

LEUKOTOXIC ACTIVITY OF *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ISOLATED FROM HUMAN AND NON-HUMAN PRIMATES

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ABSTRACT

Actinobacillus actinomycetemcomitans is a clinically relevant periodontopathogenic Gram-negative coccobacillus that produces a leukotoxin of the RTX cytolysin family. In this study, we evaluated the leukotoxic activity of *A. actinomycetemcomitans* strains isolated from human and marmosets by Trypan blue exclusion and by the chemiluminescence assays. Among eight *A. actinomycetemcomitans* human strains studied, two (P_{2,17} and P_{8,12}) were classified as high leukotoxin producers and among eight marmoset strains, one (M_{22,11}) showed high leukotoxin production, as determined by Trypan blue exclusion assay. The reference strains ATCC 29523 and FDC Y4 respectively behaved like moderate and low producers. The chemiluminescence assay was used to evaluate the leukotoxic activity of M_{22,11} and P_{2,17} strains submitted to different growth conditions. Leukotoxic activity was detected on cells at the logarithmic phase and was similar under anaerobic and microaerophilic growth conditions. It was greatly reduced when cells were grown at glucose concentrations lower or higher than 0.75% (0.25% and 1.5%) in thioglycolate medium. Leukotoxin production mainly by the M_{22,11} strain was low in BHI broth, whereas production in TSB medium showed a similar level as in thioglycolate broth medium. Sodium bicarbonate at 10 mM did not affect leukotoxin production.

Key words: *Actinobacillus actinomycetemcomitans*, periodontopathogen, leukotoxic activity, chemiluminescence assay

INTRODUCTION

Actinobacillus actinomycetemcomitans is a capnophilic Gram-negative coccobacillus related to periodontal disease, especially localized juvenile periodontitis, of considerable importance in both human and veterinary medicine (18,23,26). This organism has been associated to a variety of other infectious diseases such as endocarditis, brain abscesses, osteomyelitis and subcutaneous abscesses (26). Although a number of potential virulence factors from *A. actinomycetemcomitans* have been described (5,24), the best characterized is a heat-labile exotoxin that rapidly destroys human polymorphonuclear

leukocytes (PMNs), monocytes, natural killer cells, T lymphocytes, and monkey polymorphonuclear leukocytes (12,14).

Molecular genetic analysis have indicated that the *A. actinomycetemcomitans* leukotoxin is a member of the RTX (repeats in toxin) family including the *Escherichia coli* alpha-hemolysin and the *Pasteurella haemolytica* leukotoxin (9,10). In the RTX toxin determinants, genes for toxin synthesis, activation, and secretion are grouped together on the chromosome in an operon named *ltx* (10). Although the *ltx* operon seems to be present in all *A. actinomycetemcomitans* strains, the levels of toxin expression vary considerably among different strains (8). High levels of leukotoxin expression appear to be

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related to the presence of the JP2-like promoter (530 bp deletion), although physiological factors may be involved in this regulation (8,13,16,20).

Several methods has been proposed to obtain and evaluate the leukotoxic activity in different sensibility levels (2,24). The chemiluminescence inhibition assay has been used to determine the leucotoxic activity on PMNs in various studies (3,6,17), and is a very sensitive assay of cells functional ability (2). However it has not been used to evaluated the leukotoxic activity of *A. actinomycetemcomitans*. Studies using isolates from different geographical populations are lacking. In the present investigation, we compare the leukotoxic activity of several *A. actinomycetemcomitans* strains isolated from human and non-human primates by the Trypan blue exclusion assay. The effects of environmental conditions on the leukotoxic activity of two *A. actinomycetemcomitans* strains were evaluated by the chemiluminescence assay.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Reference strains *A. actinomycetemcomitans* FDC Y4 (Forsyth Dental Center, Boston, Massachusetts, USA), ATCC 29523 (American Type Culture Collection, Rockville, Maryland, USA), and 16 strains from our culture collection isolated from human beings (progressive rapid periodontitis – P_{2,2}, P_{2,17}, P_{7,13}, B₄, L₂ -, adult periodontitis – P_{11,18} and P_{14,21} -, and healthy periodontum – P_{8,12}) and from marmosets (gingivitis – M_{20,1}, M_{20,15}, M_{20,25}, M_{16,2}, M_{28,8}, M_{38,7} -, and healthy periodontum – M_{22,11} and M_{22,19}) were used. The organisms were grown in modified thioglycolate broth medium (21) supplemented with 0.5% yeast extract (Difco), and incubated in an anaerobic chamber (85% N₂, 10% H₂, and 5% CO₂). Routinely, 950 mL of thioglycolate medium were inoculated with 50 mL of a 24 hour culture of *A. actinomycetemcomitans* and incubated for 48 hours, at 37°C, in an anaerobic chamber.

Recovery of extracts for trypan blue exclusion assays

Extracts from whole cells were obtained as previously described (14). *Actinobacillus actinomycetemcomitans* cells were washed twice (16,000 x g for 20 min. at 4°C) in phosphate buffered saline - PBS (0.68% (w/v), NaCl, 0.12% (w/v) Na₂HPO₄, 0.07% (w/v) KH₂PO₄; pH 7.0), and resuspended in 20 mL of PBS. The suspension was submitted to sonication, in ice-water bath, at 16 KHz in a 200 W ultrasonic disrupter (Branson 450 - Model MSE), and centrifuged as above. The supernatants were concentrated as described by Ohta *et al.* (15). Ammonium sulfate was added to 50% saturation on ice (C-50 fraction), and the precipitate was collected by centrifugation (25,000 x g for 25 min. at 4°C). The supernatant was resaturated with 70% ammonium sulfate, resulting in the C-70 fraction. Both precipitates were resuspended in 5 mL PBS and dialyzed against 0.05 M ammonium bicarbonate buffer; pH 7.2 (1/200 of the suspension volume) overnight,

followed by lyophilization and storage at -86°C. Protein content was determined by the method of Lowry *et al.* (11).

Recovery of sonic extracts for the chemiluminescence assays

For the chemiluminescence assays, sonic extracts obtained from 24 hours culture of *A. actinomycetemcomitans* M_{22,11} and P_{2,17} strains were precipitated with 40% (C-40 fraction) and 80% (C-80 fraction) ammonium sulfate and dialyzed against ammonium bicarbonate buffer. These extracts were not lyophilized. The cells were submitted to different growth conditions using thioglycolate medium as follows: (1) glucose concentration - 0.25%, 0.75% and 1.50%; (2) addition of sodium bicarbonate - 10 mM; (3) atmosphere of incubation - anaerobiosis and microaerophilia; the culture media Brain Heart Infusion (BHI; Difco) and Tryptic Soy Broth (TSB; Difco) were also employed. Leukotoxic activity of sonic extracts from cells in each condition, as well from at different growth curve phases (midlog, later log and stationary) were evaluated.

Isolation of human polymorphonuclear cells

Polymorphonuclear leukocytes (PMNs) from adult donors (not pooled) were isolated from heparinized venous blood by centrifugation on Ficoll Hypaque gradients as described by Bicalho *et al.* (1). The PMNs were resuspended in RPMI 1640 medium (Trypan blue exclusion test) or in veronal buffer (chemiluminescence assay) (4). PMNs donors were adult (20-30 years old) from both gender, and had no relevant history of periodontal disease.

Assay of PMNs viability by Trypan blue exclusion

After exposure of 10⁶ PMNs to 100 µL of protein extracts (500 µg/mL), from every strains, in a final volume of 600 µL, for 60 min., a Trypan blue suspension (1.6 mg/mL in saline solution) was added to the PMNs monolayers at a final concentration of 0.8 mg/mL (14). The monolayers were kept at 37°C for 7 min. in a CO₂ chamber (5%), mounted on a hemocytometer and then observed under light microscope. The cells taking up Trypan blue (dead cells), and cells excluding the dye (viable cells) were counted.

Decrease of PMNs viability caused by leucotoxic activity

A cell death curve was determined by the Trypan blue exclusion test. PMNs (2 x 10⁶ cells/mL) were incubated with 200 µL of protein extract (250 µg/mL - C-70 fraction; from M_{20,19}) at 37°C, in 5% CO₂, in a final volume of 600 µL. After intervals of 0, 10, 20, 30, 40, 45, 60, 90 and 120 min., aliquots were taken (20 µL) and assayed for PMNs viability.

Chemiluminescence assay

Leukotoxic activity was measured by chemiluminescence (4, 6). PMNs (10⁶), suspended in 200 µL veronal buffer, were stimulated with the addition of tetradecanoyl-phorbol-acetate (TPA; 0.005 µg) in the presence of luminol (10⁻⁵ M) in a final

volume of 750 μ L in PBS; pH 7.2. The chemiluminescence emission was measured in millivolts (mV) at 1 s intervals at room temperature using a Wallac luminometer (LBK Wallac 1250). A ideal dose was determined by addition of different doses of protein extracts. For the routine chemiluminescence assays, 50 μ L of protein extract (250 μ g/mL; M_{22.11} and P_{2.17}) were added to PMNs suspension and incubated for 10 min. at room temperature, and the light emission was measured as above. Leukotoxic activity was expressed as percentage of inhibition of the peak value of the normal chemiluminescence response (control) of the PMNs to TPA. Negative controls were performed without addition of TPA.

Proteic nature determination

As we worked with crude extracts, 200 μ L of each one was submitted to different treatments for the determination of its proteic nature: (1) 70°C, for 30 min.; (2) 100°C, for 10 min.; and (3) trypsin 10 μ L (1 mg/mL), for 30 min. Leukotoxic activities were verified by chemiluminescence assays.

RESULTS

Effect of the *A. actinomycetemcomitans* protein extracts on PMNs

In serum-free cultures, eighteen strains of *A. actinomycetemcomitans* from human and non-human primates showed leukotoxic activity as indicated by the Trypan blue exclusion test (Table 1). Sonic extracts that killed less than 40%, 40 to 70% and 70% or more PMNs were considered to be low, medium and high-producers, respectively. The viability of PMNs in controls was 98.00% with human strains and 97.00% for marmoset ones. According to the Table 1, the highest leukotoxic activity was detected on C-70 fractions. The FDC Y4 strain was considered to be a medium producer inducing 54% cell death, whereas the extract from ATCC 29523 induced 34% cell death, regarding the C-70 fractions. Among human strains, 6 were medium and 2 high producers, whereas 1, 6 and 1 marmoset strains were low, moderate and high producers, respectively. Among marmoset strains, the highest and lowest leukotoxin producers were isolated from the same animal.

The decrease of PMNs viability occurred at a higher rate during the initial 20 min. of incubation with the protein extract from *A. actinomycetemcomitans* M_{20.19} strain than later on (final concentration - 83.3 μ g/mL).

Quantitative analysis of PMNs sensitivity to *A. actinomycetemcomitans* leukotoxin

Leukotoxin caused a dose-dependent inhibition of the chemiluminescence of PMNs. The highest dose of sonic extract which gave a chemiluminescence reading was 62.5 μ g/mL and the lethal average dose (LD₅₀), i.e., the dose of C-80 extract required to inhibit 50% of chemiluminescence response of PMNs, was calculated to be approximately 0.02 μ g/mL (Fig. 1).

Table 1. Leukotoxic activity of *Actinobacillus actinomycetemcomitans* strains isolated from the human and marmoset oral cavity.

Strains	% of inhibition	
	C-50 sonic extract	C-70 sonic extract
Reference strains		
ATCC 29523	6.3	34.0
FDC Y4	41.0	54.0
P _{2.2}	59.0	65.3
P _{2.17}	73.0	82.0
P _{7.13}	27.3	48.7
P _{8.12}	56.3	73.3
P _{11.18}	47.7	68.4
P _{14.21}	27.4	41.2
B ₄	29.6	63.4
L ₂	33.0	58.4
Marmosets		
M _{20.1} *	33.6	48.5
M _{20.19} *	43.1	68.4
M _{20.25} *	38.9	50.0
M _{22.11} *	58.2	90.5
M _{22.19} *	19.2	28.5
M _{16.2} †	35.3	53.0
M _{38.8} †	39.7	59.6
M _{38.3} ‡	12.2	46.0

* *Callithrix jacchus*; † *Callithrix geoffroy*; ‡ *Callithrix penicillata*.

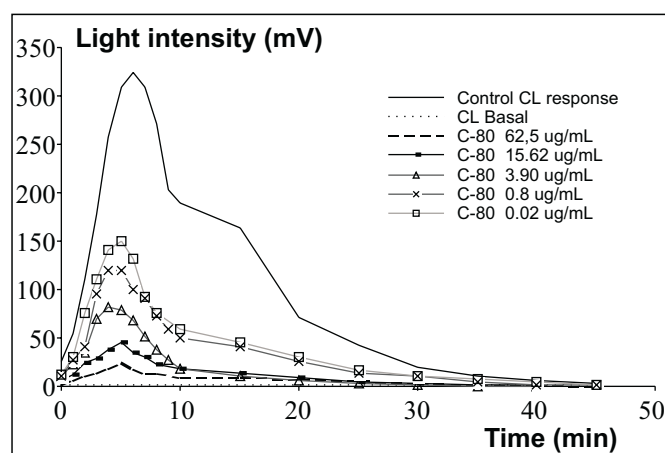


Figure 1. The effect of different concentrations of *A. actinomycetemcomitans* C-80 sonic extract on peripheral blood mononuclear cells. Several dilutions of a sonic extract from the P_{2.17} strain were added to human PMNs (10⁶ cells) and the effect was measured by chemiluminescence for 45 min. Control cells were incubated with TPA.

Chemiluminescence inhibition by sonic extracts of *A. actinomycetemcomitans* strains P_{2.17} and M_{22.11}

Results of a chemiluminescence inhibition assay with human neutrophils and samples from two strains of *A. actinomycetemcomitans* are shown in Fig. 2

Sonic extracts from strains M_{22.11} and P_{2.17} were highly toxic for human PMNs. Leukotoxic activity was detected within 6 hours of growth (midlog phase) and no significant additional increase was observed after 12 and 24 hours (data not shown). The atmosphere of incubation and the addition of sodium bicarbonate at 10 mM did not affect the toxicity of sonic extracts from both strains. The leukotoxic activity was intensely reduced by lower glucose concentration (0.25% glucose) in both P_{2.17} and M_{22.11} strains (only 10.46% and 3.76% chemiluminescence inhibition, respectively). A higher glucose concentration (1.5% glucose) also reduced the chemiluminescence response (58.51% for P_{2.17} and 68.11% for M_{22.11}, respectively), whereas in the presence of 0.75% glucose, the chemiluminescence response was inhibited by 92.75% and 93.74%, respectively (Fig. 3). When the leukotoxic activity of the two *A. actinomycetemcomitans* strains grown in different culture media was compared, higher activities were observed when the cells were cultivated in tioglycolate and TSB media (93.50% and 90.03% inhibition for M_{22.11} strain and 92.48 and 92.82 for P_{2.17} strain, respectively) than in BHI media (72.04% inhibition for the M_{22.11} strain and 90.28% inhibition for the P_{2.17} strain). Fig. 4 shows extract activity from M_{22.11} strain.

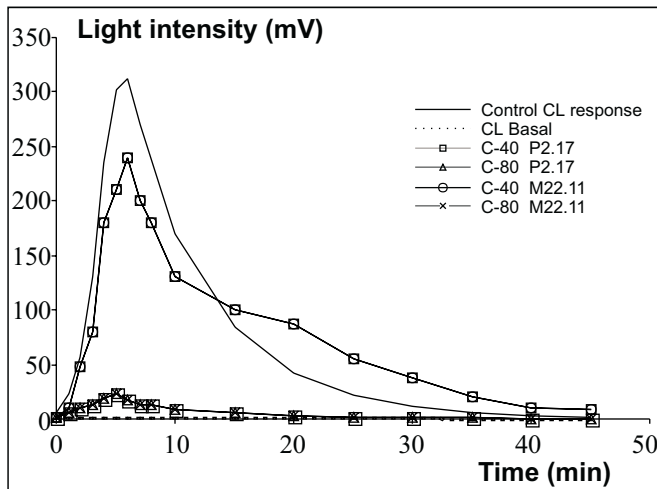


Figure 2. The effect of a sonic extract C-40 and C-80 from *A. actinomycetemcomitans* P_{2.17} and M_{22.11} strains on the chemiluminescence response of human PMNs to TPA. The inhibition curves of both strains for the C-40 and C-80 fractions, were the same. The sonic extracts were obtained from an anaerobic growth at 37°C for 24 hours. Human PMNs (10⁶ cells) were incubated with 50 mL sonic extracts (0.5 mg/mL).

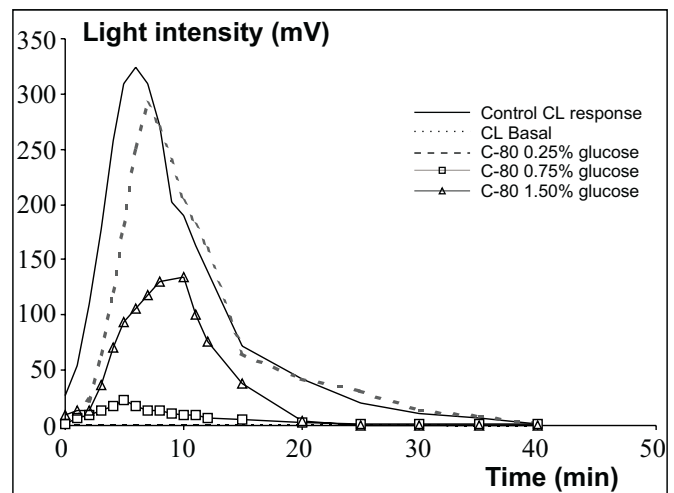


Figure 3. The effect of a sonic extract (C-80 fraction) from *A. actinomycetemcomitans* P_{2.17} strain grown at different concentrations of glucose on the chemiluminescence response of human PMNs to TPA. The sonic extracts were obtained from an anaerobic growth at 37°C for 24 hours. Human PMNs (10⁶ cells) were incubated with 50 µL sonic extracts (0.5 mg/mL).

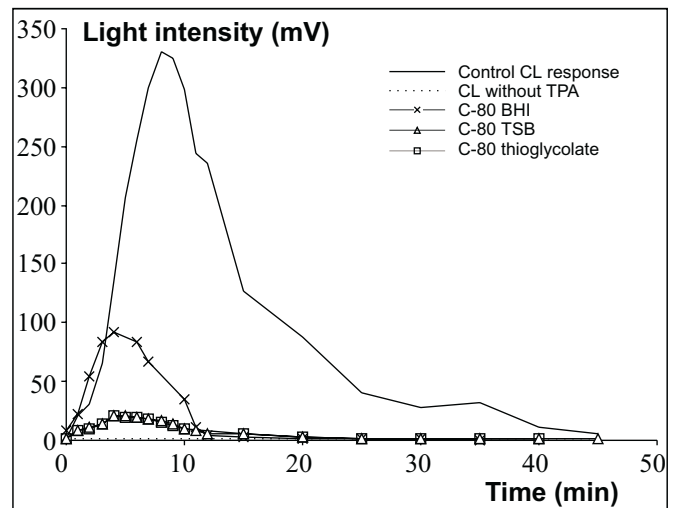


Figure 4. The effect of a sonic extract (C-80 fraction) from *A. actinomycetemcomitans* M_{22.11} strain grown in different culture media on the chemiluminescence response of human PMNs to TPA. The sonic extracts were obtained from an anaerobic growth at 37°C for 24 hours. Human PMNs (10⁶ cells) were incubated with 50 µL sonic extracts (0.5 mg/mL).

Proteic nature determination

Incubation at 70°C for 30 min. or boiling in water for 10 min., as also the treatment for 30 min. at room temperature with trypsin 10 µL (1 mg/mL) completely inactivated the leukotoxin. This

indicates that the molecules presenting leukotoxic activity have a proteic nature.

DISCUSSION

The present study was designed to investigate the leukotoxic activity of *A. actinomycetemcomitans* isolated from 7 human subjects and from 5 marmosets in Brazil, to evaluate its heterogeneity and to examine the environmental effects on this activity. Marmoset strains were included because wild primate populations may hold valuable clues to the origins and evolution of some important pathogens (28).

Although initial observations have demonstrated a strong association between high leukotoxic activity strains and periodontal disease (29), as well as high leukotoxin producer strains and younger patients (26), more recent findings suggest that highly leukotoxic strains may only be important in the pathogenesis activity, although in our study this activity was extremely heterogeneous. Interestingly, the higher and lower leukotoxin producer strains were isolated from the same animal. Moreover, the strain P_{8.12} was a high producer (73.3%) despite its healthy subject origin, and the strains from periodontal disease patients were moderate producers.

The kinetics of leukotoxic activity is similar to that reported in others studies (12,23). The maximal activity occurred after 60 min. of PMNs incubation with sonic extracts (C-70 fraction) of the *A. actinomycetemcomitans* M_{20.19} strain, although the highest rate of activity was observed during the initial 20 min.

Chemiluminescence was used in this study to evaluate the leukotoxic potential of M_{22.11} and P_{2.17} strains submitted to different growth conditions. Initially, the protein concentration was standardized. The ideal dose was 62.5 µg/mL, because higher concentrations completely inhibited the chemiluminescence response. On the other hand, a lower concentration could be confused with the decrease in leukotoxic activity (Data not shown). The lethal average dose (LD₅₀) was approximately 0.02 mg/mL (46.36% inhibition), a concentration lower than that reported by Taichman *et al.* (24) who found 0.37 µg/mL to be the LD₅₀ on PMNs for *A. actinomycetemcomitans* JP2 strain, a high leukotoxin producer. These differences could be due to methodological approaches used in each study.

Ohta *et al.* (14) reported leukotoxic activity of sonic extract obtained during the stationary phase, but no activity of extracts obtained during the exponential growth phase. In contrast, in this study leukotoxic activity was evidenced after 6 hours of growth (mid-exponential growth phase) for both strains, and no significant additional increase was observed during the later exponential phase or during the stationary phase. This data agrees with results obtained by Spitznagel *et al.* (20) who observed that the *ltxA* and *ltxB* transcripts increased during the logarithmic growth phase and decreased as the cell entered the stationary phase.

Hritz *et al.* (8) reported that the production of leukotoxin is repressed by aeration, and this oxygen-dependent regulation differs significantly between highly toxic and minimally toxic *A. actinomycetemcomitans* strains (JP2 and 652, respectively), resulting in different levels of leukotoxic protein production rather than a change in toxin activity (21). In our study, the M_{22.11} and P_{2.17} strains were not influenced by aeration, i.e., they behaved similarly to JP2 strain.

Little is known about the physiological basis of the leukotoxic activity. Ohta *et al.* (17) have studied the effects of bicarbonate concentration and fermentable sugar levels in a chemostat culture on the production of leukotoxin by *A. actinomycetemcomitans*. The addition of 10 mM bicarbonate stimulated the efficiency of leukotoxin synthesis up to about threefold, whereas a further increase of bicarbonate concentration (20-40mM) had no effect on total leukotoxin production, but increased the amount of leukotoxin in the supernatants (17). Our findings do not confirm these results since no differences in leukotoxic activity were observed among the M_{22.11} and P_{2.17} strains when bicarbonate was added to a final concentration of 10 mM, in thioglycolate medium.

Mizoguchi *et al.* (13) demonstrated that leukotoxin production is repressed by fructose and glucose in chemostat cultures, but not by a non-fermentable sugar analog. Our results revealed that higher and lower glucose concentrations (1.50% and 0.25%) had a negative effect on leukotoxin production when compared with 0.75% glucose concentration in thioglycolate medium. Interestingly, sonic extracts from both strains growing in different culture media presented varied leukotoxic activity levels. The cells growing in BHI showed the lowest leukotoxin production whereas the cells grown in both TSB and thioglycolate broth medium showed the highest leukotoxin production. These differences should be related to the glucose and protein concentration of the different culture media employed. Curiously, despite the low glucose concentration in BHI broth (<0.02%), leukotoxic activity was higher than in thioglycolate at 0.25% glucose concentration. We hypothesize that protein contents in BHI (>90%), as well as in TSB containing 0.25% glucose and approximately 60% protein, might have stimulated leukotoxin production.

In summary, the diversity of leukotoxin expression by *A. actinomycetemcomitans* strains suggests the need of detecting new parameters for a better evaluation of the role of leukotoxin in periodontal and other infectious diseases.

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RESUMO

Atividade leucotóxica de amostras de *Actinobacillus actinomycetemcomitans* de primatas humanos não-humanos

Actinobacillus actinomycetemcomitans é um cocobacilo Gram negativo, periodontopatógeno clinicamente importante, que produz uma leucotoxina pertencente à família das citolisinas RTX. Neste estudo, avaliou-se a atividade leucotóxica de amostras de *A. actinomycetemcomitans* isoladas de seres humanos e de calitriquídeos pelos métodos de exclusão de azul de Tripan e quimioluminescência. Duas (P_{2,17} e P_{8,12}) entre oito amostras de *A. actinomycetemcomitans* isoladas de seres humanos, e uma (M_{22,11}) entre 8 amostras isoladas de sagüis se apresentaram como altamente produtoras de leucotoxina, como determinado pelo teste de exclusão de azul de Tripan. As amostras de referências de *A. actinomycetemcomitans* ATCC 29523 e FDC Y4 se comportaram como média e baixa produtoras de leucotoxina, respectivamente. O teste de quimioluminescência foi usado para avaliar a atividade leucotóxica das amostras M_{22,11} e P_{2,17} submetidas a diferentes condições de crescimento. A atividade leucotóxica foi detectada em células durante a fase logarítmica e foi similar sob crescimento em anaerobiose e microaerofilia. A atividade leucotóxica foi muito reduzida quando as células foram crescidas em concentrações de glicose mais baixa e mais alta que 0,75% (0,25% e 1,5%), em meio tioglicolato. A produção de leucotoxina, especialmente pela amostra M_{22,11}, foi mais baixa em caldo BHI, enquanto em meio TSB a produção foi em nível similar a aquele em meio tioglicolato. Bicarbonato de sódio 10mM não afetou a produção de leucotoxina.

Palavras-chave: *Actinobacillus actinomycetemcomitans*; periodontopatógeno; atividade leucotóxica; ensaio de quimioluminescência

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