suggests that either not all subjects are equally susceptible or that there are variations in the virulence and the pathogenic potential of this microorganism.

Based on the presence of specific capsular proteins able to elicit a subject's differential antibody response, six serotypes (from "a" to "f") of *A. actinomycetemcomitans* have been identified.[6] This genetic variability has been associated to differential virulence since *A. actinomycetemcomitans* serotype "b" has been linked to periodontitis lesions, while serotypes "a" and "c" demonstrated a stronger association with periodontal health.[2, 7–9] A similar serotype-dependent differential virulence has also been suggested for *Porphyromonas gingivalis.* [10, 11]

The global *A. actinomycetemcomitans* serotype distribution is not homogeneous and the association between serotype and periodontal status may depend on the geographical location and/or ethnicity of the studied population. [12–15] For example, Yang (2004) found that serotype "b" was more often isolated in aggressive than chronic forms of periodontitis in a large population in Philadelphia.[16] Although most studies suggest that *A. actinomycetemcomitans* colonization occurs through one unique serotype, others have shown infected subjects with two or three serotypes.[7, 17–20]

A. actinomycetemcomitans has also demonstrated other forms of genetic diversity also linked to virulence traits, mostly in relation to its capacity to produce leukotoxin, a protein that specifically destroys human polymorphonuclear leukocytes.[21–23] There are, however, significant variations in the leukotoxin production demonstrated among different isolates.[10 ,23] The mechanism by which the most virulent species produce high levels of leukotoxin was identified by a 530-bp deletion in the region upstream of the leukotoxin gene operon (*lxt*), which encodes this protein, thus bringing a second strong promoter and producing high amounts of this toxin. The majority of strains of this highly leukotoxic clone were isolated from subjects of African descent. Haubek et al. demonstrated, in a population based longitudinal study of 700 adolescents, that the JP2 strain of *A. actinomycetemcomitans* was the etiological agent of the aggressive forms of periodontitis in adolescents living in or originating from North and West Africa.[24] Individuals who carried the JP2 clone of *A. actinomycetemcomitans* had a significantly increased risk of periodontal attachment loss, either alone (relative risk 18.0) or together with the presence of non-JP2 clones of *A. actinomycetemcomitans* (12.4). A much less pronounced disease risk was found in those carrying non-JP2 clones only (3.0).

Another identified virulence factor associated to this bacterial species is the cytolethal distending toxin (Cdt), which blocks the progression of the cell cycle in specific types of eukaryotic cells and other cell lines, specifically oral epithelial cells and T lymphocytes. This holotoxin, which has only been identified in *A. actinomycetemcomitans* isolated from the human oral cavity is made up of three subunit proteins designated as CdtA, CdtB and CdtC and different studies have reported that this cytotoxic Cdt activity is also heterogeneous among *A. actinomycetemcomitans* isolates. In a study by Ahmed et al, 86% of *A. actinomycetemcomitans* isolates presented the complete operon and its characteristic cytotoxic activity.[25,26] Tan et al. showed a close association between aggressive forms of periodontitis and *A. actinomycetemcomitans* strains positive to the Cdt genotype. [27]

In summary, numerous studies have shown that the genetic diversity in *A. actinomycetemcomitans* strains may be associated to geographical diversity, thus suggesting a differential virulence.[7,9,21,28,32] Since there are no studies in Southern

Europe evaluating the differential virulence of *A. actinomycetemcomitans* isolates in periodontitis patients, it is the objective of this investigation to study the serotype distribution of *A. actinomycetemcomitans*, the variation in the sequences of the genes that codify the leukotoxin and the distribution of the *cdt* operon in periodontitis patients in Spain.

Material and methods

Patient selection and sample reception

The bacterial isolates used in this study were obtained from a consecutive series of subgingival microbial samples from Spanish patients sent to the Laboratory of Microbiology at Faculty of Odontology, University Complutense, Madrid, Spain, for microbial diagnosis over 18 months from January 2007 to July 2008. The patient residence was Spain and ethnicity Caucasian in all cases. 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 consent was obtained from all patients.

Samples from patients with a clinical diagnosis of chronic or aggressive untreated periodontitis according to the American Academy of Periodontology 1999 classification were included.[33] Sample from patients categorized as "refractory" periodontitis as a separate group were also included, when these subjects had a recurrence of disease after treatment or when the treatment outcome was not appropriate.

These microbiological samples were collected by periodontists in private practices as well as by postgraduate students in the Postgraduate Clinic of Periodontology in the same Faculty of Odontology. Each sample was accompanied by clinical information including patient age, gender, clinical diagnosis, brief clinical and drug intake history, smoking status, sample sites and time and date of sampling. The

clinical parameters: probing pocket depth (PPD), gingival recession, presence or absence of plaque (PI), bleeding on probing (BOP) and suppuration were recorded at the four selected sites from which samples were taken. These sites represented the deepest pocket with bleeding in each quadrant and after careful removal of supragingival plaque and isolation 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 seconds. [34] The paper points were transferred to a vial containing 1.5 ml of Reduced Transport Fluid (RTF), and pooled with all the other paper points.[35] The vial was sent to the laboratory and processed within 24 hours.

Only samples were selected when belonging to patients with at least four sites with (PPD) deeper than 4 mm and clinical attachment loss (CAL) higher than 4 mm. Samples from subjects with fewer than 16 teeth and from patients who had taken any antibiotic medication within the previous three months were excluded.

Culture, isolation and storage

Samples were homogenized with a vortex and 10-fold serially diluted in Phosphate sodium buffer (PBS) and 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.[36]

Blood agar plates were studied after 7 and 14 days of anaerobic incubation (80% N_2, 10% H_2; 10% CO_2 at 37ºC). Plates were carefully examined and based on the morphology of the isolated colonies the following pathogens were identified: *P. gingivalis, Prevotella intermedia/nigrescens, Tannerella forsythia, Parvimonas micra, Capnocytophaga* spp.*, Eikenella corrodens* and *Fusobacterium* spp. Colonies from each pathogen were counted as the total number from a representative plate (between 30 to 300 colonies). The specific media, Dentaid-1 was used for the selective isolation and growth of *A. actinomycetemcomitans*. Plaques with this media were incubated at 37ºC in air with 5% CO2 and after 3-5 days were carefully examined for the identification of *A. actinomycetemcomitans*. This identification was based on its typical colony morphology, a catalase reaction and a set of specific enzymes (Rapid ID, NH system

Romel Inc, USA). One to three isolates per subject were subcultured and purified. All original samples were stored at -80º until further use. Only positive isolates for *A. actinomycetemcomitans* were included in this investigation.

Isolation of genomic DNA

Bacterial cells were collected after centrifugation and re-suspension in 1 ml of tris- HCl 10 mM, EDTA 0.8 mM (ph 8.0) and lysozyme (final concentration 5.0 mg/ml). They were then incubated at 37 ºC for 30 min and proteinase K was added until reaching a final concentration of 2 mg/µl, 1 mg/µl and 1 %, respectively. [37] 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 extracted DNA was electrophoresed through a 1.0 % agarose gel, in a tris- Acetate EDTA (TAE) buffer. The gel was stained with ethidium bromide (10 µg/ml) and visualized under UV-illumination.

Serotyping of strains

Serotypes "a" to "f" were determined by the PCR technique, based on specific sequences from the gene clusters responsible for the distinct serotypes. The PCR reaction was performed with the specific primers described by Kaplan for the identification of the serotypes "a" to "f". [6] The sequences of the primers used are listed in Table 1. The PCR reaction was performed in a 25 µl final volume containing 2.5 µl of 10x PCR buffer, 1.0 µl of 25mM $MgCl_2$, 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 is electrophoresed through a 1.0 % agarose gel in 1X TAE buffer, the PCR products are visualized by staining with ethidium bromide (10 mg/ml) and visualized under UV illumination. These PCR assays were performed including positive and negative controls in an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc). After the initial step of denaturation at 96 ºC for 3 min, a total of 35 PCR cycles were performed; each cycle consisted of 30 s of denaturation at 95 ºC, 1min of annealing at 55 ºC and 72ºC for 2 min, and a final step of extension at 72 ºC for 10 min.

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 et al. [30] The PCR reaction was performed in a 25 µl final volume containing 2.5 µl of 10x PCR buffer, 1.0 µl of 25mM $MgCl_2$, 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.

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 ºC, a total of 30 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 60ºC, and a final step of extension at 72ºC 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 were included in the PCR. 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 distribution of the *cdt* operon was also determined by means of PCR using the same procedure previously described for Ltx but using the primers that amplify the complete operon *cdt*A1 and *cdt*C2, 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.

Data analyses

Descriptive statistics, including frequency distribution were used to characterize the sample in terms of the different serotypes, the variation in the sequences of the genes that codify the leukotoxin and the distribution of the *cdt* operon. Further subgroup analysis was performed to assess the differences in serotype, leukotoxin and *cdt* gene over-production according to the different clinical diagnoses: chronic, aggressive or

refractory, as well to other clinical and patient variables.

For continuous variables, ANOVA test was used to compare different disease categories, with the multiple rank test as the post hoc test. For categorical variables, contingency tables were constructed and compared by means of chi-square test. The software used for the analyses was Statgraphics Plus 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA).

Results

Demographics

From a total of 701 patient samples processed, 396 samples were taken in 25 private practices throughout Spain (16 were located in Madrid area), and 305 from patients seeking treatment in the Section of Periodontology, Faculty of Odontology, University Complutense, Madrid, Spain. Out of these 701 patients, 40 (5.7%) fulfilled the inclusion criteria and were positive for *A. actinomycetemcomitans*. These patients had a mean age of 45.2 years (ranging 24-68) and were predominantly non-smokers 62.5% (25). They were stratified in three groups according to their periodontal condition and 19 (47.5%) were diagnosed of chronic periodontitis, 13 (32.5%) of aggressive periodontitis and 8 (20%) of "refractory" periodontitis. "Refractory" periodontitis patients had significantly more recession ($p=0.010$) and shallower pockets (tendency towards statistical significance, $p=0.051$) than chronic and aggressive periodontitis patients. Similar CAL levels (7.5-7.7 mm), BOP proportions (78-85%) and plaque index (40-45%) were observed among the three patient groups. Patients with aggressive periodontitis were significantly younger ($p<0.001$) than chronic periodontitis or refractory patients. The detailed periodontal status, clinical and demographic variables of these 40 patients are depicted in Table 3. No differences among the three groups were observed for either smoking or gender (Table 4).

Patient-based results

Serotype distribution (Table 4)

Overall, 75% of the patients were characterized by a unique serotype, while 17.5% harbored two serotypes and in 7.5% of the patients the serotype could not be characterized (although *A. actinomycetemcomitans* was confirmed by PCR using different primers). In the patients where a unique serotype was identified, "a" or "b" serotypes were detected in 60% of the patients, while in those patients with more than one serotype, 4 different co-colonization profiles were detected, with 1-3 patients each.

When assessing the serotype distribution according to clinical diagnosis, refractory patients showed a clear predominance of serotype "b" (either alone in 50% of the cases, or combined with serotype "a" in 25% of the patients) and the frequent presence of more than one serotype (37.5%). In aggressive periodontitis, mono-colonization with "a" and "b" serotypes occurred in 25% and 35% of the cases, respectively. A less clear pattern was observed for chronic periodontitis, with similar figures for serotypes "a", "b", "c", not-typeable and co-colonization.

cdt operon detection (Table 4)

Cdt positive samples were identified four times more frequently. Presence of Cdt positive and negative isolates in the same patient was observed in 28% of the cases. No Cdt negative samples were observed in refractory patients, while in chronic periodontitis there was also a clear predominance of Cdt positive profiles (67%).

lktC promoter deletion

No highly leukotoxic JP2 strains (identified by the presence of a 530-bp deletion in the promoter region of the *lkt*C gene of *A. actinomycetemcomitans)* were identified in this studied population. In all the isolates, a clear band in the 1.0 % agarose gel, equivalent to a molecular weight of 1034-bp was observed.

Strain-based results

From the 40 positive samples, 79 strains were isolated and studied corresponding to serotype "a", 24 strains (30.4%), to serotype "b", 30 strains (38%), to serotype "c", 12 samples (15.2%) and to serotype "d", 4 samples (5.1%). Mono-colonizations with serotypes "e" or "f" were not identified and the serotype could not be determined in 9 of these strains (11.4%). All strains were positive for the *ltx* gen. The *cdt* gene was

detected in 52 out of the 79 strains of periodontitis patients (65.8%).

The distribution of the *cdt* gen among different serotypes (Table 6) showed a clear predominance of *cdt* positive strains in serotype "a" and, to a lesser extent, in serotype "c", while an almost even distribution was observed for serotypes "b" and "d". The differences between serotypes "a" and "b" were statistically significant (p=0.042).

Discussion

The main findings of this investigation show that among the 40 patients where *A. actinomycetemcomitans* was detected 75% carried a unique serotype, while 17.5% were colonized by two serotypes. The most predominant serotypes were "a" and "b" and none of the isolates were highly leukotoxic strain JP2 and while 65.8% were positive for the *cdt* gen.

Using bacterial culturing the obtained prevalence of *A. actinomycetemcomitans* in this adult periodontitis population from Spain was of 5.7%. Previous reports from our research group have shown similar prevalence data using culture (6.3%) in a similar Spanish population, although using PCR methods the prevalence rose to 18.8%.[38] A similar low prevalence (3.2%) was also reported when studying a comparable population in Spain, but with the use of different culture medium (TSVB Tryptic soy-serum- bacitracin-vancomycin),[39] also reporting a low recovery rate.[36] In other geographical locations, the reported prevalence of *A. actinomycetemcomitans* in periodontitis patients varies substantially depending on the microbial detection technologies used, with proportions ranging between 3% to 38% in United States of America and 15% in Sri Lanka when using immunofluorescence.[37–40] With the use of bacterial culturing the reported prevalence of *A. actinomycetemcomitans* has varied between 5.4% in a group of aggressive periodontitis patients in Jamaica to 18% in Germany or 54.4% in Ghana.[41–43] These prevalence can rise to 83% in China and to 93% in Thailand when using DNA-DNA checkerboard hybridization.[44–45] With PCR the reported prevalence was 23.5% in United States of America, 19% in a Thai population, between 17.5% and 25% in Brazil, from 30% to 47.7% in Germany, 26.7%

in Korea, 27.5% in a Greek population and 44% in Haiti.[42,46–53]

From the 40 *A. actinomycetemcomitans*-positive samples studied, 79 strains were isolated and studied, being the serotype "b" the most frequent, followed by serotype "a", both representing 68.4% of all the strains. These findings are in accordance with other studies assessing populations from different geographical locations, which have also reported predominance in *A. actinomycetemcomitans* serotypes "a", "b", and "c", including Germany. [54] In the United States of America, serotype "b" strains were more frequently isolated from patients with localized juvenile periodontitis (LJP) when compared to other periodontal conditions or periodontally healthy subjects. [7,9] Listgarten et al. reported that elevated antibody responses to serotype "a" were more common in chronic periodontitis patients. [55] In this investigation, we have identified not only a higher percentage of serotype "a" strains in chronic periodontitis patients, but also in aggressive periodontitis patients, in which the most common is the serotype "b", like in the refractory periodontitis group. On the contrary, Dogan et al. reported that serotype "a" was the most frequent serotype in LJP subjects while in adult periodontitis subjects no differences among the serotypes "a", "b" and "c" were reported.[56] A higher frequency of serotype "b" was reported in Finnish patients with periodontitis, while "c" was more often related to periodontally healthy subjects. [9,20] In Asian populations, such as in Korea and Japan or in European countries, such as in Greece, the serotype "c" was also frequently associated with periodontally affected sites. [19,20,52,57] Strains with serotypes "d", "e" and strains without detectable serotype were also identified in Japanese subjects in proportions of 5%, 23% and 10%, respectively. [18,56] These serotypes are less frequently found than "a", "b" and "c" serotypes.[10,17,18,56,58]

This investigation demonstrated that the genes responsible for the codification of the Ltx were present in all the strains (100%), but none showed the JP2 clone responsible of the highly leukotoxic strains. These highly virulent *A. actinomycetemcomitans* strains have been detected mainly in subjects of African origin, with proportions of 8.8% reported in Ghana adolescents and 77% in Moroccan patients with a diagnosis of aggressive periodontitis and with a higher risk of periodontal attachment loss.[43,59] JP2 clone strains have also been reported in other distant

geographical populations, such as in Thailand, but has been found to be absent in Greece.[47,51] In a German population, and using PCR, the JP2 clone was found to be present in 2 out of 99 (2%) of the recruited patients and, in both cases, the subjects were immigrants of African origin suffering from chronic periodontitis.[54] Using a characterization method of point mutations within housekeeping genes and pseudogenes, Haubek et al. developed a model of global spreading of the JP2 clone. [60] According to this model, the patterns of mutation suggest that the JP2 clone emerged from a distinct genotype in the Mediterranean Africa approximately 2400 years ago and spread subsequently to West Africa and to other parts of the world. In fact, in Brazil colonization by highly leukotoxic *A. actinomycetemcomitans* was associated with increased periodontitis severity.[38] Even though JP2 clone strains have almost been exclusively detected among individuals in North-Africa or from African descent limited information is still available regarding the microbiological status of this clone in Caucasians. In fact, this study has shown that a Spanish population, although being in close relation to North African populations (namely, Moroccan populations), due to geographical and immigration factors, did not harbor any highly leukotoxic strains.

This investigation also evaluated the third factor of genetic variability of *A. actinomycetemcomitans*, the *cdt* gene, being detected in 65.8% of the strains from periodontitis patients. The reported prevalence of this toxin in *A. actinomycetemcomitans* strains is highly variable. It has been found to be present in 45.5% of a chronic periodontitis patient sample in Germany. [54] Ahmed et al. found 43 of 50 strains from periodontitis patients contained all three *cdt* genes and expressed Cdt activity. [26] Similarly, Fabris et al. reported Cdt activity in 39 out of 40 patients.[61] In another study, however, only 13 out of 106 diseased sites were positive for these genes.[28]

It is important to highlight the limitations of the present observational study, since a limited number of strains were studied and due to the low prevalence of *A. actinomycetemcomitans* in Spanish periodontitis patients the obtained associations only allowed us to generate hypothesis, rather than to establish clear conclusions. Moreover, in 22.5% of the patients, only one strain was studied and therefore, the results from the co-colonization of different strains per patient should be taken with caution. Similarly in

a small number of patients, strains with non-attributable serotype were identified, which may belong to the serotype e´ described by van der Reijden et al., since we did not use this specific primer, that was described after our samples were analyzed.[62] Furthermore, the stratification used according to the patient's clinical diagnosis should also be taken with caution since different clinicians, not using uniform criteria, did the diagnosis. In spite of this, the study showed the high variability in the genetic variance of *A. actinomycetemcomitans*, which has been also reported in similar studies. This variability may be explained, in part, by the different bacterial detection techniques used in the different laboratories, but also due to differences in ethnicity, geographic location and the periodontal condition of the populations sampled. Further studies should evaluate whether the presence of certain pathogenic bacterial species is associated with periodontitis and attachment loss in some population groups but not in others, depending on the presence of specific virulence traits.

In conclusion, this research has evidenced that, in samples from a group of Spanish patients with periodontitis, the most common *A. actinomycetemcomitans* serotypes found were "b" and "a", while "e" and "f" were not detected. The genes responsible for the codification of leukotoxin were detected in all the strains, although none belonging to the JP2 strain. The operon that codifies the *cdt* was also detected in the majority of the samples (65.8%).

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