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Characterization of *Actinobacillus actinomycetemcomitans* isolated from young Chinese aggressive periodontitis patients

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Objective: This study characterized *Actinobacillus actinomycetemcomitans* isolates from young Chinese aggressive periodontitis patients.

Methods: Subgingival plaque samples (two/subject) were collected from diseased subjects < 25 years old ($n = 9$, mean age 21.1 ± 1.6 years) and age-matched periodontitis-free controls ($n = 47$, mean age 22.0 ± 1.1 years). Selective and anaerobic culture were used. The serotype, leukotoxin gene (*ltx*) operon promoter and the cytolethal distending toxin (*cdt*) genes complex of the *A. actinomycetemcomitans* isolates were investigated. Effects of the isolates on non-keratinizing periodontal ligament epithelial cells monolayer were studied.

Results: Diseased subjects had significantly higher full-mouth bleeding score ($p = 0.002$) and total viable counts from plaque samples (7.2×10^6 vs. 2.1×10^5 CFU/paperpoint, $p < 0.005$). *A. actinomycetemcomitans* was isolated from 67%/56% or 6%/4% of diseased or controls subject/sites, respectively ($p < 0.001$). The proportion of *A. actinomycetemcomitans* isolatable from aggressive periodontitis or periodontitis-free associated subgingival plaque was low (0.7% vs. 0.1%, $p < 0.02$). The serotype of the isolates was characterized. All isolates possessed 652-like *ltx* gene promoter and all but one serotype c isolate from a diseased patient had intact *cdtABC* genes. That particular strain appeared to confer the least cellular damages on periodontal ligament epithelial monolayer compared to others.

Conclusion: This preliminary study confirmed the notion of increased prevalence and quantity of *A. actinomycetemcomitans* associated with aggressive periodontitis in young patients. The overall *ltx* promoter and *cdt* characteristics of the *A. actinomycetemcomitans* isolates, however, were similar among the diseased and control groups. A strain lacking the *cdtABC* gene appeared to be less damaging to a periodontal ligament epithelial cell model. Further studies therefore are warranted to clarify the pathogenic role and potentials of *A. actinomycetemcomitans* in aggressive periodontitis.

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In the southern Chinese city of Hong Kong, the population incidence of deep periodontal pocket in adolescents is moderate (approx. 1%) (1, 2) compared to 0.5–1.5% in the rest of the world (see review (3)). However, the proportion of 15–19 year-olds in Hong Kong with shallow periodontal pockets was 26% (1), which was comparable to the 22% of subjects of the same age, who had shallow pockets in the United States (4). Periodontal diseases observable in young individuals are believed to be caused by various periodontopathogens, as in the case for older individuals (5). The microorganism *Actinobacillus actinomycetemcomitans* is regarded as one of the key bacterial agents associated with aggressive periodontitis in young adults (6).

Six serotypes of *A. actinomycetemcomitans*, i.e. serotypes a, b, c, d, e and f, have been identified (7). *A. actinomycetemcomitans* is a special periodontopathogen in the sense that in most strains at least two complex multigene toxin systems are in operation, the leukotoxin (RTX) (8) and the later identified cytolethal distending toxin (CDT) (9). The RTX or repeats in the structural toxins comprise a family of large, heat-labile, Ca^{2+}-dependent, pore-forming cytotoxins that display different target cell specificities (10). *A. actinomycetemcomitans* leukotoxin is active against lymphocytes and granulocytes (8), triggering hyper-production of reactive oxygen intermediates, and causes degranulation and release of lysosomal contents leading to inflammation-mediated injury of infected host tissue or programmed cell death in affected host cells (11). The ultimate effect of *A. actinomycetemcomitans* leukotoxin seems to enable the pathogen to evade the host immune system, hence establishing a local infection. CDT is a multicomponent bacterial holotoxin that targets most eukaryotic cells, causing distention and cell cycle arrest (12). In brief, CdtB, the active subunit of the CDT holotoxin, through its nuclease activity, could induce limited DNA damage of infected eukaryotic cells and hence lead to arrest of cell cycle or proliferation (13). This action of CDT was suggested to be a strategy used by *A. actinomycetemcomitans* to moderate host cell functions (14).

In recent years, a series of clinical and microbiological studies on young Chinese adults of southern and western Chinese decent with poor oral hygiene and untreated gingivitis (15) showed a high prevalence of the putative periodontopathogens *Porphyromonas gingivalis* and *A. actinomycetemcomitans* in subgingival plaque samples (16). Upon examination of the leukotoxin gene *lktA* operon of the *A. actinomycetemcomitans* isolates, Mombelli and coworkers (16) could not detect the presence of a deletion in the promoter region indicative of a highly leukotoxic (or JP2 type) *A. actinomycetemcomitans* strain. Later on, the same group conducted a larger scale microbiological study, which included the above young subjects with untreated gingivitis ($n = 73$), 35–44 years-old subjects from an oral epidemiological survey in southern China ($n = 81$), and middle-aged periodontitis patients in Hong Kong ($n = 31$) (17). They concluded that *A. actinomycetemcomitans* appeared to be a common constituent of the normal oral flora in the Chinese subjects, a finding confirmed by Tan and coworkers in Singapore (18) and also shown for a rural adult population in southern Thailand (19). No highly leukotoxic strain similar to JP2 type of *lktA* promoter sequence could be isolated by Mombelli and coworkers in Chinese subjects (17), complementing previous reports about racial tropism of a highly leukotoxic *ltx* clone of *A. actinomycetemcomitans* (20, 21) shown to be pathogenic among *A. actinomycetemcomitans* detected in early onset periodontitis (22).

The prevalence of CDT in *A. actinomycetemcomitans* clinical isolates has also recently been studied. Clinical isolates from Brazil, Kenya, Japan and Sweden were analyzed (23) and three *cdt* genes, i.e. *cdtA*, *cdtB* and *cdtC*, were detectable from 34 of 40 *A. actinomycetemcomitans* strains. However, that study did not report the relationship between CDT positivity and the periodontal status of the patients and the sites from which the *A. actinomycetemcomitans* were sampled. A later report by Tan *et al.* (24), using direct polymerase chain reaction (PCR) detection of subgingival plaque samples from 146 Singaporean patients with various periodontal diseases, detected a low prevalence of the *cdt* genes complex. In that study, subjects of mean age 42.5 ± 6.5 years were sampled. Twelve per cent of diseased sites with *A. actinomycetemcomitans* were *cdt* genes complex positive, of which 77% were from adults diagnosed with aggressive periodontitis, showing a strong association between the presence of these genes with severe disease conditions. No data, however, is available yet regarding the occurrence of *A. actinomycetemcomitans cdt* genes in young adults suffering from aggressive periodontitis.

The aim of the present study was hence to investigate the *A. actinomycetemcomitans* carrier rate in young Chinese subjects with untreated aggressive periodontitis and to compare this with the carrier rate in periodontitis-free age-matched controls, and to study the prevalence and nature of the *A. actinomycetemcomitans* isolates' serotypes, and the two complex multigene toxin genotypes and their pathogenic potential in a porcine periodontal ligament epithelial cell explant model.

Material and methods

Subjects

Subgingival microbiological samples were obtained from nine untreated aggressive periodontitis patients recruited over a period of 6 months, who also fulfilled the following inclusion criteria: (i) < 25 years old, (ii) possessing pockets ≥5 mm on at least two of the first molars or incisors and these teeth should be free from > 1 surface restorations, not endodontically treated or showing signs of pulpal diseases, (iii) not taking any systemic antibiotics in the preceding 6 months, and (iv) not suffering from any debilitating systemic diseases and not pregnant. The aggressive periodontitis subjects were recruited from the University Health Service Dental Clinic and the Reception Clinic of the Prince Philip Dental

Hospital, Faculty of Dentistry, the University of Hong Kong. During the recruitment period, approximately 800 first year university students attended the University Health Service Dental Clinic for initial examination and six aggressive periodontitis patients satisfying the inclusion criteria were identified and recruited. Another second-year university student with aggressive periodontitis was recruited out of three potentially suitable cases referred from the University Health Service Dental Clinic for possible inclusion. Two more subjects were recruited during the same period from a total of 5041 adult patients who attended the Reception Clinic, Faculty of Dentistry for screening. Fifty age-matched periodontitis-free university dental students (control group) were recruited, who had (i) sound ($\leq$ 1 surface restoration, not endodontically treated or showing signs of pulpal disease) first molars and incisors; (ii) no probing pocket depth $>$ 3 mm, and (iii) no radiographic signs of alveolar bone loss on any standing tooth.

Clinical examination

The following parameters were measured at six sites on all teeth: (i) plaque recording (modified from (25)), (ii) probing pocket depth using William's 14 W periodontal probe, and (iii) bleeding on probing. Clinical attachment level was measured from the depth of the sampling pocket to the cemento-enamel junction. All aggressive periodontitis subjects, after examination and microbiological sampling, were given a comprehensive course of non-surgical periodontal therapy followed by appropriate supportive periodontal therapy. Surgical periodontal therapy was carried out for individual patients when indicated.

Microbiological sampling

In the aggressive periodontitis patient group, the deepest periodontal pockets from two non-neighbouring teeth were selected for microbial sampling. All sample sites from the aggressive periodontitis group were from first molars. For control subjects, two sites were selected randomly from any two of the four first molars for microbial sampling. The microbiological sampling was performed as described previously (26). In brief, the sites to be sampled were dried and isolated with sterile cotton wool rolls. Supragingival plaque was removal from the sample site and adjacent teeth with a sterile curette. Four sterile endodontic paper points (medium absorbent points, DiaDent, Burnaby, Canada) were inserted into the sample site two at a time until resistance was felt and left in place for 20 s. The specimens were than transferred to the laboratory in 1 ml reduced transport fluid at 4°C. All samples were processed within 1 h after sampling.

Culture

All samples were vortexed for 20 s at maximum setting (Autovortex Mixer SA2, Stuart Scientific, London, UK) and then serially diluted in 10-fold increments up to 1000-fold in trypticase soy broth (TSB). The diluted samples were plated onto enriched Columbia blood agar (CBABS, Columbia agar base with 5% defibrinated horse blood, 0.0005% hemin and 0.00005% Vitamin K) (26) and Tryptic Soy–Serum–Bacitracin–Vancomycin Agar (TSBV) (27) using a Spiroplater (Spiral Plater Model D, Spiral System Inc., Cinannati, OH, USA). CBABS and TSBV plates were incubated in an anaerobic chamber (Forma Scientific Inc, Marietta, OH, USA) under an atmosphere of 10% CO_2, 10% H_2, 80% N_2 for 5–7 days at 37°C.

TSBV or CBABS plates with appropriate number of colonies that were well separated and evenly dispersed with 30–300 colony-forming units (CFU) were chosen for counting. Total viable count measurement was performed on CBABS plates. The presumptive identification of *A. actinomycetemcomitans* on TSBV plate was based on colony morphology (transparent colonies with characteristic stellar structure) and a positive catalase reaction. Presumptive *A. actinomycetemcomitans* isolates were subcultured in TSBV plates to obtain pure cultures. Pure culture of *A. actinomycetemcomitans* was identified using the MicroSeq 500 16S rDNA-based bacterial identification system (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA). With reference to the dilution factor and the count on TSBV, the *A. actinomycetemcomitans* CFU per paper point was also calculated.

Bacterial strains

The bacteria strains used in this study and their relevant genotypic characteristics are shown in Table 1.

Table 1. Bacterial strains

Strains	Serotype	*ltx* promoter class[a]	*cdt* genotype[b]			Source[c]
			cdtA	*cdtB*	*cdtC*	
Actinobacillus actinomycetemcomitans						
ATCC 29522	b	652	+	+	+	ATCC
ATCC 29523	a	652	+	+	+	ATCC
ATCC 33384	c	652	+	+	+	ATCC
ATCC 43718	b	652	+	+	+	ATCC
JP2	b	JP	+	+	+	MK
HK 928	d	JP	+	+	+	MK
HK 929	b	JP	+	+	+	MK
HK 1037	b	652	+	+	+	MK
Haemophilus aphrophilus						
HK 329						MK

[a]Promoters were classified as 652-like or JP2-like on the basis of polymerase chain reaction amplification of the promoter region as described by Brogan *et al.* (28); promoter class was named as published previously (28, 29) and/or as detected by the current study.
[b]Genotype as described by Yamano *et al.* (30) and/or as detected by the current study.
[c]ATCC, American Type Culture Collection; MK, Dr Mogens Kilian, University of Aarhus.

DNA preparation of *A. actinomycetemcomitans*

DNA was isolated from 2-days-old *A. actinomycetemcomitans* reference strains or isolates cultured in TSB supplemented with 1% (wt/vol) yeast extract. Isolation of chromosomal DNA was carried out according to the instruction on the QIAamp DNA kit (Qiagen, Hilden, Germany) using a series of commercial lysis, wash and elution buffers. In brief, 200 μl of the bacterial culture on TSB was pelleted at 5000 *g* for 10 min, washed once in phosphate-buffered saline, pH 7.2 and then resuspended in 180 μl ATL (lysis) Buffer. Twenty microliters of Proteinase K was added to the mixture, vortexed and incubated at 56°C on a shaking water bath overnight for cell lysis. Then 200 μl AL (lysis) Buffer was added to the sample, followed by pulse-vortexing of the mixture and incubation at 70°C for 10 min. The bacterial lysate was washed in the following sequence: (i) addition of 200 μl 99% ethanol to the sample, vortexed 15 s, then carefully applied to the QIAamp spin column, centrifuged at 6000 *g*, 1 min; (ii) 500 μl AW1 (wash) Buffer at 6000 *g*, 1 min (iii) 500 μl AW2 (wash) Buffer at 13,000 *g*, 3 min, followed by a repeated spin at 13,000 *g*, 1 min. Then, 200 μl AE (elution) Buffer was added and the spin column incubated at room temperature for 1 min, followed by DNA elution at 6000 *g*, 1 min. The elution step was repeated by adding another 200 μl AE Buffer and the resultant eluted DNA were pooled, aliquoted and stored at −20°C for future PCR analysis.

MicroSeq 500 16S rDNA-based bacterial identification

DNA extract from pure culture of presumptive *A. actinomycetemcomitans* strains was amplified according to the manufacturer's instruction. In brief, the 25 μl of 2 × PCR master mix was added to 25 μl of DNA template (1 μg/ml). The mixture was amplified at 95°C for 10 min initial denaturation, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 10 min in a GeneAmp 9700 machine (Applied Biosystems). The amplified product was purified using the QIAquick gel extraction kit (Qiagen). PCR products were bi-directionally sequenced with forward and reverse sequencing mix using an ABI 310 automated sequencer according to the manufacturer's instruction (Applied Biosystems). The DNA sequences were analyzed using the database provided by the MicroSeq 500 16S rDNA-based bacterial identification system. The sequence homology analysis was performed using the BLAST algorithm in the database of the National Center for Biotechnology Information.

PCR analysis of *A. actinomycetemcomitans* genes

PCR primers — Table 2 lists the PCR primers used in this study.

Serotyping — PCRs were performed on each 16S rDNA confirmed *A. actinomycetemcomitans* isolate as described by Kaplan *et al.* (7). The primer sets used were as shown in Table 2. The 100 μl volume of the PCR master mix contained 2 μl of *A. actinomycetemcomitans* DNA template, 2 μl of 50 mM $MgCl_2$, 10 μl of 0.2 M Tris/0.5 M KCl, pH 8.4 (10 × PCR buffer), a 150 μM concentration of each dNTP, 1 μM of the corresponding forward and reverse primer and 2 U of *Taq* polymerase (Life Technologies, Frederick, MD, USA). The 30 cycles of the PCR protocol included 30 s of denaturation at 94°C, 90 s of annealing at

Table 2. Polymerase chain reaction primers used in characterization of *Actinobacillus actinomycetemcomitans*

Primer	Sequence (5′−3′)	Analysis	Product (base)	Reference
I. Serotype				
P11	TCTCCACCATTTTTGAGTGG	Serotype b	333	(7)
P12	GAAACCACTTCTATTTCTCC	Serotype c	268	
P13	CCTTTATCAATCCAGACAGC	Serotype f	232	
P14	ARAAYTTYTCWTCGGGAATG[a]			
P15	TGGGTCATGGGAGGTACTCC	Serotype a	293	
P16	GCTAGGAACAAAGCAGCATC			
P17	TGGAACGGGTATGGGAACGG	Serotype d	411	
P18	GGATGCTCATCTAGCCATGC			
P19	ATTCCAGCCTTTTGGTTCTC	Serotype e	311	
P20	TGGTCTGCGTTGTAGGTTGG			
II. Leukotoxin gene operon promoter				
Forward	TTTCTCCATATTAAATCTCCTTGT	*ltx* promoter	504 or 1034	(20)
Reverse	CAGATCAAAACCTGATAACAGTATT			
III. Cytolethal distending toxin				
cdtA-7	GATGGATCTAAGGAGAGATATAATG	*cdtA*	326	(31)
cdtA-13	AATTAACCGCTGTTGCTTCTAATACAG			
cdtA-12	AAGGAGTTTATATGCAATGGGTAAAG	*cdtB*	462	
cdtA-8	TAGCGATCACGAACAAAACTAACAG			
cdtA-1	TAGTTTTGTTCGTGATCGCTAAGGAG	*cdtC*	272	
cdtA-4	GCTACCCTGATTTCTTCGCACCG			

[a]R = A or G; Y = C or T; W = A or T.

57°C, 90 s of primer extension at 72°C. The PCR product were electrophoresed in 1.5% agarose gel in TBE buffer, stained with ethidium bromide, and visualized under UV transillumination. Isolates of the same serotype groups were noted and the analysis was repeated on at least one separate occasion with strains recovered from the stock kept at −70°C. Then the amplicon from the isolates underwent electrophoresis side-by-side with amplicon from positive *A. actinomycetemcomitans* control (strain of known serotype except for serotypes e and f, which were not available) to confirm the findings.

Characterization of A. actinomycetemcomitans ltx operon promoter — The characteristic of the *A. actinomycetemcomitans* leukotoxin gene operon promoter was studied by PCR (20, 28) with slight modifications to their methods. The corresponding custom-synthesized primers (Gibco BRL, Hong Kong) used in the study are shown in Table 2. A 100-μl volume of the PCR master mix contained 10 μl of *A. actinomycetemcomitans* DNA template, 10 μl of 0.5 M KCl/0.2 M Tris, pH 8.4 (10 × PCR buffer), a 150 μM concentration of each dNTP, 1.5 mM $MgCl_2$, 1 μM of forward and reverse primer and 2 U of *Taq* polymerase. The 30 cycles of the PCR protocol included 1 min of denaturation at 94°C, 1 min of annealing at 60°C, 2 min of primer extension at 72°C. Control tubes without template DNA or with DNA from *Haemophilus aphrophilus* were included in each run. The PCR products were analyzed by 0.8% agarose gel electrophoresis (20). Repeated PCR analysis was carried out for confirmation of results.

PCR detection of cdtABC genes — The characterization of individual *cdtABC* genes from *A. actinomycetemcomitans* was carried out as described by Ahmed *et al.* (31). PCR was performed with 5 μl of the DNA template in 50 μl reaction mixture containing 2 mM $MgCl_2$/50 mM KCl/10 mM Tris-HCl, pH 8.3, 2.5 U *Taq* polymerase, 200 μM of each dNTP and 10 μM of each primer. The amplification cycle comprised 30 cycles of denaturation at 94°C, 30 s, annealing at 57°C, 2 min and primer extension at 72°C, 2 min. The PCR products were visualized by gel electrophoresis on 1% agarose side-by-side with amplicon from positive controls, followed by staining using ethidium bromide and illumination with UV. The *cdtABC* genes analysis was repeated on one separate occasion with strains recovered from the stock kept at −70°C.

Eukaryotic cell culture

Porcine periodontal ligament epithelial cell explants (32) were cultured in minimum essential alpha medium (αMEM: Gibco BRL, Paisley, UK) with 15% fetal bovine serum (FBS, Gibco BRL) with 10,000 μg/ml penicillin G, 10 μg/ml streptomycin, 1.2% fungizone (v/v) (Gibco BRL), 30 μg/ml amphotericin B and 25 μg/ml sodium deoxycholate in the manner described previously (33). In brief, the periodontal ligament epithelial cells were cultured on an 12-well chamber slide (Nune, Naperville, IL, USA) until early confluency. Immediately prior to the assays, the culture medium was discarded and the monolayer washed twice with warm (37°C) αMEM. The washed periodontal ligament epithelial cultures were challenged with 100 μg/ml of 2.5×10^9, 5×10^8 and 1×10^8 *A. actinomycetemcomitans* per ml suspensions in 15% FBS–αMEM, 37°C, 5% CO_2 in air, 24 h. Periodontal ligament epithelial passages 5–10 were used in the experiment.

Morphometric analysis of *A. actinomycetemcomitans*-induced periodontal ligament epithelial cell damage

A. actinomycetemcomitans-treated periodontal ligament epithelial cells were fixed and stained with May–Grünwald and Giemsa stains (E. Merck, Darmstadt, Germany) as described earlier (34). After mounting, the specimens were examined under a microscope at 40 times magnification fitted with a digital camera (Leica DC 300 V 2.0, Leica, Wetzlar, Germany). The morphometric analysis was done as previously described (33) using an image analyzing software (Qwin version 2.4, Leica, Cambridge, UK). Cell number, cell area and mean cell size were measured.

Statistics

Data on patient demography, clinical conditions, *A. actinomycetemcomitans* isolation and *A. actinomycetemcomitans*-induced periodontal ligament epithelial cell damage – in terms of confluency, cell size, proportion of cells left attached – were expressed as mean ± standard deviation. Differences between the means were analyzed by unpaired *t*-test with Welch correction, or analysis of variance with Bonferroni adjustment for multiple comparison when appropriate. Fisher's exact test was also used. Groups were regarded as significantly different from each other if $p < 0.05$.

Results

Clinical findings

Nine aggressive periodontitis patients fulfilled the inclusion criteria and were recruited. Forty-seven out of 50, prescreened to meet the inclusion criteria, university dental students participated as controls. Two out of nine and 20 out of 47 of the diseased and periodontitis-free controls, respectively, were male. The age of the test (19–24 years) and control (20–24 years) subjects was well matched. On the whole, the diseased and periodontitis-free control subjects appeared to have similar plaque levels: 16–52% in aggressive periodontitis group vs. 7–60% in healthy controls. The aggressive periodontitis subjects, however, showed significantly higher bleeding on probing, 23–97%, compared to 9–76% in healthy controls (Table 3), indicating that periodontal or gingival inflammation was generalized among the diseased subjects. The diseased subjects possessed a mean of 7 pockets ≥6 mm (2–17 pockets per subject). The periodontal conditions of the 112 sample sites were as shown in Table 4. All except one site in the diseased group bled on probing, whereas only

Table 3. Demographic and periodontal conditions of subjects

	Aggressive periodontitis	Control
n	9	47
% male	22	43
Age (year, mean ± SD)	21.1 ± 1.6	22.0 ± 1.1
Plaque % (mean ± SD)	27.8 ± 15.6	26.4 ± 14.7
Bleeding on probing % (mean ± SD)[a]	56.8 ± 25.9	28.9 ± 22.5
No. of pocket ≥6 mm (mean ± SD)	6.8 ± 4.9	0

[a]Significant difference between aggressive periodontitis subjects and control group, unpaired *t*-test, $p = 0.0016$.

Table 4. Clinical and microbiological data

	Aggressive periodontitis	Control
Sample site		
Bleeding on probing %[a]	94%	43%
Pocket depth (mean ± SD)[b]	6.7 ± 1.7	1.9 ± 0.6
Clinical attachment level (mean ± SD)[b]	6.8 ± 2.0	1.9 ± 0.6
Total anaerobic viable count ($\times 10^6$ CFU/paper point, mean ± SD)[b]	7.2 ± 9.1	0.2 ± 0.5
Actinobacillus actinomycetemcomitans		
Prevalence per subject[a]	67%	6%
Prevalence per site[a]	56%	4%
No. of strains per subject[b]	1.0 ± 0.9	0.1 ± 0.4
Count (CFU/paper point)[b]	$2.1 \times 10^4 \pm 3.1 \times 10^4$	$6.3 \times 10^2 \pm 3.7 \times 10^3$
Percentage proportion[b]	0.7 ± 0.6	0.1 ± 0.8

[a]Significant difference between aggressive periodontitis and periodontitis-free groups, $p < 0.0002$, Fisher's exact test.
[b]Significant difference between aggressive periodontitis and periodontitis-free groups, $p < 0.02$, unpaired *t*-test, with Welch correction.
CFU, colony forming units.

40 sample sites (43%) from the control group bled on probing.

Microbiological findings

Significantly higher total viable counts/paper point were detected in aggressive periodontitis samples than in periodontitis-free controls, and the specific counts for *A. actinomycetemcomitans* isolates were higher in aggressive periodontitis patients than in controls (Table 4). Per subject and per site, *A. actinomycetemcomitans* isolation prevalence was significantly higher in aggressive periodontitis subjects than in controls. Overall, the percentage proportion isolation of *A. actinomycetemcomitans* detected by culture was at a level below 1%.

A total of 14 *A. actinomycetemcomitans* strains were identified and characterized (Table 5). When more than one *A. actinomycetemcomitans* isolates were recovered from a subject, the genotypic profile of the isolates appeared identical. Isolates of serotype a, c and f were isolated from aggressive periodontitis sites, whereas serotypes b and c were isolated from subgingival plaque of controls (Table 5, Fig. 1). Serotype c, which was isolated in five of nine *A. actinomycetemcomitans* positive subjects or eight of 14 *A. actinomycetemcomitans* positive sites, was the predominant serotype isolated.

All *A. actinomycetemcomitans* isolates possessed the 652-like *ltx* promoter (Table 5, Fig. 2). Only from one strain of an aggressive periodontitis patient was the *cdt* genes complex not detectable (Table 5, Fig. 3).

Table 5. Genotypic characteristics of *Actinobacillus actinomycetemcomitans* isolates[a]

Strains[b]	Sample site	Serotype[c]	*Cdt* genotype[d] A	B	C
Aggressive periodontitis					
P1a	16ML	c	+	+	+
P1b	46DB	c	+	+	+
P4a	26MB	c	+	+	+
P4b	36DB	c	+	+	+
P5a	26ML	a	+	+	+
P5b	46ML	a	+	+	+
P7a	16MB	f	+	+	+
P7b	26MB	f	+	+	+
P8	46MB	f	+	+	+
P9	36MB	c	–	–	–
Controls					
P-F12	16DL	c	+	+	+
P-F33	46MB	b	+	+	+
P-F45a	26DB	c	+	+	+
P-F45b	46DL	c	+	+	+

[a]Identity of all *A. actinomycetemcomitans* isolates were confirmed by 16S rDNA partial sequencing (MicroSeq 500, Applied Biosystems) and all of them process the 652-like *ltx* promoter (20).
[b]*A. actinomycetemcomitans* strains designation: P, isolates from aggressive periodontitis patients; P-F, isolates from periodontitis-free controls; the Arabic numeral following the letters P or P-F denotes the code for an individual subject; the small letter a or b denotes isolates from different sample sites of the same subject.
[c]Serotype detection as per Kaplan *et al.* (7).
[d]+, the *cdt* A, B, or C gene detectable by polymerase chain reaction; –, the *cdt* A, B, or C gene not detectable by PCR (31).

A. actinomycetemcomitans induced periodontal ligament epithelial cell monolayer changes

A. actinomycetemcomitans reference strains and isolates induced changes in periodontal ligament epithelial cell monolayers (Table 6). When used in concentrations of 2.5×10^9 or 5×10^8 bacteria/ml, all specimens showed close to or 100% detachment of periodontal ligament epithelial cells (data not shown). At a concentration of 1×10^8 bacteria/ml, all *A. actinomycetemcomitans* strains caused a reduction in cells remaining attached, a general increase in cell size and various levels of change in periodontal ligament epithelial cell confluency. *A. actinomycetemcomitans* strain HK 928, which possessed the *cdtABC* gene and the JP2 *ltx* promoter (Table 1), appeared to induce the most damage to the periodontal ligament epithelial monolayer culture, whereas isolate P9, which possessed the 652-like *ltx* promoter and lacked the *cdtABC* gene

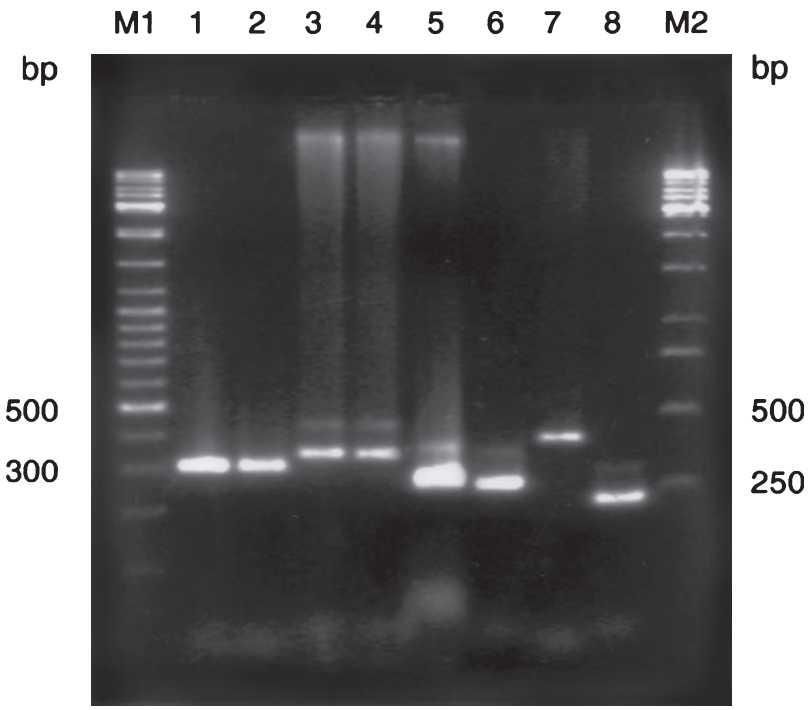

Fig. 1. Polymerase chain reaction (PCR) determination of *Actinobacillus actinomycetemcomitans* isolates serotype. The primers listed in Table 2 were used (7). Amplicons were visualized by gel electrophoresis in 1.0% agarose. Shown are examples from different *A. actinomycetemcomitans* strains/isolates. Lane M1, DNA ladder molecular weight standard (MBI Fermentus); lanes 1 and 2, *A. actinomycetemcomitans* serotype a: ATCC 29523 and strain P5a; lanes 3 and 4, serotype b: ATCC 29522 and strain P-F33; lanes 5 and 6, serotype c: ATCC 33384 and strain P1a; lane 7, serotype d: HK 928; lane 8, serotype f: strain P7a; lane M2, 1 kb DNA ladder molecular weight standard (MBI Fermentus). Note that (i) PCR amplicon sizes for various serotypes: a = 293 base pairs, b = 333 base pairs, c = 268 base pairs, d = 411 base pairs, f = 232 base pairs; (ii) no serotype d nor e was detected from the isolates recovered; (iii) no reference strain profiles for serotype e or f is shown.

caused least damage to the monolayer (Table 6).

Discussion

A. actinomycetemcomitans prevalence from subgingival plaque samples reported in earlier surveys of young subject groups, who were of comparable age to the present study's groups, ranged from around 10% for Europeans (35, 36) to more than 30% in Asians (16, 37). These studies, however, did not provide breakdown of the *A. actinomycetemcomitans* prevalence data in subgingival plaque with respect to clinical conditions, i.e. periodontal health, presence or absence of shallow, moderate or deep pockets, at sample sites. A German study of young adults with minimal periodontal disease, in which each tooth was sampled, found *A. actinomycetemcomitans* to be associated with some deviation from gingival health (36). An Italian study reported *A. actinomycetemcomitans* subject/site prevalence of 4.2%/1.0% from subgingival plaque of young periodontally healthy individuals before commencement of orthodontics (38). The background of the subjects was similar to those of the controls of this report, i.e. individuals with good to fair oral hygiene without periodontitis. Both the Italian study and the current study showed similar low levels of *A. actinomycetemcomitans* prevalence in young individuals free from periodontitis. The university dental students who formed the control group of the present study were not all in their clinical years of study and their plaque control levels were not optimal, but their general bleeding on probing levels were lower than those found in the aggressive periodontitis patients. In young adults with *A. actinomycetemcomitans* it has been suggested that a heavy load of *A. actinomycetemcomitans* might suppress gingival inflammation (36), an effect not evident in the present study. An 8 year longitudinal study from Norway followed up the periodontal status of 206 14-year-olds until they were 22 years-old. A total of 13 were found to have experienced periodontal bone loss at the end of the study. Thirteen periodontally healthy individuals were then randomly selected to be the control subjects and one (7.7%) of them carried subgingival *A. actinomycetemcomitans* detectable at their last review appointment (39). The reported *A. actinomycetemcomitans* prevalence from control subjects was at a similar level to the prevalence in periodontitis-free controls in the current study.

The findings regarding subgingival *A. actinomycetemcomitans* prevalence in young adults with aggressive periodontitis of the present report are similar to those reported by other studies (39, 40). The mean proportion of the *A. actinomycetemcomitans* isolated from aggressive periodontitis associated plaque in the present study, however, appeared to be slightly lower than what has been reported previously (40). The study subjects themselves displayed the clinical conditions compatible with a classification of aggressive periodontitis (41), although no enquiry was made about a family history of periodontitis to confirm any familial aggregation.

It has been reported that serotypes a, b, c and f occur more frequently among oral isolates than serotypes d and e (29, 42). Serotype c seems to be associated more often with clinically healthy situations, although *A. actinomycetemcomitans* of serotypes a, b, c and f could all be detected from diseased subgingival plaque specimens (7), suggesting that phylogenetically diverse strains carry pathogenic potential (43). Serotypes a, b, c and f of *A. actinomycetemcomitans* strains were isolated from both periodontitis-free control and aggressive periodontitis plaque samples in the present study (Table 5), recon-

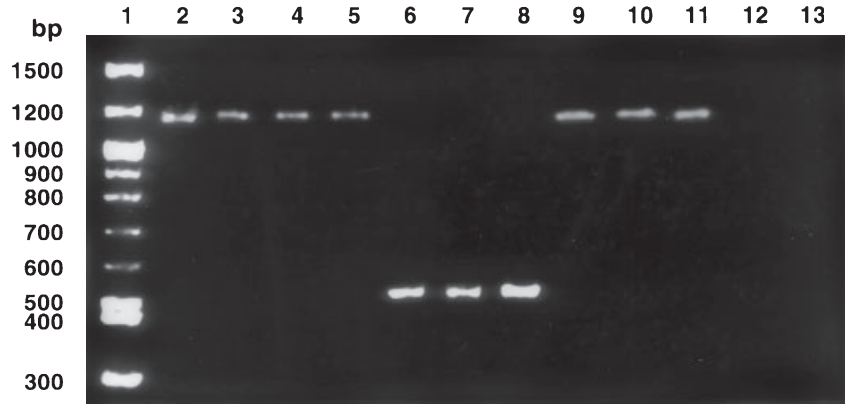

Fig. 2. Polymerase chain reaction (PCR) determination of the leukotoxin gene (*ltx*) promoter size of *Actinobacillus actinomycetemcomitans* isolates. The primers listed in Table 2 were used (20). Amplicons were visualized by gel electrophoresis in 0.8% agarose. Shown are examples from *A. actinomycetemcomitans* strains/isolates carrying 652-like (1034 base pairs, lanes 2–5, 9–11) or JP2-like (504 base pairs, lanes 6–8) promoters. *A. actinomycetemcomitans* strains isolated in the current study all carried 652-like promoter. Lane 1, DNA ladder molecular weight standard (MBI Fermentus); lanes 2–11, *A. actinomycetemcomitans* strains P-F33, P1a, P5a, P7a, JP2, HK 928, HK 929, ATCC 29522, ATCC 29523 and ATCC 33384, respectively; lane 12, *H. aphrophilus* HK 329; lane 13, milli Q water control.

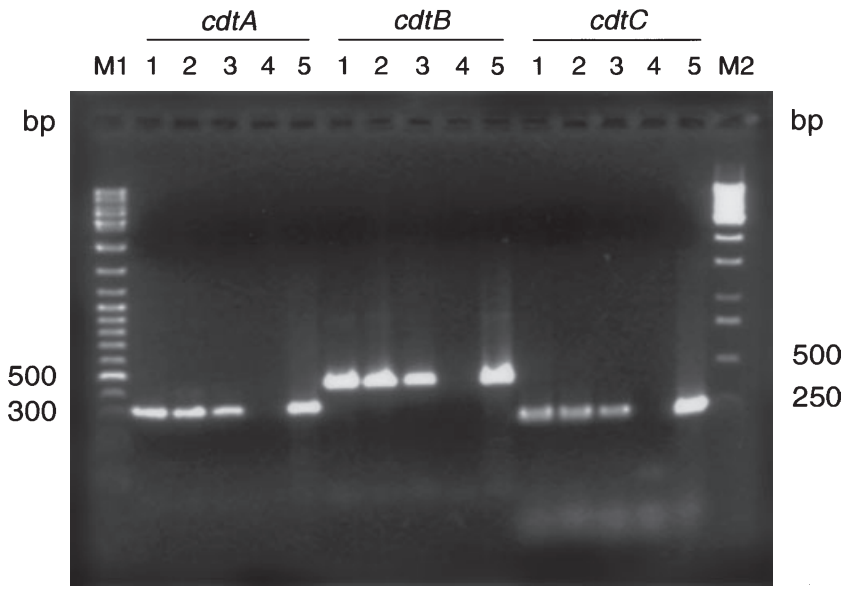

Fig. 3. Detection of cytolethal distending toxin (*cdt*) genes complex of *Actinobacillus actinomycetemcomitans* isolates by polymerase chain reaction (PCR). The primers listed in Table 2 were used (31). Amplicons were visualized by gel electrophoresis in 1.0% agarose. Shown are examples from different *A. actinomycetemcomitans* strains/isolates. Lane M1, DNA ladder molecular weight standard (MBI Fermentus); lane 1, *A. actinomycetemcomitans* strain P-F33; lane 2, strain P1a; lane 3, strain P7a; lane 4, strain P9; lanes 5, ATCC 29523; lane M2, 1 kb DNA ladder molecular weight standard (MBI Fermentus). Note that no *cdtABC* genes complex is detectable from isolate P9, whereas the remaining *A. actinomycetemcomitans* strains all showed typical sized PCR products, i.e. *cdtA* = 326 base pairs, *cdtB* = 462 base pairs and *cdtC* = 272 base pairs.

firming what was observed earlier (29, 42). For Japanese adults it was suggested that serotype c, which was the most common serotype found in the periodontitis-free controls, may differ from other serotypes (44). In one out of eight serotype c *A. actinomycetemcomitans* isolates, one strain from a diseased patient lacked the *cdtABC* gene and was found to be less damaging to the periodontal ligament epithelial monolayer (Tables 5 and 6). The other serotype c isolates, be it from healthy or disease patients, however, possess similar pathogenic properties in periodontal ligament epithelial monolayer. Further investigation therefore is needed to clarify the role of serotype c in periodontal health and disease.

As was found previously with respect to *A. actinomycetemcomitans* isolated from Chinese subjects of different ages and clinical status (16, 18), a highly leukotoxic genotype, considered to be a specific association with aggressive periodontitis in many populations (45) was not found in the present study, further confirming the belief that this highly leukotoxic genotype may be population specific (17, 21, 22). The per subject prevalence of 652 *ltx* promoter positive *A. actinomycetemcomitans* in subgingival plaque of Chinese migrant workers (16) and Singaporean Chinese adults (18), however, is very much different than reported in the current study. We postulate that the high plaque level (< 25% tooth-site plaque free) of the migrant Chinese workers (15) might reflect less healthy gum condition and hence higher chances for *A. actinomycetemcomitans* detection (35, 36), whereas the discrepancies between the *A. actinomycetemcomitans* prevalence in current control group than in the older healthy Singaporean Chinese remain to be elucidated.

The current study shows high prevalence of CDT genotype in *A. actinomycetemcomitans* from plaque in aggressive periodontitis or periodontitis-free young individuals (Table 5). This contrasts with an earlier report showing lower per site subgingival plaque PCR detection rate (30% vs. 50% in the present study) of the *A. actinomycetemcomitans* CDT genotype from generalized early onset and refractory periodontitis patients, mean age 43 years, in Singapore (24). The small number of young aggressive periodontitis subjects in the present study, however, precludes a definitive explanation for these different

Table 6. Effect of *Actinobacillus actinomycetemcomitans* isolates on porcine periodontal ligament epithelial (PLE) cell monolayer cultures[a]

Strains[b]	PLE[c] Cell size (μm^2)	Proportion of cells remaining attached (%)[d]	Culture confluency (%)[e]
Untreated monolayer	1974 ± 1629	100.0 ± 13.5	98.0 ± 4.6
Aggressive periodontitis			
P1a/b	3638 ± 3181	55.7 ± 15.1	96.3 ± 4.8
P4a/b	2746 ± 1995	67.8 ± 19.6	98.7 ± 3.5
P5a/b	5146 ± 3268	40.9 ± 12.9	85.8 ± 16.6
P7a/b	4133 ± 3174	47.5 ± 12.2	93.8 ± 2.9
P8	4001 ± 3252	51.0 ± 15.0	94.5 ± 5.8
P9	2436 ± 1784	78.4 ± 14.9	97.5 ± 8.8
Controls			
P-F12	2864 ± 1999	46.6 ± 14.7	92.3 ± 9.3
P-F33	3518 ± 3009	54.1 ± 14.5	96.1 ± 12.4
P-F45a/b	3103 ± 2185	53.1 ± 8.1	94.8 ± 6.7
Reference strains			
ATCC 29522	3716 ± 3197	52.8 ± 17.5	94.7 ± 13.9
ATCC 29523	5430 ± 4209	36.5 + 13.6	77.5 + 12.9
ATCC 33384	3915 ± 3239	53.1 ± 9.9	93.3 ± 10.8
HK 928	2158 ± 1536	18.5 ± 5.3	23.9 ± 6.2

[a]PLE monolayers were treated with 1×10^8 cells/ml in 15% FBS-αMEM for 24 h.
[b]*A. actinomycetemcomitans* strains designation: please refer to Table 5 for details. Isolate a or b from the same patient, i.e. P1, P4, P5, P7 or P-F45 were found to possess identical serotype, *ltx* promoter, *cdt* genotype and similar effects on PLE monolayers and their data were pooled.
[c]PLE stained with May–Grünwald and Giemsa stains (34); shown are mean ± SD; $n = 6$ or 12 (pooled data) obtained from two independent experiments.
[d]Significantly different ($p < 0.05$, Bonferroni multiple comparison) data values between: untreated monolayer vs. all except strain P9 treated PLE; P9 treated PLE vs. P5a/b, P7a/b, P-F12, ATCC 29523 and HK 928 treated PLE; HK 928 treated PLE vs. all except P5a/b, P-F12 and ATCC 29523 treated PLE.
[e]Significantly different ($p < 0.05$, Bonferroni multiple comparison) data values between HK 928 treated PLE vs. the rest.

observations. The small number of aggressive periodontitis subjects recruited resulted from the difficulties in detecting untreated cases who met all the inclusion criteria listed. However, these were young patients with a mean age of 21 years, compared to the patients of Tan and coworkers (24) who had a mean age of 43 years. A recent report studying *A. actinomycetemcomitans* in an older Hong Kong Chinese periodontitis patient cohort showed comparable per subject but lower per site (38% vs. 56%) *A. actinomycetemcomitans* prevalence (46). These considerations might help in explaining why there was a lower per site *A. actinomycetemcomitans* CDT gene complex detection by Tan and coworkers (24) than in the current study.

To elucidate the exact relation of the different *A. actinomycetemcomitans* genotypes isolated in association with periodontal health and disease, one needs to study the *in vivo* expression of the two virulent genes studied, i.e. *ltx* and *cdtABC*, which was not done in the present study. Rather, the *in vitro* effects of *A. actinomycetemcomitans* whole cell were studied. The periodontal ligament epithelial cell monolayer model enabled *in vitro* evaluation of pathogenic potentials of the *A. actinomycetemcomitans* isolates. The extent of periodontal ligament epithelial cells damage appeared to correlate somewhat with the characterics of the *ltx* operon promoter and *cdtABC* genes complex (Tables 5 and 6). Caution must be exercised in interpretation of the preliminary cytopathic data of this present study. Recently reported pathogenic mechanisms of *A. actinomycetemcomitans* were not investigated. The adherence functions of the *tad* gene locus (47) or *Aae* gene (48), the apoptotic effector *cagE* homologue (49), T-cell apoptotic GroEL-like molecules (50), and other yet unknown virulent genes or factors of the periodontopathogen, could also contribute to the whole bacterium induced *in vitro* cell damage, which were not characterized in the present study.

It is well known that periodontitis develops when a susceptible host is challenged by periodontopathogens (5). It is likely that a consortium of bacteria participate in the induction of both chronic and aggressive periodontitis. The current investigation studied the factors of a single peridontopathogen in isolation, without considering the contributions of any other periodontopathogens that might coexist in the clinical situation. Obviously, colonization patterns by *A. actinomycetemcomitans* and characterization of their *ltx* and *cdtABC* genes alone are not enough to explain differences in possible pathogenic potentials of this microbe. Therefore, the results of the current study can only explain part of the contribution of only one pathogen in aggressive periodonitits. Furthermore, the influence of a susceptible host towards the pathogen–human interaction was not evaluated in this current study. The reason why one *cdtABC* deficient *A. actinomycetemcomitans* strain was isolated from an aggressive periodontitis individual while the other periodontitis-free or disease associated strains with intact *cdtABC* gene could perhaps be explained by the above arguments. Nevertheless, the present study did provide preliminary evidence showing variations of *in vitro* pathogenic potentials related to the two virulent genes of the periodontopathogen *A. actinomycetemcomitans*.

The present preliminary study has shown an elevated prevalence and quantity of the periodontopathogen *A. actinomycetemcomitans* detected in subgingival plaque using selective culture in young untreated aggressive periodontitis individuals. This finding indicates that the bacterium is associated with the clinical situation and further confirms the common belief

that it plays a part in the aggressive periodontitis disease process. The investigated three genotypic characteristics among the *A. actinomycetemcomitans* isolates, however, did not pin-point an association of any particular *A. actinomycetemcomitans* genotype studied with periodontitis-free status or aggressive periodontitis. The present study focused on two widely studied virulent genes of this periodontopathogen, whereas the genotypes in relation to adherence abilities (47, 48) or host cell apoptosis induction factors (49, 50) of the isolates were not studied. The *in vitro* effects of the whole-cell *A. actinomycetemcomitans* isolates on periodontal ligament epithelial monolayer in the present study appeared to correspond to the characteristics of these two virulent genes studied. However, the possibility still exists of variations in carriage of other virulent genes in *A. actinomycetemcomitans* isolates from different clinical situations, which might underpin differences in pathogenic potential of this pathogen. To fully elucidate the pathogenic role in periodontitis of the two *A. actinomycetemcomitans* virulent genes, including the two investigated in the present report, study of the control and/or *in vivo* expression of the genes in subgingival plaque associated with different clinical states is required. Laboratory study of various purified virulent genes' products and their *in vitro* actions are often required for understanding exact pathogenic mechanisms. Therefore, further studies are needed before the pathogenic potentials of *A. actinomycetemcomitans* in various periodontal disease states could be further clarified.

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