

Università degli Studi di Napoli “Federico II”

**Scuola di dottorato in medicina clinica e sperimentale
Dipartimento di Scienze Odontostomatologiche e Maxillo-Facciali**



**Dottorato di ricerca in
Scienze Odontostomatologiche
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XXII CICLO

TESI DI DOTTORATO

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

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SUMMARY

Objective:

To investigate the serotype distribution of *Aggregatibacter actinomycetemcomitans* (*A. a.*) and the polymorphism of the genes that codify the leukotoxin (*ltx*) and the operon of the cytolethal distending toxin (*cdt*) in periodontitis patients in Spain.

Materials and methods:

Forty *A. a.*-positive periodontitis patients were included in the study (mean age 45±7.8 years, 62.5 % female) and their clinical periodontal status was assessed. On average, 1 to 2 isolates from each patient were subcultured and characterized by specific PCR reactions.

Results:

Serotype distribution analysis concludes that the most frequent serotype was *a* and that 29 patients showed monoinfection. The 11 patients that showed coinfection were infected mostly with serotypes *a* and *b*.

From these 40 isolates 15 corresponded to serotype *a*, 8 to serotype *b* and 6 to serotype *c*. Monoinfection with serotypes *d*, *e* or *f* was not found.

Further characterization of these samples with leukotoxin specific PCR reaction showed that none of the 40 samples presented the 530-bp deletion called $\Delta 530$ in the leukotoxin's promoter region, which characterizes the JP2 strain.

As for the *Cdt*, the operon that codifies for the toxin was present in 85 % of the samples.

Conclusions:

The most common serotypes were *a* and *b*. Serotype *a* was the most frequent in

monoinfections, while serotypes *d*, *e* and *f* were not detected. In the majority of samples, operon that codifies the *cdt* (85%) and the genes responsible for the codification of leukotoxin (100%) were found. None of the isolates belonged to the JP2 strain.

INTRODUCTION

***Aggregatibacter actinomycetemcomitans* as a periodontal risk factor**

Periodontal diseases are caused by microorganisms colonizing the subgingival biofilm¹. One of these pathogenic microorganisms, *Aggregatibacter actinomycetemcomitans* (*A. a.*), has received particular attention and is regarded as a major pathogen in periodontal diseases^{2,3,4,5} and as a key factor in the etiology of early onset and refractory forms of periodontal disease. This notion is based primarily on association studies, conducted in North America and Europe, linking the organisms to the disease and showing a correlation between worse than expected treatment outcomes and high levels of *A. a.* after therapy, but its presence has also been identified as a risk factor for the onset of periodontitis in a study performed recently by Van der Velden and coworkers⁶. On the other hand, it has been reported that *A. a.* can be detected with considerable frequency also in subjects without clinical evidence of periodontal disease. If a putative pathogen can be detected frequently in healthy subjects, this may suggest that not all humans are equally susceptible or that there is variation in virulence and pathogenic potential of the pathogen.

Serotype characterization of *Aggregatibacter actinomycetemcomitans*

An intra-species division of this microorganism is the one termed as *serotypes*. The species is represented by 6 serotypes (from *a* to *f*)⁷ that were, at first, identified with monoclonal antibodies. Nowadays a more simple and fast technique to analyze the serotypes of *A. a.* is represented by the polymerase chain reaction (PCR), performed

with specifically designed primers. Serotype-dependent variance in virulence has been suggested for *A. a.* and *Porphyromonas gingivalis*, since both microbial species have several serotypes that differ in virulence^{8,9}. *A. a.*'s serotype *b* has been more frequently associated to periodontitis lesions, while serotypes *a* and *c* have shown a stronger association with periodontal health^{9,10,11,12}. Yang found out that serotype *b* was more often found in aggressive than chronic forms of periodontitis in a big population in Philadelphia, PA - USA¹³. The global serotype distribution is not homogeneous^{14,15} and the association between serotype and periodontal status may be depending on the geographical location and/or ethnical status of the study population^{16,17}. Most studies defend the concept of monoinfection putting forward that one subject tends to be colonized by one unique serotype¹⁰ while several studies found that, on the contrary, subjects with two or three serotypes can also be detected^{18,19,20,21}.

***Aggregatibacter actinomycetemcomitans*'s toxins: leukotoxic and cytolethal distending toxin**

A. a. shows a high degree of genetic diversity^{22,23} but almost all the identified clones have demonstrated *in vitro* and *in vivo* capability of inducing periodontitis, and that is particularly obvious when assessing its main virulence factor: this bacteria produces a leukotoxin that specifically destroys human polymorphonuclear leukocytes and macrophages²⁴. The toxic activity of this factor, which belongs to the group of the so called Repeats in Toxin (RTX), depends on its capability of forming pores in the membrane of macrophages and leukocytes²⁵, but it doesn't affect epithelial or endothelial cells, nor fibroblasts.

Previous studies have revealed variations in leukotoxin's production among different isolates^{24,8}. The molecular mechanism of high-level production was identified as a 530-bp deletion in the region upstream of the leukotoxin gene operon (*lxt*) encoding the toxin, thereby bringing a second strong promoter into context. The vast majority of strains of this highly leukotoxic clone were isolated from subjects of African descent. Recently, a study by Haubeck and coworkers²⁶ demonstrated, in a population based longitudinal study of 700 adolescents, that the JP2 strain of *A. a.* can be considered as an aetiological agent of the aggressive form of periodontitis in adolescents living in or originating from north and west Africa. Individuals who carried the JP2 clone of *A. a.* had a significantly increased risk of periodontal attachment loss, either alone (relative risk 18.0) or together with non-JP2 clones of *A. a.* (RR 12.4). A much less pronounced disease risk was found in those carrying non-JP2 clones only (RR 3.0).

Another virulence factor of this bacterial species is the cytolethal distending toxin (*Cdt*) which is a recently characterized genotoxin that blocks cell cycle progression in specific classes of eukaryotic cells and cell lines. This holotoxin is made up of three subunit proteins designated Cdt A, CdtB and CdtC. The Cdt has, to date, only been found in *A. a.* among bacterial species indigenous to the human oral cavity²⁷. Several types of oral epithelial cells and T lymphocytes are particularly susceptible to the *A. a.*'s Cdt. Therefore, expression of this relatively unique secreted bacterial virulence factor may, in part, play a role in the pathogenic capacity of this microorganism. Literature reveals once more, that not every *A. a.* presents cytotoxic Cdt activity. Based on a study by Ahmed²⁸ the 86% of *A. a.* isolates presented the complete operon and its characteristic cytotoxic activity. A work by Tan and coworkers showed a close association between

aggressive forms of periodontitis and *A. a.* strains that belonged the Cdt genotype²⁹.

In summary, numerous studies show the wide range of genetic diversity of *A. a.* strains isolated throughout the world^{10,22,12,23,30,31,32,33,34}

These genetic variation among natural isolates suggest a variability in the virulence potential of strains. Actually Kaplan, evaluating six characteristics of *A. a.* (serotype, 16S rRNA, *ltx*, *flp-1*, *cdt*, and AP-PCR genotypes) identified three phylogenetic clusters. One cluster consisted of serotype *b* strains, a second cluster consisted of serotype *c* strains, and a third cluster consisted of serotype *a*, *d*, *e*, and *f* strains. Serotype *b* and *c* strains formed distinct, monophyletic groups within the serotype {*b,c*} cluster while serotypes *a*, *d*, *e* and *f* formed an independent group. On the bases of these findings Kaplan suggested the hypothesis that LJP may have two different etiologies and epidemiologies: in Caucasians LJP may be associated with diverse *A. a.* clones acting as opportunistic pathogens, whereas in some Africans and African-Americans LJP may be associated with JP2-like strains acting as exogenous pathogens. It is also possible that JP2-like strains are acting as opportunistic pathogens that are simply more prevalent in Africans and African-Americans, who have an increased susceptibility to LJP for other reasons, perhaps related to host determinants⁷.

Rationale for research

In Spain, to the best of our knowledge, no single study has been conducted with the aim to characterize the *A. a.* isolates in Spanish periodontitis patients. For this reason, our aim was to investigate the serotype distribution of *A. a.* and the polymorphism of the genes that codify the leukotoxin and the operon of the cytolethal distending toxin in

periodontitis patients in Spain.

MATERIALS AND METHODS

Patients' selection and samples' reception

The plaque samples used in this study were obtained from a consecutive series of subgingival microbial samples forwarded to the Laboratory of Microbiology, Faculty of Dentistry, University Complutense, Madrid, Spain, over 18 months from January 2007 to July 2008.

Microbiological samples were collected by periodontists in private practices as well as postgraduate students in the Postgraduate Clinic of Periodontology in the same Faculty of Dentistry. Each sample was accompanied by clinical information including patient age, gender, clinical diagnosis, brief clinical history, smoking status, sample sites and time and date of sampling. Pocket depth, recession and presence or absence of plaque, bleeding on probing and suppuration were also recorded on the 4 selected sites per patient, from which samples were taken.

Samples were taken from the deepest pocket in each quadrant³⁵. At selected sites, supragingival plaque was carefully removed to avoid bleeding using sterile gauze and / or curettes. Then, these sites were dried with sterile cotton rolls and gentle air blowing. Two consecutive sterile medium size paper points (Maillefer, Ballaigues, Switzerland) were inserted as deep as possible in the pocket, and left in place for 10 seconds. The paper points were transferred to a vial containing 1.5 ml of Reduced Transport Fluid (RTF)³⁶, and pooled with all the other paper points. The vial, kept at room temperature, was sent to the laboratory and processed within 24 hours.

Recording of clinical data

Only patients presenting 4 locations with probing depth deeper than 4 mm and at least 4 locations with clinical attachment loss higher than 4 mm were included in the study.

Subjects with less than 16 teeth and patients who had taken any antibiotic medication during the previous 3 months were excluded.

A periodontal diagnosis was established for each patient according to the clinical diagnostic classifications approved by the American Academy of Periodontology in 1999³⁷. Patients with untreated periodontitis were divided into chronic and aggressive form. In addition, patients already treated but showing signs of disease or a less than optimal treatment outcome, were placed in an additional category named as “refractory”.

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 medium³⁸.

The blood agar plates were studied after 7 and 14 days of anaerobic incubation (80% N₂; 10% H₂; 10% CO₂ at 37°C). Plates were carefully examined for the identification of *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, *Tannerella forsythia*, *Parvimonas micra*, *Capnocytophaga* spp., *Eikenella corrodens* and *Fusobacterium* spp., 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. a.*, were incubated for 3-5 days at 37°C in air with 5% CO₂. After this period, plaques were carefully examined to determine the presence of *A. a.*. The identification of *A. a.* was based on three typical features: the colonies' morphology, a positive response to catalase test and the results of a set of specific enzymes (*RapID NH system*, Innovative Diagnostics Systems, Inc., Decatur, GA - USA). Some example of cultured *A.a.* on DENTAID-1 plates is shown at Figures 1 and 2. A maximum of 2 isolates per subject were subcultured and purified. All original samples were stored in cryogenic vials containing polypropylene beads (CryoBeads™, Hardy Diagnostics, Santa Maria, CA - USA) at -80°C until further use.

Culture techniques

Cells proceeding from stored samples were sowed in DENTAID-1 agar plates and incubated at 37°C in air with 5% CO₂. After 3 days a single colony from every plate was passed into a new, sterile DENTAID-1 agar plate. This step was repeated for at least two times, then a single colony per plate was passed a into a sterile plastic tube filled with 10 ml of brain heart infusion (BHI) broth (BBL™ Brain Heart Infusion, Oxoid, Basingstoke, England).

Isolation of genomic DNA

Cells were harvested by 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). After incubation at 37°C for 30 min, proteinase K, RNAase and sodium dodecyl sulfate (SDS), were added to

a final concentration of 2 mg/μl, 1 mg/μl and 1 %, respectively. The mixture was incubated at 37°C for 30 minutes. 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 resuspended in 50 μl of sterile distilled water.

The phenol-chloroform technique was chosen after comparing three different methods for extracting DNA: extraction of DNA by using the Boiling Lysis procedure, phenol-chloroform method and G-NOME[®] DNA isolation kit (MP Biomedicals LLC, CA, USA). For this purpose, three random plates of cultivated reference strains of *A. a.* (ATCC 29523 and ATCC 29524) were chosen. Then, the three DNA extraction techniques were performed for each plate. The G-NOME[®] DNA isolation kit was used according to the manufacturer's instruction. For the Boiling Lysis procedure every sample was passed to a new sterile Eppendorf vial with 200 μl of sterile distilled water and dispersed by vortexing at high-speed for 3 minutes. After vortexing, the paper points were removed and were incubated at 103°C for 10 minutes in a thermal block, to obtain cell wall lysis. Then, the samples were frozen at -20°C for 10 minutes. The samples were centrifuged at 12000 rpm for 10 minutes, and the supernatant recuperated. The raw DNA extracted with this method was immediately purified by using the GFX Purification kit (GE Healthcare, UK), and was eluted in 50μl of sterile distilled water and stored at -20°C pending PCR analysis

The phenol-chloroform method proved to be the most efficient technique in terms of costs-benefits, as it provided a sufficient amount of high quality DNA. However this

technique is highly operator-dependent and it required a larger apprenticing period to obtain standardized results.

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 Tris- Acetate EDTA (TAE) buffer. The gel was stained with ethidium bromide (10 µg/ml) and visualized on an UV transilluminator.

Serotyping of strains

Serotypes *a* to *f* were determined by PCR technique, based on specific sequences from the gene clusters responsible for the O-polysaccharide that characterizes the distinct serotypes. The PCR reaction was performed with the specific primers described by Kaplan⁷. For serotypes *a* to *f* a PCR was performed. 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 MgCl₂, 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. The PCR assays are performed with a Veriti® 96-Well Thermal Cycler (Applied Biosystems Inc., Foster City, CA - USA). 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 seconds of denaturation at 95°C, 1 minute of annealing at 55°C and 72°C for 2 minutes, and a final step of extension at 72°C for 10 minutes.

Positive and negative controls were included for each performed PCR: as positive

controls reference strains ATCC 29523 and ATCC 29524 were used, while sterilized distilled water was used as negative control.

A 15 µl aliquot of each PCR was added to 2 ml of gel loading dye (0.25% bromophenol blue, 50% glycerol, 100mM EDTA pH 8.0) and electrophoresed through a 2.0 % agarose gel in 1X TAE buffer. The PCR products are visualized by staining with ethidium bromide (10 mg/ml) and visualized under UV illumination.

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.¹⁴. The PCR reaction was performed in a 25 µl final volume containing 2.5 µl of 10x PCR buffer, 1.0 µl of 25mM MgCl₂, 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'-CAGATCAAACCTGATAACAGTATT-3', and the primer downstream from the deletion had the sequence 5'-TTTCTCCATATTCCTCCTTCTGT-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. a.*'s leukotoxin operon. Positive and negative controls were included in the PCR: as positive controls reference strains ATCC 29523 and ATCC 29524 were used, while sterilized distilled water was used as negative control. The molecular weights of the PCR products were determined by visualization and comparison with standard molecular weights markers using agarose gel electrophoresis.

Detection of Cdt's polymorphism in *A. a.*

The presence of the operon that codifies for the Cdt toxin was also determined by means of PCR using the same procedure previously described for Ltx 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

Descriptive statistics, including frequency distribution, were used to characterize the sample in terms of the different serotypes, the polymorphism of the genes that codify the leukotoxin and the operon of the cytotoxic distending toxin.

Different subgroup analyses were performed to compare patients with different clinical diagnosis chronic, aggressive or refractory, serotype, the presence of leukotoxin overproducers and the *cdt* gene.

These groups were related to other variables including age, gender, periodontal attachment level, probing pocket depth, bleeding on probing (0/1), plaque index (0/1) (at sampled sites), suppuration (0/1) and smoking habits.

For parametric variables ANOVA test with Bonferroni adjustment was used to determine the presence of statistically significant differences between groups, while the Pearson's chi-square test was used in case of non-parametric variables. In case of dichotomous variables and small samples the Fisher's exact test was preferred.

All statistical analysis were performed using SPSS v. 13.0 software (SPSS Inc., Chicago, IL – USA)

RESULTS

In total, 720 samples were received in that period, and 696 fulfilled the predetermined inclusion criteria. Among them, 42 (6 %) were positive for *A. a.* All the samples evaluated proceeded from Caucasian individuals. Two of the *A. a.* strains were contaminated and lost during the purification phase and only 1 to 2 isolates from 40 patient samples were kept at -80°C and one strain per patient was further analyzed.

Serotype distribution

From the 40 isolates, the predominant serotype was type *a* (37.5%), whereas serotypes *b* and *c* showed a distribution of 20 % and 15 % respectively. Serotypes *d*, *e* and *f* were not detected. More than one serotype was found in 27.5% of the patients, most of them were coinfections of serotypes *a* and *b* (54%). None of the isolated strains were non-typeable. Results are showed in table 3. Some of the products of PCR with serotypes' primers are shown in figures 3 and 4.

LtxC promoter deletion

The results for the presence of a 530-bp deletion in the promoter region of the LtxC gene of *A. a.* show that none of the Spanish isolates belonged to the highly leukotoxic strain JP2. A clear band equivalent to a molecular weight of 1034-bp is appreciable in the 1.0 % agarose gel for every strain. Results are showed in table 4. Figure 5 shows a picture of an electrophoresed gel with products of PCR with *ltx* primers

Cdt operon detection

The PCR products determined by visualization in 1% agarose gel showed that 34 of the 40 isolates presented an amplified segment of 2016 bp.

85 % of the isolates presented the complete operon of the Cdt gene while in 15 % the operon that codifies for the Cdt toxin was not present. Results are shown in table 5.

Figure 6 shows an electrophoresed gel with products of PCR with *cdt* primers

Subgroup analyses

Data used for the subgroup analysis based on periodontitis groups, *cdt* and serotypes are reported in tables 6, 7 and 8 respectively.

a) periodontitis

32.5% patients were diagnosed with aggressive periodontitis, which is characterized by rapid attachment loss, bone loss and familial aggregation³⁹. 47.5% were diagnosed with chronic periodontitis characterized by having at least 4 sites with pockets depth > 4mm, and at least four sites with attachment level measurements < 4mm.

As we can observe from the subgroup analysis, refractory patients showed less probing pocket depths, more suppuration, and more coinfections (4 out of 8 patients). Aggressive periodontitis patients were younger and harbored half of the 8 serotype *b* strains. Chronic periodontitis patients demonstrated the lower percentage of bleeding on probing.

The ANOVA test with Bonferroni adjustment revealed a statistically significant difference for patients' age between the aggressive periodontitis groups and the chronic

periodontitis group.

Gingival recession was statistically significant higher in the refractory group when compared with the aggressive group, while there was no significant difference between chronic and aggressive groups, or refractory and chronic groups.

The frequency of non smokers versus smokers (including former smokers) was homogenous between patients with chronic periodontitis and refractory periodontitis while it was significantly increased in patients presenting aggressive periodontitis.

b) Cdt operon

A positive result for *cdt* was largely present in the analyzed samples, and this result doesn't seem to depend on serotypes' distribution. Nevertheless while every serotype *c* patient presented a positive *cdt* result, only 62,5% of the patients with coinfection presented *cdt* genes. No differences are present regarding clinical variables between *cdt* positive and *cdt* negative patients.

c) serotypes

While there is a significantly higher percentage of females with serotype *a*, a higher percentage of men shows coinfections, although this data is not statistically significant. No other significant differences could be evicted from these data, but there seems to be a tendency toward a higher plaque index and suppuration scores for those patients with serotype *c* or coinfections.

The proportion of serotype *b* in subjects with cultivable *A. a.* and aggressive periodontitis was 10 % higher than that in subjects with cultivable *A. a.* and chronic

periodontitis.

A study by Yang reported that serotype *b* accounted for the majority of serotypes in patients with aggressive periodontitis whereas there was no difference in the distribution of serotypes *a*, *b* and *c* in patients with chronic periodontitis in a sample of 115 subjects in the University of Pennsylvania¹³.

DISCUSSION

The cumulative frequency detection of *A. a.* in Spanish adult periodontal patients by culture techniques was estimated around the 6 %.

In a previous study performed in Spain a prevalence of 6.3 % was also detected in periodontitis patients with culturing techniques and the same specific medium Dentaid-1, while a prevalence of 18.8% was reported when using PCR methods⁴⁰. A similar value (3.2%) was also reported in another study of our group with a comparable population in Spain⁴¹ but, in this case, a different culture medium was used (Tryptic Soy Serum Bacitracin Vancomycin Agar or TSBV) which demonstrated a lower recovery rate³⁷.

Epidemiologic studies in adults showed that the prevalence of these microorganism varies substantially. The prevalence of *A. a.* was estimated around 3% to 38% in the United States^{42,43,44,45}, 15% in Sri Lanka⁴⁶, 83% in China⁴⁷, 93% in Thailand⁴⁸, 47.7% in Germany and 26.7% in Korean periodontal patients⁴⁹.

Serotypes' distribution

This study examined the distributions of the different serotypes in culture positive plaque samples from Spain. We observed that from these 40 isolates, 15 corresponded to serotype *a*, 8 to serotype *b* and 6 to serotype *c*. Monoinfection with serotypes *d*, *e* or *f* was not found, this is in accordance with previous studies which have demonstrated that the prevalence of *A. a.* serotypes in populations from geographically distant regions show three predominant serotypes (*a*, *b*, *c*).

In the United States, serotype *b* strains were more frequently isolated from patients

with localized juvenile periodontitis (LJP) than other disease-types or periodontally healthy subjects^{10,12}. According to Listgarten, elevated responses to serotype *a* were more common in chronic periodontitis⁵⁰. Our study confirms those data, since we found a higher percentage of serotype *a* strains in chronic periodontitis patients.

On the contrary, Dogan reported that serotype *a* was a major serotype in LJP but the results for adult periodontitis showed no differences between the distributions of serotypes *a*, *b* and *c*⁵¹.

A higher frequency of serotype *b* was reported in Finnish patients with periodontitis, while serotype *c* was more related to periodontally healthy subjects^{19,12}.

Predominance of serotypes other than *b*, however, has been reported from other parts of the world. In Korea¹⁸ and Japan⁵², for example, serotype *c* was frequently observed in plaque samples from sites with pathology. Yamamoto reported that serotypes *d*, *e* and untypeable strains were found in Japanese subjects at the following rates, 5%, 23% and 10% respectively⁵³. These serotypes have been recognized more recently^{50,21} and they are less frequently found than *a*, *b* and *c*^{8,20,54}.

Distribution of *ltx* gene

The highly leukotoxic strain of *A. a.* has not been found in any of our patients. This result confirms the literature's reports. Previous studies have demonstrated, in fact, that JP2 clone of *A. a.* infects more frequently people of African descent^{30,55} than northern Europeans²³, Chinese^{56,21} or people of Asian descents in the United States³¹.

In a cross sectional study in Brazil, greater clinical attachment loss was associated with

infection by highly leukotoxic *A. a.*. This result is consistent with the recent longitudinal study performed by Haubek in Morocco were individuals who carried the JP2 clone of *A. a.* had a significantly increased risk of periodontal attachment loss²⁵.

Distribution of *cdt* gene

In 34 out of the 40 strains of periodontitis patients (85 %) *cdt* gene polymorphism has been detected. The prevalence in *A. a.* of this toxin is extremely variable, Ahmed et al. found 43 of 50 strains from periodontitis patients contained all three *cdt* genes and expressed Cdt activity²⁷. In another study, PCR revealed that only 13 out of 106 diseased sites were positive for this genes⁵⁷, Fabris also reported that 39 out of 40 were present in another patient cohort⁵⁸.

Evaluation of subgroup analysis

Data proceeding from our population confirm the previous studies. In periodontal patients infected with *A. a.*, aggressive periodontitis has a higher prevalence (32,5 %) than in the total population of periodontal patients; moreover, patients with aggressive periodontitis were younger than patients with chronic and refractory periodontitis. The fact that serotype *b* seems to be more common in patients with aggressive periodontitis could confirm the data that suggest a stronger association with aggressive periodontitis for this strain. Nevertheless most of the patients with aggressive periodontitis presented serotype *a* *A. a.*. This findings may strengthen the hypothesis suggested by Kaplan of a different aetiological pattern for aggressive periodontitis in Caucasian individuals.

As for the other differences in clinical data, lower PD and higher REC values in refractory periodontitis patients reflect the effects of past periodontal therapies, while the lower

tendency to bleeding (lower BOP) in patients with chronic periodontitis may depend on the effects of smoking, which is more frequent in these group.

CONCLUSIONS

In general we can see the great variation among the different studies, this may be due to differences in bacterial detection techniques, ethnicity, geographic location and the periodontal condition of the population samples.

This kind of studies, where the clonal types of bacteria are studied, may help to explain why the presence of certain bacterial species is associated with periodontal disease in one population group but not in others.

FUTURE RESEARCH PROPOSALS

These data, all proceeding from a Spanish patients, only give us partial information, although the sample was well dimensioned and representative of the whole population.

To allow us a more complete analysis our research team is conducting a new study, in collaboration with the University of Rabat, with patients proceeding from Morocco, in order to confront data from Spanish and Moroccan patients.

To complete these analysis another research has been proposed and designed, including subjects of Moroccan descents living in Spain, in order to evaluate the presence of variations in the distribution of *A. α*. due to environmental factors.

FIGURES AND TABLES

Primer	Sequence (5'-3')	Serotype	PCR product size
P11	TCTCCACCATTTTTGAGTGG	<i>b</i>	333 pb
P12	GAAACCACTTCTATTTCTCC	<i>c</i>	268 pb
P13	CCTTTATCAATCCAGACAGC	<i>f</i>	232 pb
P14	AAAACCTTCTCATCGGGAATG		
P15	TGGGTCATGGGAGGTACTCC	<i>a</i>	293 pb
P16	GCTAGGAACAAAGCAGCATC		
P17	TGGAACGGGTATGGGAACGG	<i>d</i>	411 pb
P18	GGATGCTCATCTAGCCATGC		
P19	ATTCCAGCCTTTTGGTTCTC	<i>e</i>	311 pb
P20	TGGTCTGCGTTGTAGGTTGG		

Table 1 Primers selected for the PCR serotyping of *A. α*.

Primer	Sequence (5'-3')	Gen	PCR product size
Cdt A ₁	ATGAAGAAGTTTTTACCTGGTC	CdtA	669
Cdt A ₂	TAATTAAGGGGTCAAGCTTCT		
Cdt B ₁	TGCAATCCCATTTTGAATTA	CdtB	851
Cdt B ₂	TTAGCGATCACGAACAAAAC		
Cdt C ₁	CTTTAGGTACATGTATTGAA	CdtC	560
Cdt C ₂	GATGCTTAGTGGGGCGAGGG		

Table 2 Primers used for the detection of the presence of the cdt operon

SEROTYPE	Monoinfection 72,5 % (29)	Coinfection 27,5% (11)	
Serotype a	37.5 % (15)	a + b	54%(6)
Serotype b	20 % (8)	a + c	28% (3)
Serotype c	15 % (6)	a + d	19% (2)
Serotype d	0% (0)	Others	0% (0)
Serotype e	0% (0)		
Serotype f	0% (0)		

Table 3 Distribution of serotype strains for A. a.

Ltx gene	% (N)
Ltx C deletion (504- bp PCR product)	0% (0)
Absence of Ltx deletion (1034- bp PCR product)	100% (40)

Table 4 Distribution of *ltx* gene

CDT operon	% (N)
Cdt gene presence (2016-bp PCR product)	85% (34)
Cdt gene absence (less than 2016-bp PCR product)	15% (6)

Table 5 Distribution of *cdt* gene

Periodontitis	Age		Gender		Serotype			Genes			Tobacco		PPD	CAL	Rec	BOP (%)	Pli (%)	SUP (%)
	M	F	a	b	c	co	ftx	cdt	Y	N	F							
Chronic (N=19)	7	12	8	3	3	5	19	16	12	7	0	6,97±0,89	7,85±0,9	0,91±0,64	71,05±39,32	46,05±45,08	17,53±25,28	
Aggressive (N=13)	5	8	6	4	1	2	13	12	11	2	0	6,94±1,43	7,52±1,67	0,6±0,57	84,62±19,2	46,15±41,89	13,46±29,96	
"Refractory" (N=8)	3	5	1	1	2	4	8	6	4	2	2	5,88±0,97	7,67±1,68	1,78±1,42	84,38±22,9	40,63±42,13	31,25±45,81	

Table 6 Forms of periodontitis

CDT	Age	Gender		Serotype			Genes		Periodontitis			Tobacco			PPD	CAL	Rec	BOP (%)	PII (%)	Sup (%)
		M	F	a	b	c	CO	Itx	C	A	R	N	Y	F						
CDT+ (n=34)	44,68±9,96	13	21	13	7	5	8	34	16	12	6	23	9	2	7,76±1,37	1,01±0,58	78,68±9,6	45,59±41,96	20,09±33,08	
CDT- (n=6)	48,67±9,37	2	4	2	1	0	3	6	3	1	2	4	2	0	7,38±1,03	0,79±0,4	75±41,83	41,67±49,16	12,5±20,32	

Table 7 Presence of *cdt* gene

Serotype	Age	Gender		Genes		Periodontitis			Tobacco			PPD	CAL	Rec	BOP (%)	PH (%)	Sup (%)
		M	F	fts	cdt	C	A	R	Y	N	F						
a (n=15)	44±12,55	3	12	15	13	8	6	1	10	5	0	6,68±1,05	7,41±1,22	0,76±0,83	75±35,36	33,33±44,99	15,53±21,08
b (n=8)	47,38±10,06	2	6	8	7	3	4	1	7	1	0	6,03±0,87	7,31±0,75	1,31±0,86	75±37,8	21,88±36,44	12,5±35,36
c (n=6)	45,17±9,43	3	3	6	6	3	1	2	4	2	0	7,33±1,27	8,33±1,31	1±1,27	83,33±25,82	66,67±37,64	25±38,73
Co (n=11)	45,55±6,11	7	4	11	8	5	2	4	6	3	2	7,02±1,31	8,05±1,7	1,02±0,9	81,82±25,23	65,91±34,05	25±38,73

Table 8 Serotypes

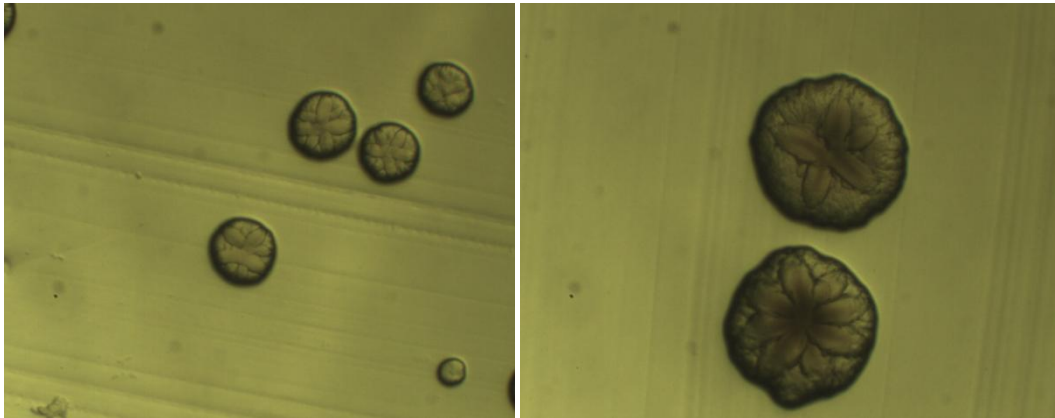
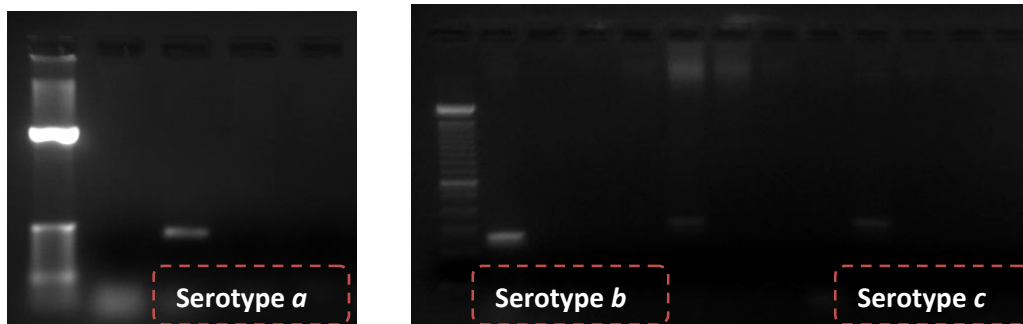


Figure 1 and 2 *Agaritibacter actinomycetemcomitans* on DENTAID-1 plates

Figures 3 and 4 Examples of different results for PCR performed with serotypes' primers. Electrophoresis on 2.0 % agarose gel with λ -Hind-III and 1kb dimensional markers, respectively.



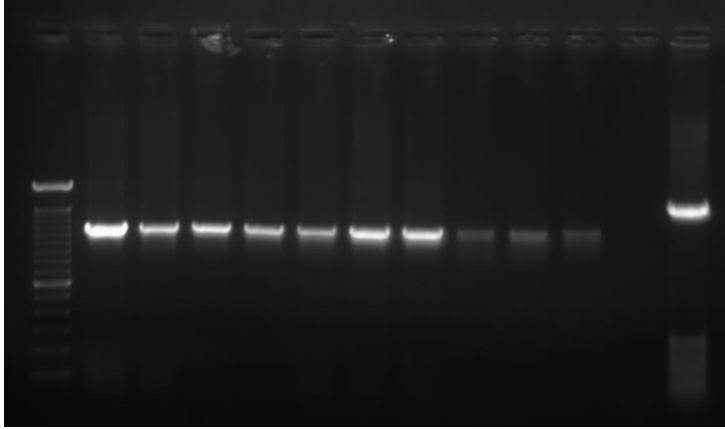


Figure 5 Examples of results for PCR performed with */tx* primers. Electrophoresis on 1.0 % agarose gel with 1kb dimensional marker.

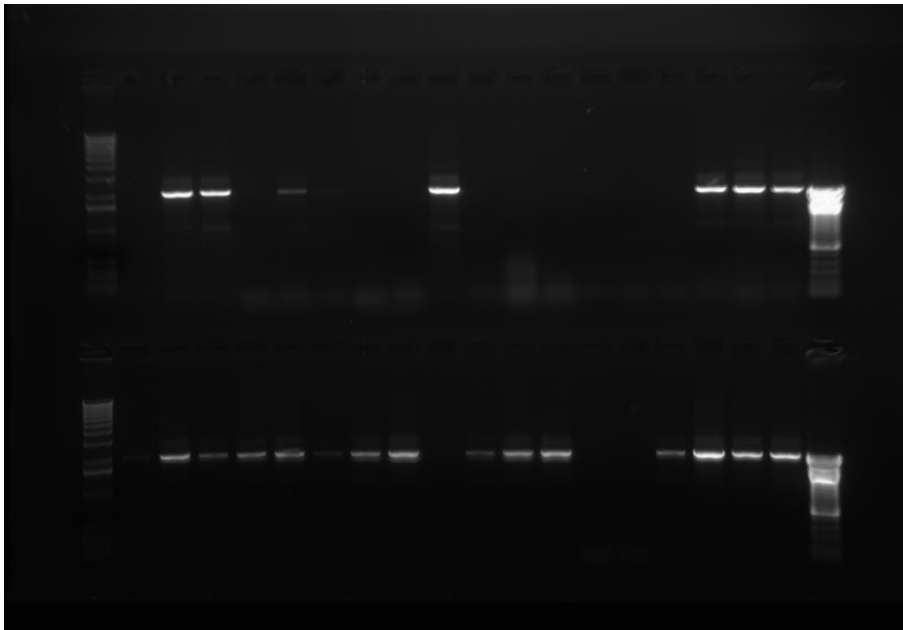


Figure 6 Examples of results for PCR performed with *cdt* primers. Electrophoresis on 1.0 % agarose gel with 1kb dimensional marker.

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