

## DISTRIBUTION OF BIOTYPES AND LEUKOTOXIC ACTIVITY OF *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* ISOLATED FROM BRAZILIAN PATIENTS WITH CHRONIC PERIODONTITIS

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### ABSTRACT

*Aggregatibacter actinomycetemcomitans* is an important etiologic agent of the periodontitis and is associated with extra-oral infections. In this study, the detection of the *ltxA* gene as well as the *ltx* promoter region from leukotoxic *A. actinomycetemcomitans* isolated from 50 Brazilian patients with periodontitis and 50 healthy subjects was performed. The leukotoxic activity on HL-60 cells was also evaluated. Leukotoxic activity was determined using a trypan blue exclusion method. The 530 bp deletion in the promoter region was evaluated by PCR using a PRO primer pair. *A. actinomycetemcomitans* was detected by culture and directly from crude subgingival biofilm by PCR using specific primers. By culture, *A. actinomycetemcomitans* was detected in nine (18%) of the periodontal patients and one (2%) healthy subject. However, by PCR, this organism was detected in 44% of the periodontal patients and in 16% of the healthy subjects. It was verified a great discrepancy between PCR detection of the *ltx* operon promoter directly from crude subgingival biofilm and from bacterial DNA. Only one periodontal sample harbored highly leukotoxic *A. actinomycetemcomitans*. Moreover, biotype II was the most prevalent and no correlation between biotypes and leukotoxic activity was observed. The diversity of leukotoxin expression by *A. actinomycetemcomitans* suggests a role of this toxin in the pathogenesis of periodontal disease and other infectious diseases.

**Key-words:** *Aggregatibacter actinomycetemcomitans*, biotype, leukotoxin, periodontitis.

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### INTRODUCTION

*Aggregatibacter actinomycetemcomitans* is a capnophilic gram-negative microorganism, member of the indigenous oral microbiota, and it is found in periodontal lesions, especially in young adults. This organism has been associated with several infectious diseases, such as septic endocarditis, brain and lung abscesses, osteomyelitis, and cardiovascular pathologies (8,27), and aggressive and chronic periodontal diseases (25,32,33).

This microorganism is grouped into 10 different biotypes, and their distribution depends on several factors, such as,

geographic location and clinical condition of the patients (3,28). However, there is a need to confirm this statement in Brazilian population, which presents several peculiarities, such as racial miscegenation and cultural aspects. In addition, there are no available data to support the relationship virulence-biotype in this microorganism.

The virulence of *A. actinomycetemcomitans* is not well understood, but this organism produces virulence factors including a heat-labile leukotoxin (7,9) that belongs to the repeat-in-toxin (RTX) family. The gene *ltxA* encodes the leukotoxin, genes *ltxB* and *ltxD* encode proteins presumably required for the toxin secretion, and gene *ltxC* encodes the acyl-transferase

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production that is responsible for the toxin transformation, from protoxin to the active form (20).

Genetic determinants for the synthesis, activation, and secretion of leukotoxin are localized on *ltx* operon, which seems to be present in all *A. actinomycetemcomitans* strains (16,31), however levels of toxin expression vary considerably in different clones. It has been observed that the leukotoxin expression is related to the presence of a 530 bp deletion in the promoter region, although physiological factors may also be involved in this regulation (18).

The presence of leukotoxin has been associated with the ability of *A. actinomycetemcomitans* to evade the main defense line into periodontal pocket and it may contribute to the pathogenesis of periodontal disease (17). The leukotoxic activity is determined by a cytolytic action that kills human polymorphonuclear leukocytes, T lymphocytes and macrophages. In contrast, epithelial and endothelial cells, fibroblasts, and platelets are resistant to this action (8,17).

Due to the variation in the leukotoxin genes transcriptional regulation, *A. actinomycetemcomitans* have been classified into high and low leukotoxin-producing strains. The occurrence of high leukotoxin-producing *A. actinomycetemcomitans* strains has showed variations in different ethnic populations (12,16) and the presence of low leukotoxin-producing strains in patients with aggressive periodontal disease, as well as, in healthy subjects (5,26) suggest that genetic and environmental factors may interfere with the leukotoxin expression and the host's response (10). Therefore, in this study, the biotypes distribution and the *ltx* gene presence in *A. actinomycetemcomitans* isolated from Brazilian patients with advanced periodontitis were determined.

## MATERIAL AND METHODS

### Patients and subgingival samples

Fifty patients with chronic periodontitis visiting the Clinic of Periodontology of the Dental School of the University of São Paulo, São Paulo, Brazil, and 50 periodontally healthy subjects were selected. Patients with periodontitis were 27 females and 23 males aged between 18 to 50 years old (mean age  $37.72 \pm 10.63$ ), while healthy subjects were 30 females and 20 males aged from 18 to 35 years old (mean age  $23.76 \pm 5.79$ ). All patients displayed  $\geq 25$  teeth, did not require pre-medication with antibiotics for a periodontal examination, and were not under any periodontal therapy during the last 6 months. Periodontal patients and healthy subjects were submitted to a full-mouth periapical radiographic examination with a Kodak film (Ektaspeed plus). Patients with chronic periodontitis showed clinical and radiographic evidences of bone loss and periodontal pocket depth exceeding 5mm, while healthy subjects did not show evidences of bone loss and gingival inflammation. Exclusion criteria included pregnancy, history of self-

medication, nursing, diabetes, autoimmune diseases and other systemic pathology.

Subgingival samples from both groups were obtained by using two sterile paper points (Dentsplay, Ind. Co. Ltd., RJ, Brazil) inserted to the apical portion of the periodontal pocket or gingival crevice for 60 s, and transported in VMGA III medium (23). Samples were plated, in duplicate, onto selective trypticase soy-serum-bacitracin-vancomycin agar (30) and after 72 h of incubation in anaerobiosis (90% N<sub>2</sub> + 10% CO<sub>2</sub>) at 37°C, characteristic colonies of *A. actinomycetemcomitans* were identified by biochemical methods (29). Ethic Committee of the Institute of Biomedical Sciences, University of São Paulo (ICB 087/CEP), approved this study.

### Biotyping

The biotyping of all *A. actinomycetemcomitans* isolated was based on dextrin, maltose, mannitol and xylose fermentation (28).

### DNA extraction

Bacterial DNA was obtained from clinical samples and from colonies. Subgingival samples were homogenized and 500 µl were transferred to tubes containing 500 µl of sterile Milli-Q water. After centrifugation at 14,000 x g for 10 min, the pellet was suspended in 300 µl of sterile Milli-Q water and boiled for 10 min. In addition, two colonies were picked from brain heart infusion agar supplemented with 0.5% yeast extract and 5% horse blood and resuspended in 500 µl of sterile Milli-Q water, homogenized and boiled for 10 min (2). Finally, both clinical samples and colonies after boiling were harvested by centrifugation at 14,000 x g for 10 min, and the supernatants were stored at -20°C.

### PCR assay

PCR amplification for detecting *A. actinomycetemcomitans* from clinical samples was performed in final volumes of 25 µl, containing 10 X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 200 µM dNTP (Invitrogen do Brasil, São Paulo, Brazil), 1.5 U Platinum *Taq* DNA polymerase (Invitrogen), 0.4 µM each primer and 10 ng of DNA template. In all the PCR reactions this mixture was used. Table 1 shows the primer pairs used in PCR reactions. Amplifications were performed in a Perkin Elmer Amp PCR System 9700. The temperature profile included an initial step of 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing temperature of primer pair for 30 s, and 72°C for 30 s and a final step of 72°C for 5 min (Table 1).

Twenty microliters of the amplified products were analyzed by electrophoresis in 1% agarose gel. Gels were stained with 0.5 µg/ml ethidium bromide and photographed under UV transilluminator with a Kodak Eletrophoresis Documentation and Analyses System-120. *A. actinomycetemcomitans* JP2 with a 530-bp deletion was used a positive control, and *A. actinomycetemcomitans* ATCC 33383 as negative control.

**Table 1.** Primers used for detection of *ltx* (promoter) and *ltxA* leukotoxin genes in clinical samples and culture.

| Primer pair      | Oligonucleotide<br>5' → 3'                | Amplicon<br>(bp)         | Amplification<br>cycles    | Ref. |
|------------------|---|--------------------------|----------------------------|------|
| Promoter         |   |                          |                            |      |
| PRO1             | GTAATG AAAATT GCC GCTTT                   | 1076 without<br>deletion | 30 cycles:<br>94°C 30 sec  | 24   |
| PRO2             | CCA TAT TAA ATC TCC TTG TTAA              | 545 with deletion        | 52°C 30 sec<br>72°C 30 sec |      |
| <i>ltxA</i> gene |   |                          |                            |      |
| LKT1             | GGAATT CCT AFF TAT TGC GAA ACA ATT TGA TC | 284                      | 30 cycles:<br>94°C 30 sec  | 19   |
| LKT2             | GGAATT CCT GAA ATT AAG CTG GTA ATC        |                          | 55°C 30 sec<br>72°C 30 sec |      |

### Leukotoxin assay

Leukotoxin activity was determined by using trypan blue exclusion method, with modifications (14). Briefly, promyelocytic leukemic cell line HL-60 was grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), penicillin G (100 UI/ml), and streptomycin (100 µg/ml) in an atmosphere of 5% CO<sub>2</sub> at 37°C. 100 µl of bacterial inoculum (10<sup>6</sup> UFC) or supernatant from *A. actinomycetemcomitans* cultures were added and incubated at 37°C for 2 h. The proportion of HL-60 cells and bacterial cells was estimated in 1/100.

HL-60 cells without bacteria or supernatant were used as negative controls. The assay was performed in microtubes without antimicrobials. After incubation, 100 µl of 0.4% trypan blue were added to mixtures, placed on hemocytometer and then observed under a light microscope. The cells taking up the trypan blue (death cells) and cells excluding the dye (viable cells) were counted in a Neubauer chamber. Percentages of lyses were determined by dividing the number of surviving cells by the number of cells in the negative control, and values were given as the average of assays. The percentage of viable cells as the average of five viability measurements, each requiring the scoring of 100 cells was determined. Measurements were repeated five times to assess the reproducibility of viability measurement.

Highly leukotoxic strains were able to lyses ≥ 70%; strains showing medium toxicity produced cell lyses values from 70% to 50%; strains with low toxicity produced cell lyses values from 50% to 25%, and non-toxic strains < 25%. Highly producer *A. actinomycetemcomitans* JP2 was used as positive control, *A. actinomycetemcomitans* ATCC 43718 (Y4), a medium leukotoxin-producing strain, and *A. actinomycetemcomitans* SUNY AB 67 or ATCC 33383, non-leukotoxic strains, used as negative control.

### Statistical analysis

The results were expressed as median, standard deviation and percentages. Chi-square ( $\chi^2$ ) and multiple comparison tests

were performed to check differences in detection of *A. actinomycetemcomitans*, with a significant level at 5%.

## RESULTS

### Bacterial detection and leukotoxin-producing strains

Probing depth in patients with periodontitis was  $6.24 \pm 1.39$  mm, while healthy subjects presented probing depth of  $1.78 \pm 0.54$ . A total of 91 *A. actinomycetemcomitans* strains were obtained from 9 (18%) patients with periodontitis and three isolates were obtained from one (2%) healthy subject.

Seven biotypes from periodontal patients were identified, I, II, V, VI, VIII, IX and X. Biotype II was found in 12% of the periodontal patients and it was the most prevalent, followed by biotype X found in 8% of the patients. Only one healthy subject harbored three strains (two biotypes II and one X). Most of strains isolated from clinical samples generally belonged to the same biotype, but some clinical samples harbored from two to five biotypes as observed in Table 2.

This microorganism was detected in 44% of the periodontitis clinical samples and in 16% of the healthy subjects by using the LKT primer pairs. Moreover, by using the PRO primers, *A. actinomycetemcomitans* was detected in 6% of the clinical samples, but it was not observed in samples from healthy individuals. The presence of the highly leukotoxic strains showing the 530 bp deletion was verified in 13 (14.2%) of the 91 *A. actinomycetemcomitans* obtained from periodontitis patients. The three strains recovered from healthy subjects produced a 1.0 kb amplicon characteristic of low leukotoxin-producing strains. Fig. 1 shows highly leukotoxic and medium to low leukotoxic strains.

### Leukotoxin assay

As presented in Table 3, some patients were colonized by strains producing different levels of leukotoxin. The toxic activity in most of the isolates showed to be cell bound, since supernatants of the highly leukotoxic strains did not produce significant damage on HL-60 cells.

**Table 2.** Distribution of 91 *A. actinomycetemcomitans* biotypes isolated from periodontal patients (50) and healthy subjects (50).

| Clinical samples<br>(No. of strains) | Biotypes<br>(No. of strain) |       |      |      |      |      |      |
|--------------------------------------|-----------------------------|-------|------|------|------|------|------|
|                                      | I                           | II    | V    | VI   | VIII | IX   | X    |
| <b>Periodontal patients</b>          |                             |       |      |      |      |      |      |
| P6 (24)                              | -                           | +(24) | -    | -    | -    | -    | -    |
| P7 (13)                              | +(1)                        | +(1)  | -    | +(4) | -    | +(4) | +(3) |
| P8 (1)                               | -                           | +(1)  | -    | -    | -    | -    | -    |
| P15 (5)                              | -                           | -     | +(1) | -    | +(1) | -    | +(3) |
| P29 (8)                              | -                           | -     | +(2) | -    | -    | -    | +(6) |
| P42 (8)                              | -                           | +(8)  | -    | -    | -    | -    | -    |
| P43 (20)                             | -                           | +(16) | -    | +(1) | +(2) | -    | +(1) |
| P46 (8)                              | -                           | +(8)  | -    | -    | -    | -    | -    |
| P47 (4)                              | -                           | -     | -    | -    | +(4) | -    | -    |
| <b>Healthy subjects</b>              |                             |       |      |      |      |      |      |
| C1 (3)                               | -                           | +(2)  | -    | -    | -    | -    | +(1) |

**Table 3.** Leukotoxin action of 13 whole *A. actinomycetemcomitans* and supernatant on HL-60 cells from isolates with deletion

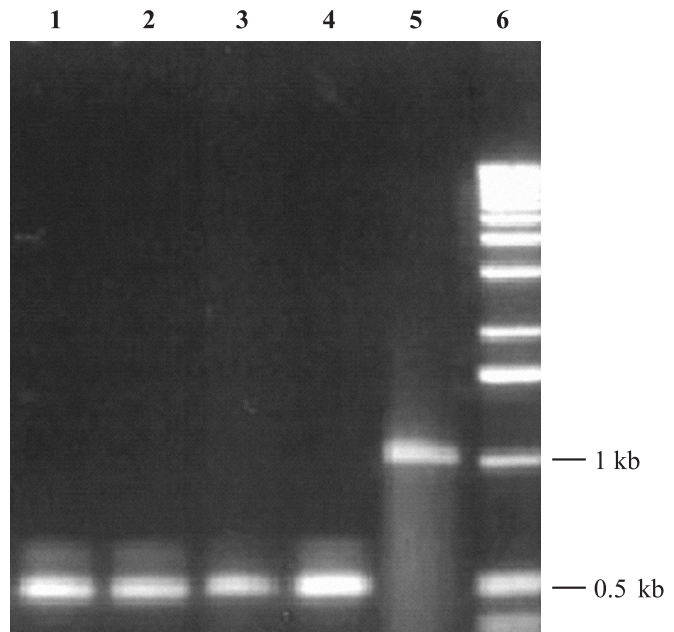
| Strain                       | Rate (%) of dead<br>HL-60 cells |             | Level of leukotoxic<br>activity |             |
|------------------------------|---------------------------------|-------------|---------------------------------|-------------|
|                              | Whole<br>cells                  | Supernatant | Whole<br>cells                  | Supernatant |
| P7a                          | 78                              | 65          | High                            | Medium      |
| P7b                          | 73                              | 63          | Medium                          | Medium      |
| P7c                          | 55                              | 55          | Medium                          | Medium      |
| P6                           | 26                              | 25          | No                              | No          |
| P8                           | 100                             | 18          | High                            | No          |
| P15                          | 100                             | 22          | High                            | No          |
| P15a                         | 76                              | 75          | High                            | High        |
| P15c                         | 77                              | 77          | High                            | High        |
| P29                          | 100                             | 9           | High                            | No          |
| P42                          | 100                             | 0           | High                            | No          |
| P43                          | 100                             | 0           | High                            | No          |
| P43a                         | 75                              | 75          | High                            | High        |
| P43c                         | 60                              | 45          | Medium                          | Medium      |
| JP2 <sup>1</sup>             | 83                              | 75          | High                            | High        |
| ATCC 43718 (Y4) <sup>2</sup> | 42                              | 35          | Medium                          | No          |
| SUNY AB 67 <sup>3</sup>      | 23                              | 26          | No                              | No          |
| ATCC 33383 <sup>3</sup>      | 25                              | 22          | No                              | No          |

<sup>1</sup>*A. actinomycetemcomitans* highly leukotoxic;

<sup>2</sup>*A. actinomycetemcomitans* medium leukotoxic;

<sup>3</sup>*A. actinomycetemcomitans* non-leukotoxic strain.

Levels of leukotoxin-producing strains were defined as follow: High: ≥75% to 100%; Medium: 50% ≤ K < 75%; Non-leukotoxic: 0 to 40%.



**Figure 1.** PCR products from *A. actinomycetemcomitans* strains. Columns: 1, JP2; 2, P8; 3, P15; 4, P43; 5, ATCC 43718 (Y4), 6, 1kb DNA ladder.

## DISCUSSION

Human subgingival microbiota displays a great heterogeneity in populations from different geographic locations (11). In this study, by culture, *A. actinomycetemcomitans* was isolated from 18% of the periodontal patients and 2% of the healthy subjects, in accordance with Asikainen *et al.* (1) and Malheiros and Avila-Campos (22). Moreover, studies have shown that the occurrence of *A. actinomycetemcomitans* varied from 20% to 80% in adult periodontitis patients (3,22,33). On the other hand, by using a PCR method, this microorganism was detected in 44% of the periodontal patients, and in 16% of the healthy subjects. Yang *et al.* (33) detected this microorganism in 64.4% and 64% of the patients with chronic periodontitis and healthy subjects, respectively. In Brazil, is observed a multi-ethnic population, with strong race mixture and peculiar habits, and the occurrence of different oral pathogens in dental or subgingival biofilms may reflects this characteristic.

In this study, *A. actinomycetemcomitans* strains were grouped in seven biotypes, but no relationship among any biotype and leukotoxin-producing strains or presence or absence of 530-bp deletion was observed ( $P = 0.278$ ). Haubek *et al.* (13) have suggested that highly leukotoxic *A. actinomycetemcomitans* comprise a single clone, and in our study, only 13 strains isolated from a single patient were considered highly leukotoxic strains, which belonged to five different biotypes I, II, VI, IX or X.

In addition, biotype II and X were the most prevalent colonizing 12% and 8% of the periodontal patients, respectively. In healthy subjects, biotypes II, VI and X were observed. Few studies have shown the presence of biotypes in *A. actinomycetemcomitans* and the biotype II appears to be the most prevalent in Brazilian periodontal patients (3,22).

Some findings have suggested that highly leukotoxic strains may be important in the pathogenesis of the periodontal disease in certain populations (16). Previous reports have demonstrated a strong correlation between leukotoxic strains and periodontal breakdown (12), especially in younger patients with localized early bone loss (6,21).

Different populations seem to show highly variable frequency of the 530-bp leukotoxin promoter deletion (6,12,14,16), which is associated with the maximum leukotoxin expression. The frequency of this genetic deletion in the promoter is especially relevant, since subjects harboring highly leukotoxic *A. actinomycetemcomitans* are 22.5 times more likely to convert from health status to localized aggressive periodontitis than those colonized by strains harboring full-length leukotoxin promoter region (4). A strong correlation between highly leukotoxic strains of *A. actinomycetemcomitans* and periodontitis has been reported in Brazilian population (6).

Leukotoxin produced by *A. actinomycetemcomitans* is bound to bacterial and vesicle surfaces by weak interactions mediated by electrostatic forces or by hydrophobic protein epitopes (31). Data of the cytotoxicity assay on HL-60 cells are in accordance with this statement, since supernatant of cultures showed reduced cytolytic activities (15).

In summary, the diversity of leukotoxin expression by *A. actinomycetemcomitans* strains suggests the need to a better evaluation about the role of this leukotoxin in periodontal diseases and other infections, obtaining biological data to be considered in epidemiological studies.

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#### RESUMO

##### **Distribuição de biótipos e atividade leucotóxica de *Aggregatibacter actinomycetemcomitans* isolados de pacientes brasileiros com periodontite crônica**

*Aggregatibacter actinomycetemcomitans* é um importante agente etiológico da periodontite e produz infecções extra-buciais. Neste estudo, foram detectados os biótipos, o gene *ltxA* associado à produção de leucotoxina e o promotor *ltx* em

*A. actinomycetemcomitans* de pacientes com e sem periodontite. A atividade leucotóxica sobre células HL-60 também foi avaliada. A atividade leucotóxica foi determinada através do método de exclusão do azul de tripam. A deleção de 530 bp no promotor *ltx* foi avaliada usando-se o par de iniciadores PRO. *A. actinomycetemcomitans* foi detectado por cultura e por PCR. Por cultura, *A. actinomycetemcomitans* foi detectado em nove pacientes com periodontite (18%) e em um indivíduo sadio (2%). Por PCR esse microrganismo foi detectado em 44% dos pacientes com periodontite e em 16% dos saudáveis. Verificou-se diferença estatística entre a detecção do promotor do operon *ltx*, por PCR, diretamente do biofilme subgingival e do DNA bacteriano. Somente uma amostra clínica apresentou *A. actinomycetemcomitans* altamente leucotóxico. O biótipo II foi o mais prevalente e não foi observada correlação biótipo-atividade leucotóxica. A expressão da leucotoxina por *A. actinomycetemcomitans* na doença periodontal e outras doenças infecciosas necessita ser avaliado.

**Palavras-chave:** *Aggregatibacter actinomycetemcomitans*, biótipo, leucotoxina, periodontite.

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