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Phenotypic and genotypic features of *Aggregatibacter actinomycetemcomitans* isolated from patients with periodontal disease ☆

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ABSTRACT

Aggregatibacter actinomycetemcomitans is strongly implicated in the pathogenesis of periodontitis. In this study, the phenotypic and genotypic features of *A. actinomycetemcomitans* and the presence of genes involved in toxicity were determined. Sixty-five patients with periodontal pocket and 48 healthy subjects were evaluated. Biotyping, adherence and invasion, neuraminidase and biofilm production, presence of capsule and fimbria, as well as the presence of *flp-1*, *apaH*, *ltx*, and *cdt* genes were determined. Biotype II was the most prevalent. Sixty-six strains were adherent and 33 of them were able to invade KB cells. Sixty strains produced neuraminidase, and 55 strains biofilms. Strains showed capsule but not fimbriae. Forty-six strains were cytotoxic, and most strains harbored the *apaH* and *flp-1* genes. LTX promoter and the *ltxA* gene were observed in all strains from periodontal patients. The *cdtA* gene was observed in 50 (71.4%) strains, *cdtB* in 48 (68.6%) strains, *cdtC* in 60 (85.7%), and *cdtABC* in 40 (57.1%) strains. The presence of *A. actinomycetemcomitans* harboring the *cdtC* gene from healthy subjects may represent a transitory microorganism in the oral microbiota. More studies are necessary to understand the real role of this microorganism in the pathogenesis of periodontal disease.

1. Introduction

Bacterial adherence is the first step in invading cells. Adherence of pathogens on eukaryote cells is mediated by adhesins, lipopolysaccharide, or fimbriae, which may be regulated either by the host's immune system or by environmental factors (Xie et al., 1997). *Aggregatibacter actinomycetemcomitans* is a capnophylic Gram-negative coccobacillus, is nonmotile, is non–spore forming, and has been strongly implicated in the pathogenesis of periodontitis, mainly in aggressive periodontitis, and in systemic infections such as endocarditis and soft tissue abscesses (Nakano et al., 2007). Aggressive periodontitis often occurs in young people and causes a rapid loss of attachment and alveolar bone affecting teeth, particularly first molars and incisors (Slots and Ting, 1999).

An important virulence factor produced by *A. actinomycetemcomitans* is the leukotoxin that is genetically composed of a cluster of 4 genes that gives to this species the ability to kill human polymorphonuclear leukocytes (Lally et al., 1999). Leukotoxic *A. actinomycetemcomitans* strains are grouped by the variation in the transcriptional regulation of the leukotoxin genes into highly and minimally leukotoxic strains. Highly leukotoxic strains show a deletion (530 bp) in the promoter region of the operon that enhances transcription (Brogan et al., 1994). The cytolethal distending toxin (CDT) is another implicated virulence factor in the pathogenesis of periodontal disease. The prevalence of CDT-expressing clones can vary among populations of the same bacterial species (Tan et al., 2002; Whitehouse et al., 1998).

This microorganism produces adhesins used in early events of infectious processes by attaching to specific receptors on oral mucosa and dental plaque. The bacterial ability of adherence to oral surfaces is an important property of biofilm production which is a pili- or fimbriae-mediated process (Kachlany et al., 2001).

Fives-Taylor et al. (1996) showed that the adhesive property of *A. actinomycetemcomitans* could be associated with the outer membrane, extracellular amorphous material, or extracellular vesicles. In addition, this microorganism produces fimbriae that are composed of repeating subunits of the 6.5-kDa Flp1 protein (fimbrial low-molecular-weight protein). This Flp1 protein represents a novel type IV pilin that is homologous to type IVB found in *Vibrio cholerae* and *Escherichia coli*. In addition, this protein is encoded by *flp-1* and *flp-2* genes (Kaplan et al., 2003), and is the major structural component of fibrils (Kachlany et al., 2001). *A. actinomycetemcomitans* produces rough and smooth colonies which are characterized for producing fimbriae, and smooth colonies produce extracellular

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microvesicles that are associated with the attachment process (Meyer et al., 1996; Saito et al., 2010).

Bacterial sialidases are considered virulence factors in several pathogenic organisms which colonize mucosal surfaces, since this enzyme catalyzes the sialic acid (*N*-acetyl neuraminic acid) hydrolysis from glycoproteins, glycolipids, and polysaccharides of the cell membrane, exposing the β-galactosyl determinant which acts in the adherence process among bacteria and host's mucosa. Several bacterial strains show a neuraminidase activity, and it has been suggested that this activity plays a role in the bacterial attachment to animal cells, as well as in the hemagglutination process (Moncla et al., 1990; Nakano et al., 2006).

A. actinomycetemcomitans is a biofilm-forming bacterium on dental surfaces and epithelial cells using its own extracellular polymer. This biofilm contains bacterial cells producing adhesive type IV pili. In addition, this microorganism also produces a polysaccharide, poly-β-1,6-*N*-acetyl-D-glucosamine, which is also associated with adherence and invasion. In this study, the phenotypic and genotypic features of *A. actinomycetemcomitans* and the presence of genes involved in toxicity were determined.

2. Materials and methods

2.1. Patients and sample collection

Thirty-five male and 30 female patients between 20 and 50 years old, with clinical and radiographic evidence of alveolar bone loss confined to the molar and incisor teeth, and periodontal pocket depth equal to or exceeding 5 mm, were evaluated. Forty-eight healthy subjects (30 men and 18 women between 20 and 45 years old) were also included, and none of them showed any gingival inflammation or bleeding on probing. None of them had used antibiotics 3 months prior to the sample collection. This study was approved by the Ethic Commission of the Institute of Biomedical Sciences, USP (Process No. 662/CEP). Supragingival biofilm was removed from the mesial surface of 2 affected teeth by using sterile cotton pledgets. Subgingival samples were obtained by 2 fine sterile paper points (Dentsply, RJ, Brazil), inserted to the depth of the gingival site and left in place for 60 s, and then placed into 2 mL of viability maintaining microbiostatic medium (VMGA III) transport medium and processed within 2 h of collection.

2.2. Bacterial isolation and identification

Clinical samples were 10-fold diluted and plated onto a trypticase soy-serum bacitracin–vancomycin (TSBV) agar (Slots, 1982). After 72 h of incubation at 37 °C in 90% N_2 + 10% CO_2, 1 to 2 suspected colonies from each subgingival sample were cultured on Brucella blood agar supplemented with 0.5% yeast extract, 5 mg/mL haemin, and 1 mg/mL menadione, and then identified by Gram staining, catalase production, susceptibility to NaF, and biochemical tests by fermenting glucose, fructose, and mannose but not lactose, starch, sucrose, or trehalose (Slots et al., 1980). All the *A. actinomycetemcomitans* strains were stored at −80 °C in 20% skimmed milk.

2.3. Biotyping

All isolates were biotyped on the basis of the variable fermentation of dextrin, maltose, mannitol, and xylose (Slots et al., 1980).

2.4. Adherence assay

Adherence assays using 1.0×10^5 KB cells (human oral epidermoid carcinoma cells) were performed as previously described (Cravioto et al., 1979; Nakano et al., 2008). Briefly, 960 μL of RPMI 1640 medium (Cultilab, SP, Brazil) with fetal bovine serum (2% v/v) were inoculated with 40 μL of bacterial culture (ca. 1.5×10^8 cells/mL). Plates were incubated (5 h in 5% CO_2), washed 3 times with 0.1 mol/L phosphate-buffered saline (PBS) (136 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, pH 7.4), fixed (absolute methanol), and stained with May-Grünwald-Giemsa stain. The enteropathogenic *Escherichia coli* O127:H6 (E2348/69) strain displaying a localized adhesion pattern was used as control. All assays were performed in duplicate.

2.5. Invasion assay

Invasion assays were performed as described by Nakagawa et al. (2002), with modifications. Aliquot of 100 μL of a 24-h culture with ca. 1.5×10^8 cells/mL was added to ca. 1.0×10^5 KB cells and then incubated (2 h in 5% CO_2). Nonadherent bacteria were killed by incubation (1 h in 5% CO_2, at 37 °C) in 1 mL of Dulbecco's Modified Eagle's Medium containing 2% fetal bovine serum with 300 μg/mL of gentamycin. Then, cells were washed 3 times in PBS and lysed with 400 μL of Triton-X-100 (1%, v/v). After mixing, 100 μL was plated on Brucella blood agar (Difco Laboratories, UK), incubated in anaerobiosis at 37 °C for 48 h, and then the colony-forming units were determined. An enteroinvasive *E. coli* strain serotype O124:NM and the noninvasive *E. coli* HB101 were respectively used as positive and negative controls. All assays were performed in duplicate. Invasion was expressed as the percentage of bacteria recovered from the initial inoculum after antibiotic treatment and lyses of epithelial cells, according to Tang et al. (1993).

2.6. Cytotoxicity assay

Bacterial cytotoxicity was assayed on KB tissue culture monolayer (Gaetti-Jardim et al., 2008). Cells were grown in a 96-well microtitration plate (Corning, USA) with L15 minimal medium (Cultilab), added to 2% fetal bovine serum, and incubated in air–5% CO_2 at 37 °C for 48 h. *A. actinomycetemcomitans* strains were grown in brain heart infusion (BHI) broth and then centrifuged (13,000 × *g*, 5 min). Supernatants were filtered through 0.45-μm membrane filters (Millipore, Sao Paulo, SP, Brazil), and 50 μL was added, in duplicate, to the KB cells. After 4 h of incubation (air–5% CO_2 at 37 °C), the results were compared with control cells (without bacterial supernatant). Cytotoxicity was considered when the presence of affected cells was detected. A highly cytotoxic *A. actinomycetemcomitans* JP2 strain was used as control.

2.7. Detection of the neuraminidase

Neuraminidase production was determined by using fresh normal human erythrocytes drawn from healthy volunteers as described by Nakano et al. (2006). The erythrocytes were collected in Alsever's solution (10%), washed 3 times in 0.01 mol/L of PBS (Na_2HPO_4, KH_2PO_4, NaCl, pH 7.4), and kept at 4 °C during 1 week. *A. actinomycetemcomitans* grown in BHI broth was resuspended to 1.5×10^8 bacteria/mL. Bacterial suspension was washed 3 times by centrifugation (12,000 × *g*, 10 min). Then, 1 mL of bacteria was added to 10 μL of washed erythrocytes, achieving a final concentration of 1%, and carefully homogenized and incubated in anaerobiosis at 37 °C for 4 h. Serial dilutions of peanut lectin (2.5 μg/μL) (*Arachis hypogaea*, PNA, Sigma, Sao Paulo, SP, Brazil) in PBS were performed, and 20 μL of each dilution was added to a mixture of bacteria–erythrocytes and then homogenized and incubated at room temperature for 1 h, followed by an additional incubation at 4 °C overnight. The presence of neuraminidase was noticed when an agglutination (bacteria-erythrocytes-lectin) was observed. A mixture of bacteria–erythrocytes without lectin was used as negative control (precipitation). The agglutination titer was defined as the reciprocal of the end point dilution.

Inhibition of the neuraminidase production was verified in neuraminidase-positive strains. Briefly, bacteria were treated with 1

mL of 1 mmol/L galactose and then incubated at room temperature for 30 min. Then, 2 μL of diluted lectin was mixed with 18 μL of bacteria treated with 1 mmol/L galactose and then added to 20 μL of 1% erythrocytes. Plates were lightly homogenized and incubated at 37 °C for 1 h, and enzyme inhibition was noticed by precipitation of erythrocytes. A mixture of bacteria-erythrocytes-lectin was used as negative control.

2.8. Biofilm formation assay

Biofilm formation was quantified by means of a crystal violet binding assay, as previously described by Kaplan et al. (2003). From *A. actinomycetemcomitans* grown in BHI broth, 100 μL was inoculated into wells of 24-well plates with and without circular glass cover slips (13 mm diameter) containing 1000 μL of DMEM medium (4–5 g glucose without antibiotic) (Cultilab) with a 1:10 dilution of bacterial inoculum. After 48 h of incubation at 37 °C, in a 5% CO_2 atmosphere, medium containing unattached cells was removed. Biofilms were rinsed 3 times with 1× PBS to remove loosely attached cells, fixed with 75% ethanol (1 mL per well) for 10 min, and stained with 0.5% crystal violet for 5 min. The dye bound to the biofilm was extracted with 1 mL of 95% ethanol for 2 min, and the absorbance of the crystal violet solution was measured by using a BioRad Benchmark microtiter plate-reader (Sao Paulo, SP, Brazil) set at $A_{595\ nm}$. All tests were performed in triplicate.

2.9. Detection of capsule and fimbriae

Capsules were detected by Hiss staining with modifications (Nakano and Avila-Campos, 2004). A drop of bacterial suspension, from a culture in peptone-yeast extract with 1% glucose (1%, w/v), was deposited on a microscope slide, covered with crystal violet, and heated until the emission of vapors. Each slide was washed with copper sulfate (20%, w/v) and allowed to air dry. Capsules were identified under light microscopy as clear areas around the bacteria. The presence of fimbriae was investigated by negative staining with phosphotungstic acid (2%) and uranyl acetate (2%), and examined with a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Germany) operated at 80 kV (Nakano et al., 2008).

2.10. Bacterial DNA

DNA from *A. actinomycetemcomitans* was extracted according to Avila-Campos et al. (1999). Briefly, bacterial cells grown in BHI broth were harvested and lysed with 20% sucrose, 1.4 mol/L Na_2PO_4, and 10 mg/mL lysozyme, and incubated at 37 °C for 15 min. The lysate was mixed with 0.5 mol/L Tris HCl, 0.5 mol/L EDTA, 10 mg/mL proteinase K, and 25% SDS. DNA was extracted with phenol, sodium perchlorate, and chloroform, and after centrifugation (5000 × *g*, 5 min) it was precipitated with cold ethanol. DNA was resuspended in 150 μL of sterilized TE (10 mM Tris, 1mM EDTA, pH 8.0).

2.11. Detection of *flp-1*, *apaH*, *ltx*, and *cdt* genes

Polymerase chain reaction (PCR) assays were performed by using specific primer pairs (Table 1). Amplifications were performed in volumes of 25 μL containing 10× PCR buffer, 50 nmol/L $MgCl_2$, 0.2 nmol/L dNTP mixture, 0.5 U *Taq* platinum DNA polymerase, 0.4 μmol/L of each primer and 1 ng template DNA.

DNA amplifications were performed in a thermal cycler (Amp PCR System 9700; Perkin Elmer, Sao Paulo, SP, Brazil), programmed for 94 °C (5 min) followed by 30 cycles of 94 °C (1 min), 50 °C to 65 °C (1 min), 72 °C (1 min), and then 72 °C (5 min) to allow DNA extension (Table 1). Ultrapure water instead of DNA was used as negative control. PCR products were analyzed by electrophoresis in 1% agarose gel, stained with ethidium bromide (0.5 μg/mL), and photographed on a UV light transilluminator (Electrophoresis Documentation and Analysis System 120; Kodak Digital Science, Sao Paulo, SP, Brazil). One-kilobase DNA ladder was used as marker.

2.12. Statistical analyses

All statistical analyses were performed with GraphPad InStat statistical analysis software (version 3.05, Graphpad Software, San Diego, CA, USA). Data were calculated from 2 experiments and analyzed by the chi-square and Fisher's exact test. A $P < 0.05$ was considered significant.

3. Results

3.1. Recovery and biotyping of *A. actinomycetemcomitans*

From 65 periodontal patients and 48 healthy subjects, 70 and 3 strains, respectively, were recovered. From 70 strains, 45 belonged to biotype II, 9 to biotype X, 5 to biotype VI, 5 to biotype VIII, 3 to biotype IX, 1 to biotype I, 1 to biotype III, and 1 to biotype V. Of the 3 strains from healthy subjects, 2 belonged to biotype II and 1 to biotype X.

3.2. Adhesion assay

Adherence to KB cells was observed in 64 (91.4%) of 70 strains from periodontal patients and in 2 of 3 strains from healthy subjects

Table 1
Oligonucleotides, annealing temperature, and amplicon sizes used in gene detection.

Genes	Oligonucleotides (5′→3′)	Annealing temperature (°C)	Amplicon (bp)	Reference
flp1	AAC AAC AAT AGG AGC ATT AAG ACA GTA TTT AAT ATT TAA GTT GTT ACT TATT	55	300	Kachlany et al. (2001)
apaH	ATT TAA TCG GCG ACC TGC AC TGT CTT CCC AAC GTA GCA TG	52	825	Dogan et al. (1999)
cdtA	GGT TTA GTG GCT TGT CAC GTA ATG GTT CTG TT	50	583	Fabris et al. (2002)
cdtB	GGT TTT CTG TAC GAT GT GGA TGT AAT TTG TGA GCG T	50	790	Fabris et al. (2002)
cdtC	GAC TTT GAC GAG TCA TGC A CCT GAT TTC TCC CCA	50	512	Fabris et al. (2002)
cdtABC	GGT TTA GTG GCT TGT CCT GAT TTC TCC CCA	50	2065	Fabris et al. (2002)
ltxA	TCG CGA ATC AGC TCG CCG GCT TTG CAA GCT CCT CAC C	65	285	Tan et al. (2002)
Ltx (promotor)	GCA GGA TCC ATA TTA AAT CTC CTT GT GCG GTC GAC AAC CTG ATA ACA GTA TT	55	530[a] 1022[b]	Cortelli et al. (2003)

[a] Highly leukotoxic strain.
[b] Minimally leukotoxic strain.

Table 2
Adhesion and invasion to KB cells by *Aggregatibacter actinomycetemcomitans* isolated from periodontal and health sites.

Isolates (no.)	Adhesion		Invasion		CFU/mL (10^3) average
	n	%	*n*	%	
Periodontal patients (70)	64	91.4	32	50	0.2 to 1.4
Health subjects (3)	2	66.6	1	50	0.1 to 10

(Table 2). These values showed statistically significant differences ($P = 0.002$). Adherent bacteria displayed nonlocalized clusters and were easily differentiated from enteropathogenic *E. coli* O127:H6 (E2348/69) that displayed a localized adhesion pattern (Fig. 1).

3.3. Invasion assay

Thirty-two of the 64 adherent strains from periodontal patients and 1 of 2 adherent strains from healthy subjects were able to invade KB cells. None of the nonadherent isolates was able to invade KB cells (Table 2). Most of *A. actinomycetemcomitans* isolated from periodontal patients were able to invade KB cells, but no statistically significant differences were observed ($P = 0.110$).

3.4. Neuraminidase

Sixty (82.2%) of all 73 strains produced neuraminidase with values from 2 to 8. Galactose was able to inhibit the sialidase activity in all strains.

3.5. Biofilm

Fifty-two of 70 strains from periodontal patients and 2 of 3 strains from healthy subjects produced biofilms with values from 0.5 to >3. In addition, 18 (25.7%) produced biofilms with values from <0.1 to 0.4.

3.6. Detection of capsule and fimbriae

Capsule was observed by light microscopy in all strains and by transmission electron microscopy as an electron dense layer. No strain showed any fimbriae-like structures, although vesicles on the bacterial surface were observed.

3.7. Bacterial cytotoxicity on KB cells

Of the 70 and 3 *A. actinomycetemcomitans* isolated from periodontal patients and healthy subjects, 44 and 2 strains, respectively, were cytotoxic, producing elongated to rounded cells.

3.8. Detection of genes

The *apaH* and *flp-1* genes were detected in 51 (72.9%) and 38 (54.3%) strains from periodontal patients and in 1 and 2 strains from healthy subjects, respectively (Fig. 2). All the 70 *A. actinomycetemcomitans* from periodontal patients harbored the LTX promoter. The *ltxA* gene was observed in 64 (91.4%) strains producing bands of 530 bp and in 6 strains producing bands of 1022 bp. In addition, 50 (71.4%) strains carried the *cdtA* gene; 48 (68.6%) strains, the *cdtB* gene; 60 (85.7%) strains, the *cdtC* gene; and 40 (57.1%) strains, the *cdtABC* gene. The 3 strains isolated from healthy subjects harbored the *ltxA* gene and 1 of them the *cdtC* gene (Table 3).

4. Discussion

Aggregatibacter actinomycetemcomitans has been implicated in periodontitis in young subjects, particularly in aggressive periodontitis (Asikainen et al., 1997). This microorganism produces several virulence factors that are involved in oral cavity colonization, destruction of periodontal tissues, and interference with host's defense mechanism (Wilson and Henderson, 1995). Specific biotypes and serotypes of *A. actinomycetemcomitans* may assume particular importance for disease development (Dogan et al., 1999). In this study, all the 73 strains were grouped into 8 different biotypes; however, most of them belonged to biotype II. *A. actinomycetemcomitans* biotyping is useful for epidemiologic studies, and, in general, individuals from the same family harbor the same biotype and serotype, suggesting that an intrafamilial route of infection would be partly responsible for the familial nature of the periodontitis (Genco and Loos, 1991).

Several factors have been related to the virulence of *A. actinomycetemcomitans*, such as fimbriae, sialidases, amorphous structures, and adhesin production, but the role of these factors in adherence is still unclear. Besides, each member of these organisms synthesizes a significant amount of multiple capsular polysaccharides, which are structurally different, suggesting distinct functional properties (Diaz et al., 2006; Fives-Taylor et al., 1996). Surface vesicles have been observed in oral bacteria, such as *Porphyromonas gingivalis*, and it has been suggested that there is an association between those structures and biological properties, such as proteolysis, hemagglutination, and

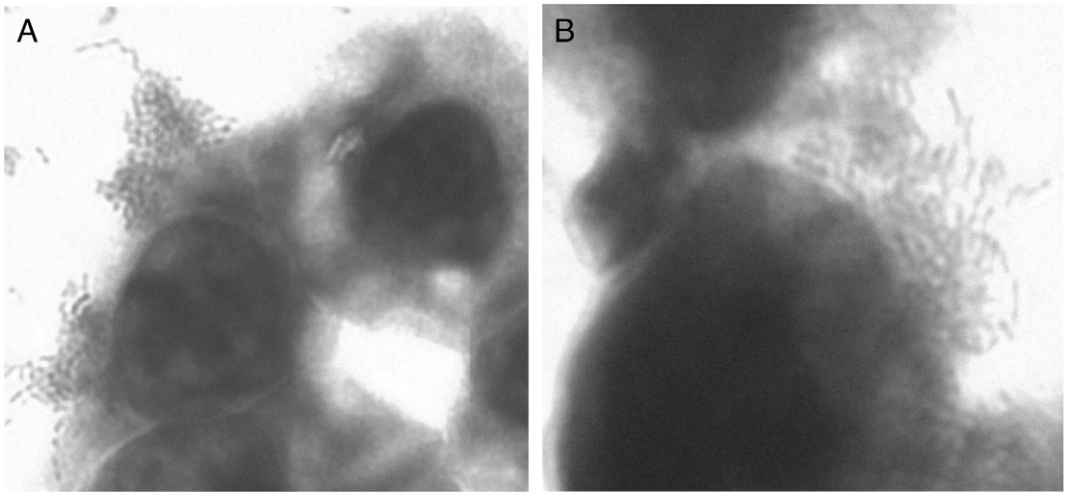

Fig. 1. Adherence to KB cells of *A. actinomycetemcomitans* (A) and *Escherichia coli* O127:H6 (E2348/69) (enteropathogenic) (B).

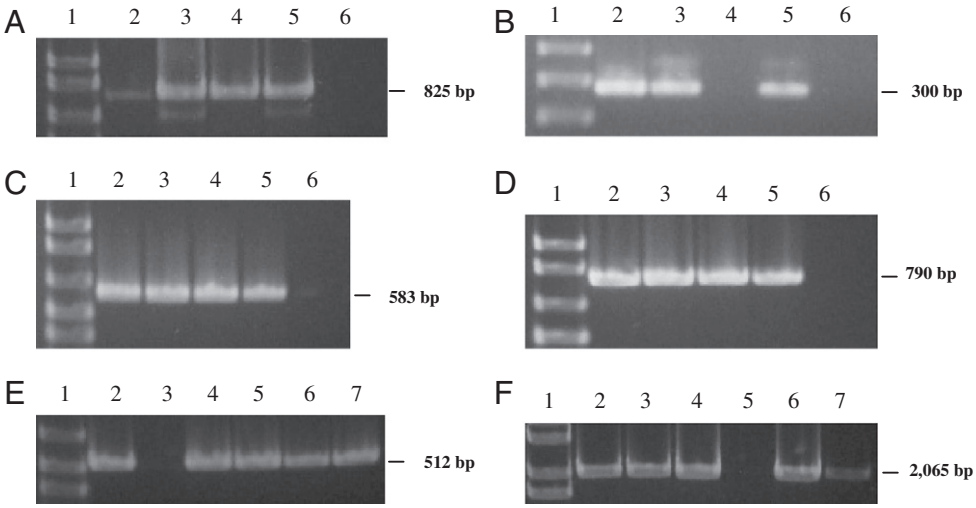

Fig. 2. Detection of the *apaH* (A), *flp1* (B), *cdtA* (C), *cdtB* (D), *cdtC* (E), and *cdtABC* genes (F) in *A. actinomycetemcomitans*.

coaggregation. A possible role of vesicles in the virulence includes their ability to disseminate enterotoxin and endotoxin, and also to penetrate epithelial cell barriers.

Encapsulated species are often found in bacterial strains belonging to the resident oral microbiota (Nishihara et al., 1995). Capsule was observed in all the strains; although we did not see a direct correlation with adhesion and invasion, it suggests that, maybe, the capsule is a necessary but not a sufficient factor in this process. The capsular polymer can first protect *A. actinomycetemcomitans* from phagocytosis, and when produced in high amounts, it stabilizes its adhesion to epithelial cells.

Several studies have demonstrated the pathogenicity of encapsulated oral bacteria and their ability to induce abscesses in experimental animals even when inoculated alone. Little is known about the role of the *A. actinomycetemcomitans* capsular polysaccharide on the host's cells (Henderson et al., 2003; Suzuki et al., 2000).

The KB cell line has been used as a model for gingival epithelial cells on the basis of studies that have shown selective invasion of *A. actinomycetemcomitans* into these cells (Meyer et al., 1991). In this study, fimbriae-like structures were not observed in any of the 66 *A. actinomycetemcomitans* showing an adherent phenotype, and it is suggested that more than 1 adhesin may be involved and/or outer membrane proteins could be responsible for this process. Pruzzo et al. (1984) affirm that the poor knowledge about this structure is due to the fact that fimbriae require a precise growth condition to be assembled and in low iron concentration and different temperatures affect the expression of this structure.

Fresh clinical strains of *A. actinomycetemcomitans* are able to adhere tightly to themselves or to substrates, such as glass, plastic, and hydroxyapatite (Fine et al., 1999a; Kachlany et al., 2000). Studies have shown that amorphous extracellular material produced by *A. actinomycetemcomitans* collaborates to convert a minimally adherent strain to a highly adherent strain.

It is well established that invasiveness is a prerequisite for the pathology of certain bacterial pathogens (Wilson and Henderson, 1995). *A. actinomycetemcomitans* displays many factors (fimbriae, hemagglutinin, capsule, lipopolysaccharide, outer membrane vesicles, and enzymatic activities) that can perturb the host's defense mechanisms and initiate tissue destruction; however, no specific invasion mechanisms have been identified. Studies have shown that invasion of human cells by *A. actinomycetemcomitans* has been limited to some bacterial strains, representing approximately 25% of invasive *A. actinomycetemcomitans* (Meyer et al., 1991). On the other hand, it is not known whether the Flp-1 protein is involved in the adhesion to epithelial cells.

The invasion rate of *A. actinomycetemcomitans* was low compared to other periodontal pathogens such as *Porphyromonas gingivalis* (Nakagawa et al., 2002), and the unusual behavior of leaving and moving from one cell to another within a host cell needs further studies to characterize the mechanisms involved in the invasion process (Meyer et al., 1996). In this study, 50% of the 66 adherent strains were able to invade epithelial cells and, of these, 38 strains harbored the *flp-1* gene. This result suggests that this gene may be in an active or inactive state or that this microorganism needs to express the *flp-1* gene by other mechanisms not determined here.

Studies have shown that the *flp-1* gene is linked to the *tadABCDEFG* genes, and this gene is found upstream of the *tad* operon in a low G + C region along with 2 other genes (*rcpA* and *rcpB*) expressed specifically in rough adherent bacteria but not in smooth mutants (Fine et al., 1999b). Our results confirm the findings showing that the level of adhesion is not an indication of the bacterial invasion phenotype. Data support the hypothesis that adhesion and invasion are separate events, and they are mediated by different macromolecules expressed on the outer membrane (Mintz and Fives-Taylor, 1994).

The leukotoxin operon consists, in transcription order, of 4 genes: *ltxC*, *ltxA*, *ltxB*, and *ltxD*. The LtxA protein needs to be acylated by LtxC and an acyl carrier protein to become biologically active (Lally et al., 1999). Analysis has revealed that adherent or rough colonies of *A. actinomycetemcomitans* do not secrete leukotoxin but nonadherents or smooth colonies do (Kachlany et al., 2000).

Haubek et al. (2007) have suggested that highly leukotoxic *A. actinomycetemcomitans* strains comprise a single clone, and, in our study, only 64 strains were considered highly leukotoxic and 6 minimally leukotoxic, which belonged to 8 different biotypes: I, II, III, V, VI,VIII, IX, and X. In addition, biotype II and X were the most

Table 3
Presence of genes in *Aggregatibacter actinomycetemcomitans* isolated from periodontal and healthy subjects.

Isolate no.	No. of isolates carrying genes							
	LTX promoter[a]	*ltxA*	*cdtA*	*cdtB*	*cdtC*	*cdtABC*	*apaH*	*flp-1*
Periodontal	64[b]	64	50	48	60	40	51	38
Patients (70)	6[c]	6	0	0	0	0	0	0
Healthy (3)	3[c]	3	0	0	1	0	1	2
Strain JP2[b]	+	+	+	+	+	+	+	+

[a] Leutotoxin promoter.
[b] Highly leukotoxic strains (530 bp).
[c] Minimally leukotoxic strains (1022 bp).

prevalent, colonizing 12% and 8% of the periodontal patients, respectively. In healthy subjects, biotypes II and X were observed. Few studies have shown the presence of biotypes in *A. actinomycetemcomitans*, and biotype II appears to be the most prevalent in Brazilian periodontal patients (Avila-Campos et al., 1995; Gaetti-Jardim et al., 2008).

Our results show that all the strains were leukotoxic, but no relationship with any biotype was observed. In addition, it is well known that the presence of highly leukotoxic *A. actinomycetemcomitans* is associated with ecologic and immunologic conditions of the examined population (Haubek et al., 1997). On the other hand, the presence of minimally and highly leukotoxic strains observed in this study was not associated with the development of periodontitis in the analyzed patients. Also, the presence of minimally leukotoxic strains in healthy subjects suggests its possible transitory stage in oral cavity.

The cytolethal distending toxin (CDT) is a cycle-modulatory protein with immunosuppressive function produced by *A. actinomycetemcomitans*. This toxin is the product of a 3-gene operon (*cdtA*, *cdtB*, *cdtC*) which is found in a range of bacteria including *E. coli*, *Shigella* spp., *Campylobacter* spp., and *Helicobacter* spp. (Smith and Bayles, 2006). The mechanism of action of this toxin is believed to be due to the nuclease activity of CdtB (Lara-Tejero and Galán, 2000). The CDT of *A. actinomycetemcomitans* and other bacteria requires all 3 CDT proteins for cell cycle inhibition; however, Shenker et al. (2001) have reported that purified or recombinant *A. actinomycetemcomitans* CdtB is enough to block human lymphocyte cells.

In this study, 20 of the evaluated strains lacked at least one of the *cdt* genes as determined by PCR analysis. Forty of these 70 isolated exhibited amplicons when the whole *cdtABC* gene operon was amplified using primers homologous to the *cdtA* and *cdtC* genes. CDT activity was observed with cell lysates from 5 strains in which *cdtA*, *cdtC*, or *cdtABC* was not detected by PCR. However, the toxic effect of these strains was lower than that observed with lysate from *A. actinomycetemcomitans* JP2 strain. The absence of amplicons in strains may be explained by the deletion of the genes or the lack of homology with the primers, but it is also possible to observe the presence of a partial deletion without loss of toxin activity. In addition, it has been reported that *cdtC* is essential for the cytotoxic effect of *A. actinomycetemcomitans* (Shenker et al., 1999). In this study, only 1 strain obtained from healthy subjects harbored the *cdtC* gene, and it is suggested that this microorganism could carry an active or inactive gene and it was temporary in the oral microbiota.

Thus, in the present study, we describe the phenotypic and genotypic features of *A. actinomycetemcomitans* strains isolated from periodontal disease, as well as the in vitro adhesion and cytotoxicity to epithelial cells, and neuraminidase enzyme production. Moreover, 2 of 3 strains from healthy individuals showed potential virulence factors which could cause periodontitis, and, certainly, more studies are necessary to understand the real role of this microorganism in periodontal disease.

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