Relationship of Serotypes of *Aggregatibacter actinomycetemcomitans* and Its Virulence Factors

Nuntiya Pahumunto

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy (Oral Health Science)

Prince of Songkla University

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ชื่อวิทยานิพนธ์ ความสัมพันธ์ของซีโรไทป์ของ *Aggregatibacter actinomycetemcomitans* และปัจจัยก่อความรุนแรง
ผู้เขียน นางสาวนันทิยา พาหุมันโต
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บทคัดย่อ

Aggregatibacter actinomycetemcomitans (*A. actinomycetemcomitans*) สามารถแบ่งออกเป็น 7 ซีโรไทป์ (serotype) ประกอบด้วยซีโรไทป์เอถึงซีโรไทป์จี อย่างไรก็ตามสามารถพบ *A. actinomycetemcomitans* บางสายพันธุ์ที่ไม่ถูกจัดเป็นซีโรไทป์หรือเรียกว่านอน-ซีโรไทป์ (non-serotype) ซึ่งสามารถพบสายพันธุ์นอน-ซีโรไทป์ได้ในหลายกลุ่มประชากรและกลุ่มอายุ แต่ยังไม่มีความชัดเจนของการกระจายของสายพันธุ์นอน-ซีโรไทป์นี้ และยังไม่มีความชัดเจนของความรุนแรงของ *A. actinomycetemcomitans* ในกลุ่มประชากรไทยที่เป็นโรคปริทันต์อักเสบเรื้อรัง (chronic periodontitis) ด้วยเช่นกัน วัตถุประสงค์ของงานวิจัยนี้คือ 1) ศึกษาความสัมพันธ์และความชุกของซีโรไทป์ของ *A. actinomycetemcomitans* และโรคปริทันต์อักเสบเรื้อรังในประชากรไทยและ 2) ตรวจสอบความสัมพันธ์ของซีโรไทป์ของ *A. actinomycetemcomitans* และการแสดงออกของยีนลิวโคท็อกซิน (leukotoxin; *ltx*) และยีนซัยโตลีทัลดิสเท็นดิ้งท็อกซิน (cytolethal distending toxin; *cdt*) ความเป็นพิษของลิวโคท็อกซิน ความสามารถในการฆ่าเซลล์ไฟโบรบลาสท์ (fibroblast) และความสามารถในการเข้าสู่เซลล์

การศึกษานี้ทำการเก็บตัวอย่างจากผู้ป่วยโรคปริทันต์อักเสบเรื้อรัง 44 คน ซึ่งทุกคนจะได้รับการตรวจช่องปากและบันทึกค่า clinical attachment level, periodontal pocket depth และ bleeding on probing สามารถแยกเชื้อ *A. actinomycetemcomitans* จำนวน 79 สายพันธุ์ได้จากร่องลึกปริทันต์ (periodontal pocket) ที่ลึกและ 17 สายพันธุ์แยกได้จากร่องลึกปริทันต์ที่ตื้นโดยใช้วิธีการเพาะเลี้ยงบนอาหาร TSBV (tryptic soy serum bacitracin vancomycin agar) ซึ่งมีความจำเพาะต่อ *A. actinomycetemcomitans* เชื้อ *A. actinomycetemcomitans* ทุกตัวจะถูกนำมาหาซีโรไทป์โดยใช้เทคนิค polymerase chain reaction และซับไทป์ (subtype)โดยใช้เทคนิค denaturing gradient gel electrophoresis (DGGE) ส่วนปัจจัยในการก่อความรุนแรงของเชื้อ ได้ทำการตรวจสอบยีน *ltx* และ *cdt* ความสามารถในการแสดงออกของลิวโคท็อกซิน ความเป็นพิษของลิวโคท็อกซิน การตายของเซลล์ไฟโบรบลาสท์และการเข้าสู่เซลล์ไฟโบรบลาสท์

จากการศึกษาพบว่าความชุกในการพบเชื้อ *A. actinomycetemcomitans* คิดเป็นร้อยละ 84.1 โดยพบว่าสายพันธุ์นอน-ซีโรไทป์เป็นสายพันธุ์ที่พบบ่อยเทียบเท่ากับสายพันธุ์ที่สามารถหาซีโรไทป์ได้ (ร้อยละ 54.5)โดยพบซีโรไทป์เอร้อยละ 18.2 ซีโรไทป์ซีร้อยละ 15.9 ซีโรไทป์อีร้อยละ 9.1 และซีโรไทป์เอฟร้อยละ 11.4 แต่ไม่พบซีโรไทป์บีและซีโรไทป์ดี สามารถแยกสองสายพันธุ์ที่คล้ายกับ JP2 (JP2-like) ได้จากผู้ป่วยโรคปริทันต์อักเสบเรื้อรัง 2 คนและพบว่าเป็นซีโรไทป์ซี และสามารถแยก *A. actinomycetemcomitans* ที่มีคู่เบส (base pair) จำนวน 886 คู่เบสแทรก (insertion) ในส่วนโปรโมเตอร์ (promoter) ของยีน *ltx* จากผู้ป่วยโรคปริทันต์อีก 2 คน ผลจาก DGGE แสดงให้เห็นถึง 16 ซับไทป์ของสายพันธุ์นอน-ซีโรไทป์ของ *A. actinomycetemcomitans* โดยพบว่า 2 ซับไทป์ได้แก่ NS1 (ร้อยละ 12.7) และ NS2 (ร้อยละ 10.1) เป็นสายพันธุ์ที่พบได้บ่อยกว่าซับไทป์อื่นๆ อีก 14 ซับไทป์ (น้อยกว่าร้อยละ 5.1) นอกจากนั้น พบเพียงหนึ่งซับไทป์ในผู้ป่วยโรคปริทันต์จำนวน 12 คน (ร้อยละ 32.4) ในขณะที่พบสองและสามซับไทป์ที่แตกต่างกันในผู้ป่วยโรคปริทันต์ 11 คน (ร้อยละ 29.7) และพบได้ถึงสี่ซับไทป์ในหนึ่งร่องลึกปริทันต์ที่ลึกในผู้ป่วยโรคปริทันต์ 3 คน (ร้อยละ 8.1) พบยีน *ltx* ในทุกสายพันธุ์ของเชื้อ *A. actinomycetemcomitans* ที่แยกได้จากผู้ป่วยโรคปริทันต์ ในขณะที่พบยีน *cdt* ได้เพียงร้อยละ 84.4 การแสดงออกลิวโคท็อกซินที่รุนแรงถึง 595.9 ± 33.0 เท่าและเป็นพิษต่อเซลล์ไฟโบรบลาสท์ร้อยละ 54.7 ± 9.3 มีความเกี่ยวข้องกับการขาดหาย (deletion) ไปของบริเวณโปรโมเตอร์ของยีน *ltx* จำนวน 530 คู่เบส นอกจากนี้ พบว่าสายพันธุ์นอน-ซีโรไทป์ 2 สายพันธุ์ได้แก่ NS1 และ NS2 มีความรุนแรงมากกว่าเมื่อเปรียบเทียบกับสายพันธุ์ที่สามารถหาซีโรไทป์ได้ โดยทั่วไปเชื้อ *A. actinomycetemcomitans* ที่แยกได้จากร่องลึกปริทันต์ที่ลึกจะมีความรุนแรงมากกว่าสายพันธุ์ที่แยกได้จากร่องลึกปริทันต์ที่ตื้น

สรุปได้ว่าการศึกษานี้แสดงให้เห็นถึงความหลากหลายของซับไทป์ของเชื้อ *A. actinomycetemcomitans* มากกว่าการศึกษาก่อนหน้านี้ในกลุ่มประชากรไทย และเป็นครั้งแรกที่มีการแยกเชื้อ *A. actinomycetemcomitans* ที่มีการขาดหายของ 530 คู่เบสหรือการแทรกของ 886 คู่เบสตรงส่วนโปรโมเตอร์ของยีน *ltx* ซึ่ง *A. actinomycetemcomitans* ทั้งสองสายพันธุ์นี้เป็นซีโรไทป์ซี ซับไทป์ของ *A. actinomycetemcomitans* แสดงให้เห็นตัวแปรที่ส่งผลต่อความรุนแรงไม่ว่าจะเป็นการแสดงออกของลิวโคท็อกซิน ความเป็นพิษของลิวโคท็อกซิน ความสามารถในการเข้าสู่เซลล์และการฆ่าเซลล์ไฟโบรบลาสท์ ซึ่งคุณสมบัติเหล่านี้อาจจะถูกควบคุมด้วยทั้งปัจจัยทางพันธุกรรมและสิ่งแวดล้อม

Thesis Title Relationship of Serotypes of *Aggregatibacter actinomycetemcomitans* and Its Virulence Factors

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ABSTRACT

Aggregatibacter actinomycetemcomitans (*A. actinomycetemcomitans*) has been traditionally classified into seven serotypes (a-g). However, the non-serotypable strains have also been reported, but their distribution in various populations and age groups is unclear. Moreover, the virulence of different strains of *A. actinomycetemcomitans* in Thai patients with chronic periodontitis has not been completely demonstrated. Therefore, the aims of this study were: 1) to investigate the prevalence and the relationship of different serotypes of *A. actinomycetemcomitans* among Thai patients with chronic periodontitis and 2) to examine the relationship between different serotypes of *A. actinomycetemcomitans* and their virulence factors, including expression of leukotoxin (*ltx*) and of cytolethal distending toxin (*cdt*), internalization ability and cytotoxicity to fibroblast.

Forty-four adult Thai patients with chronic periodontitis were examined by evaluating clinical attachment level, periodontal pocket depth and bleeding on probing. Seventy-nine strains of *A. actinomycetemcomitans* were isolated from deep periodontal pockets and 17 strains were isolated from shallow pockets by selective culture on TSBV (tryptic soy serum bacitracin vancomycin agar) agar. The strains were serotyped by polymerase chain reaction and subtyped by denaturing gradient gel electrophoresis (DGGE). The virulence factors were examined for the presence of the *ltx* gene and the *cdt* gene (*cdtBC*) and tested for leukotoxin expression, leukotoxin activity, cytotoxicity to fibroblast, and internalization into fibroblasts.

The prevalence of *A. actinomycetemcomitans* in Thai patients with chronic periodontitis was 84.1%. The non-serotypeable strains of *A. actinomycetemcomitans* were detected as frequently as the serotypeable strains (or around 54.5%), including the serotype a 18.2%, serotype c 15.9%, serotype e 9.1%, and serotype f 11.4%. However, the serotypes b and d were not

detected at all. In addition, two JP2-like strains with a characteristic of 530-bp deletion on their *ltx* promoter were isolated from two patients and serotyped as c, whereas the other two strains showed 886-bp insertion on the *ltx* promoter of their *A. actinomycetemcomitans* isolates. DGGE subtyping revealed 16 different subtypes among the non-serotypeable strains. Two of them (NS1 and NS2) were more commonly found (12.7% and 10.1%) than the other 14 subtypes (< 5.1%). Twelve patients (32.4%) had only one subtype, eleven patients (29.7%) had two and three different subtypes, and three patients (8.1%) surprisingly had four subtypes in one deep pocket. The *ltx* gene was present in all isolates, while the *cdtBC* gene was shown only 84.4%. An increase in leukotoxin expression by 595.9 ± 33.0 fold and in its activity by 54.7 ± 9.3% was associated with the 530-bp deletion on the *ltx* promoter. Furthermore, higher virulence was found in the two non-serotypeable subtypes (NS1 and NS2) than in the serotypeable strains. Higher virulence was generally found for the strains isolated from deep periodontal pockets than those from shallow non-bleeding pockets.

In conclusion, this study demonstrated a greater subtype diversity of *A. actinomycetemcomitans* than other previous studies conducted in Thai population. The non-serotypeable strains were found to be predominant in this study. The isolates with 530-bp deletion and 886-bp insertion on their *ltx* promoter were demonstrated for the first time and serotyped as c. Different subtypes of *A. actinomycetemcomitans* showed highly variable virulence, ranging from leukotoxin expression, leukotoxicity to internalization and cytotoxicity to fibroblast. These virulence factors may be regulated both genetically and environmentally.

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LIST OF ABBREVIATIONS AND SYSBOLS

ATCC	American Type Culture Collection
BOP	bleeding on probing
bp	base pair
CAL	clinical attachment loss
CFU	colony forming unit
CCUG	Culture Collection, University of Göteborg
cDNA	complementary deoxyribonucleic acid
Cdt	cytolethal distending toxin
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
FGM	fibroblast growth medium
HAD1-GC	forward primer
HAD2	reverse primer
HL-60	promyelocytic leukemic cell line
KB cells	oral carcinoma (cervical adenocarcinoma)
L929	mouse fibroblast cell line
LFA-1	lymphocyte function-associated receptor 1
ltx	leukotoxin
ltxA	leukotoxin A gene
ltx3	forward primer for leukotoxin gene
ltx4	reverse primer leukotoxin gene
non-JP2 clone	complete leukotoxin promoter
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PMNs	polymorphonuclear leukocytes
PPD	probing pocket depth

LIST OF ABBREVIATIONS AND SYSBOLS (CONTINOUED)

rRNA	ribosomal RNA
SD	standard deviation
THP-1	human acute monocytic leukemia cell line
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

LIST OF PAPERS AND PROCEEDING

International Journal Papers

1. Nuntiya Pahumunto, Praphansri Ruangsri, Mutita Wongsuwanlert, Supatcharin Piwat, Gunnar Dahlen and Rawee Teanpaisan. *Aggregatibacter actinomycetemcomitans* serotypes and DGGE subtypes in Thai adults with chronic periodontitis. ***Arch Oral Biol 2015***; 60: 1789-96.
2. Nuntiya Pahumunto, Praphansri Ruangsri, Mutita Wongsuwanlert, Supatcharin Piwat, Gunnar Dahlen and Rawee Teanpaisan. Virulence of *Aggregatibacter actinomycetemcomitans* serotypes and DGGE subtypes isolated from chronic adult periodontitis in Thailand. ***Anaerobe*** 2015; 36: 60-4.

Conference Proceeding

1. Pahumunto N. and Teanpaisan R. 2012. Search for protein from lactobacilli inhibit growth of *Aggregatibacter actinomycetemcomitans*. The 2nd Current Drug Development International Conference. 2nd-4th May 2012.

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With best regards,

Teerapol Srichana
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1. INTRODUCTION

Background and rationale

Periodontitis is a chronic inflammatory disease associated with loss of the supporting connective tissue and alveolar bone around the teeth. An etiological relationship between *Aggregatibacter actinomycetemcomitans* and chronic periodontitis has been found.[1,2] *A. actinomycetemcomitans*, a gram-negative, capnophilic coccobacillus has been associated with several forms of periodontitis including aggressive and chronic periodontitis.[1, 2] This bacterium is also involved in some systemic infectious diseases such as endocarditis, meningitis, osteomyelitis, glomerulonephritis, and arthritis.[3] However, it is known that the species is genetically heterogeneous and can be grouped into six serotypes (a–f) based on a surface polysaccharide antigen that can be are disclosed phenotypically by immunodiffusion assay[4] or genotypically by PCR.[5, 6] Moreover, a novel serotype g has recently been shown.[7]

It has been reported that there is an increase in the proportion of serotype b among patients with aggressive periodontitis (formerly known as juvenile periodontitis), whereas serotypes a and c have been associated with periodontal health.[8] In addition, a highly virulent subtype of *A. actinomycetemcomitans* (the JP2 genotype) has been classified as serotype b.[9] This particular strain has been categorized as racial tropism due to oral colonization exclusively in individuals from an African origin[9-11] and has not yet been found in the Asian population.[12-15] Interestingly, not all the isolates can be serotyped, and the non-serotypeable *A. actinomycetemcomitans* isolates have been previously reported in a frequency rate ranging from 1.2% to 11.4%.[1, 16] A higher rate of non-serotypeable strains was reported to occur in Asian populations (14.7%).[17] Thus it was hypothesized for the present study that we might face yet another example of racial tropism involving the non-serotypeable *A. actinomycetemcomitans* strains that calls for another subtyping strategy rather than traditional serotyping.

Denaturing Gradient Gel Electrophoresis (DGGE) has been used as a diagnostic tool to detect putative periodontal pathogens including *A. actinomycetemcomitans* in dental plaque samples.[18-20] Genotyping using DGGE has been used to disclose various serotypes of *A. actinomycetemcomitans* has detected in only 3 out of the 6 serotypes (1. serotype a, d, f, 2. serotype

b, 3. serotype c). However, DGGE has not yet been used for subtyping non-serotypeable *A. actinomycetemcomitans* isolates.

The virulence of *A. actinomycetemcomitans* has been shown to be associated with leukotoxin production.[3, 21] Other virulence factors that may play a crucial role in the pathogenesis of periodontal diseases, including cytolethal distending toxin (CDTs), internalization and cytotoxicity in fibroblast, known as there have not been many studies about them. [21-26] CDTs are multi-component bacterial toxins, which by targeting and entering eukaryotic cells cause a distention and cell cycle arrest. The production of CDTs is based on a specific *cdtABC* gene.[24] Toxin activity is based on the *cdtB* unit but flanking of the gene by *cdtA* and *cdtC* significantly increases the toxicity. Moreover, *A. actinomycetemcomitans* could inhibit fibroblast proliferation and undergo internalization in cell cultures.[23,26]

Serotype b has been claimed to be associated with pathogenicity due to its recovery in cases with aggressive periodontitis in young individuals (earlier called, localized juvenile periodontitis).[21] However, the virulence and toxicity have not been found to strictly follow the serotype pattern.[21, 27] Leukotoxin of *A. actinomycetemcomitans* is encoded in an operon consisting of four genes, which typically cause cytotoxicity in *polymorphonuclear* granular leucocyte (PMN), macrophages, and endothelial cells.[21, 28] A 530-bp deletion in the promoter region of the leukotoxin (*ltx*) gene in strains of the JP2 clone results in a high level of leukotoxin.[21,22] The JP2 clone of *A. actinomycetemcomitans* is identified as serotype b and is isolated frequently in young individuals of an African origin.[9, 21] The leukotoxin level of this genotype has been found to be expressed over 10- to 20-fold compared with the non-deletion strains.[21, 22] However, a number of non-JP2 genotype strains (complete *ltx* promoter and 640 bp deletion on *ltx* promoter), mainly serotype b but also serotype c and a, have shown high leukotoxic activity similar to the JP2 genotype.[27, 29] The relationship between virulence and serotypes/subtypes of *A. actinomycetemcomitans*, other than the JP2 clone, is unclear. [24 23, 26]

The hypothesis inspiring the thesis was that individual serotypes/subtypes of A. actinomycetemcomitans could be qualitatively different in their association with periodontitis, and thus have different roles in periodontitis development and progression. The ability of bacteria to colonize and persist in a particular environment depends, in part, on their ability to modify or express their genes as a response to variation in local environment conditions or stress.[2,24] The main

properties of periodontopathogenics bacteria include internalization and leukotoxin production, which enables them to survive and reproduce in the subgingival tissue. Assessment of bacterial genetics and their properties have been mostly performed on *A. actinomycetemcomitans* from African and American populations. In Asia there have been fewed studies on the relationship of serotypes/subtypes of *A. actinomycetemcomitans* and their periodontitis status, or on the role of these virulence levels of *A. actinomycetemcomitans* in Thai adults with chronic periodontitis.[2,12-14]

The current study therefore aimed to investigate the prevalence and distribution of serotypes and DGGE subtypes of *A. actinomycetemcomitans* isolated from deep and shallow pocket sites of chronic periodontitis cases in Thai adults, and the virulence of these strains with respect to the *ltx* gene, leukotoxin expression, leukotoxin activity, *cdt* gene, internalization, and killing activity on fibroblasts were with studied, special attention to *A. actinomycetemcomitans* subtypes.

Review of literature

1. Periodontal disease

Periodontitis is an inflammation of the periodontium that is usually caused by specific pathologic bacteria that grow in the gingival sulcus and in the host response to an infection. Periodontal disease includes several disease entities with different clinical presentation. The diagnosis of periodontal disease is based on a visible amount of tissue destruction due to the ongoing or proceeding inflammatory process in the periodontal tissue and these criteria are classified as the type of periodontitis (Fig. 1). The classification system for periodontal disease and conditions was proposed at the 1999 International Workshop.[30] The types of periodontitis include chronic periodontitis, gingivitis, aggressive periodontitis, periodontitis as a manifestation of systemic disease, necrotizing periodontitis, abscesses of the periodontium, periodontitis associated with endodontic lesions, and developmental or acquired deformities and conditions.

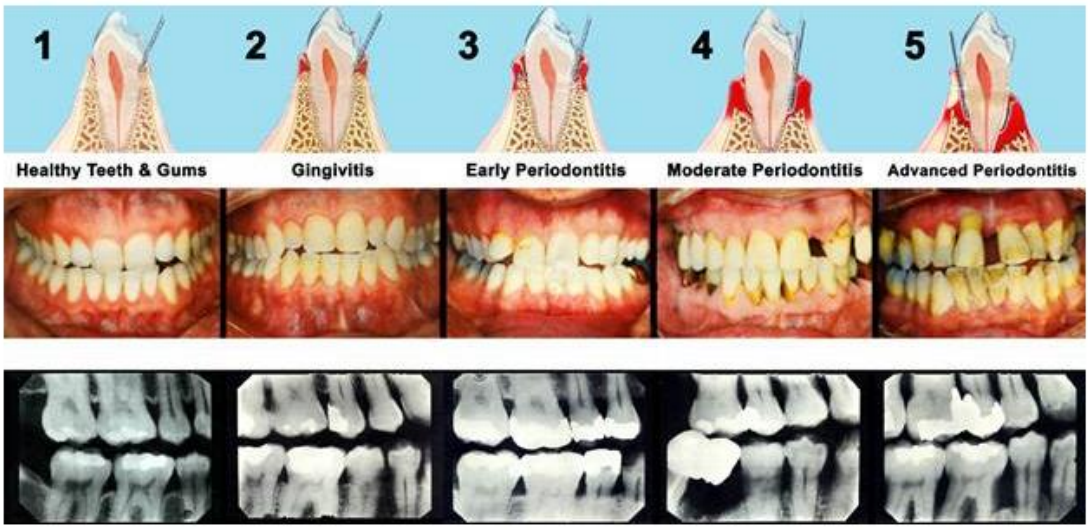


Fig. 1 Periodontitis stages (http://mizar5.com/periodontal_stages_of_gum_disease.html)

It is well established that bacteria in the dental plaque play a key role in the initiation of periodontitis, which can eventually lead to tooth loss. Periodontitis is formed from aberrant inflammation resulting from a complex biofilm of friendly commensal bacteria and their products, stimulating the human inflammatory response. However, the relationship between the contribution of these bacteria and the disease progression is still poorly understood. A shift in microbial species in the gingival sulcus such as anaerobic gram negative and facultative gram negative has been strongly associated with periodontal tissue breakdown.[31-33] Bacterial exotoxins are a diverse collection of proteins responsible for many of the biological effects caused by pathogens.

The prevalence and distribution of periodontitis may reflect differences in oral hygiene and socio-economic status rather than differences in genetic susceptibility or in occurrence of periodontal pathogens. Nevertheless, there seems to be agreement among several investigators on certain distinct patterns of prevalence and distribution of periodontal disease in different parts of the world. The prevalence of aggressive periodontitis was found to be < 1% (usually 0.1–0.2% in Caucasian populations).[2,10,21] This does not apply to other ethnic groups where the prevalence may show striking variations related to geography and/or ethnicity. In any case, chronic periodontitis is generally a slowly progressing form of periodontitis, which at any stage may undergo acute exacerbation with associated periodontal attachment loss. From prevalence studies, chronic

periodontitis in mild to moderate forms is the most commonly occurring form of periodontitis worldwide ranging in prevalence from 13% to 57% in different populations depending on oral hygiene and socio-economic status.[21] The disease is characterized by loss of clinical attachment due to destruction of the periodontal ligament and loss of the adjacent supporting bone.[34] Chronic periodontitis occurs in localized and generalized forms.[34, 30] Chronic and aggressive periodontitis are further classified into (i) localized periodontitis; when less than 30% of sites assessed in the mouth demonstrate attachment and bone loss, and (ii) generalized periodontitis; when 30% or more of the sites in the mouth demonstrate attachment and bone loss. Based on severity, chronic periodontitis is defined as "slight periodontitis" when the clinical attachment loss is between 1 and 2 mm or "moderate periodontitis" if the attachment loss is between 3 and 4 mm or "severe periodontitis" when the clinical attachment loss is 5 mm or greater.[35]

The aetiology of chronic periodontitis is polymicrobic with complex interactions among many microbes, making the study disease a challenge. In particular, the involvement of *Aggregatibacter actinomycetemcomitans* which is an opportunistic pathogen in humans that is present in the oral cavity in aggressive and chronic forms of periodontitis need to be elucidated.[24]

2. Taxonomy, identification, and prevalence of *Aggregatibacter actinomycetemcomitans*

2.1 Taxonomy of *A. actinomycetemcomitans*

Aggregatibacter actinomycetemcomitans (previously *Actinobacillus actinomycetemcomitans*) is a capnophilic gram-negative bacterium (Fig. 2) that prefering environments enriched with CO_2 (5–10%). It is a non-sporulating, non-motile, non-hemolytic coccobacillus and is oxidase and catalase positive. Growth is optimal at 37°C over a pH range of 7.0–8.5. *A. actinomycetemcomitans* can grow in the absence of X (hemin) or V (nicotinamide adenine dinucleotide). The cells are straight or curved rods (0.4–0.5 µm x 1.0–1.5 µm in size) with crinkled adherent (rough) colonies (1–2 mm diameter) on tryptic soy serum bacitracin vancomycin agar (TSBV) with a star-shaped structure in the center (Fig. 2).[36] Recent studies have shown a phylogenetic similarity of *A. actinomycetemcomitans* and *Haemophilus aphrophilus*, *H. paraphrophilus* and *H. segnis*, suggesting the new genus, *Aggregatibacter*, for them. *A.*

actinomycetemcomitans was characterized into the Pasteurellaceae family. This bacterium has also been reported to be involved in periodontitis based on prevalence studies in healthy (0-16%) and periodontally diseased subjects (22-100%).[2,4,11,13] It is also involved in some systemic infectious diseases, such as endocarditis, meningitis, osteomyelitis, glomerulonephritis and arthritis.[3]

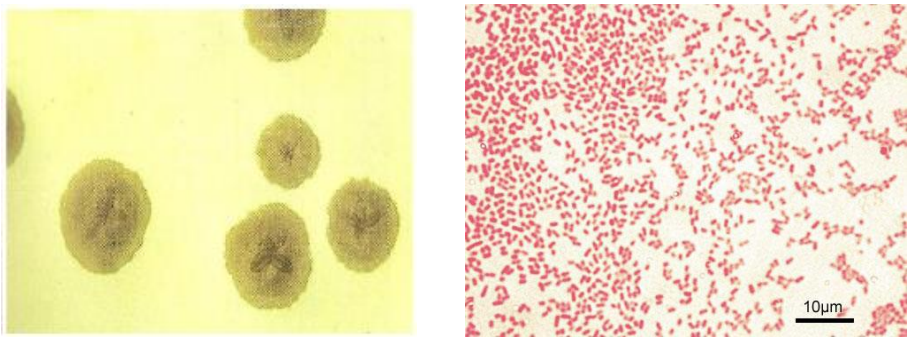


Fig. 2 Colony morphology and gram stain of *A. actinomycetemcomitans* (http://www.atsu.edu)

2.2 Identification of *A. actinomycetemcomitans*

The frequency of *A. actinomycetemcomitans* in subgingival samples is assessed by traditional cultivation methods on selective media, as well as by molecular techniques. Traditional techniques for *A. actinomycetemcomitans* identification in samples consisted of culture techniques with biochemical testing and gram staining. The biochemical test included catalase, esculin hydrolysis, indole, nitrate reduction and fermentation of glucose, lactose, maltose, mannitol, sucrose, and xylose.[37] Slot (1982) developed media for *A. actinomycetemcomitans* detection being a selective agar for *A. actinomycetemcomitans* (TSBV).[36] Detection of *A. actinomycetemcomitans* in subgingival samples was high in cultures on TSBV in previous studies.[13, 38] Although, traditional techniques are low cost, but its limitations include specificity, sensitivity and it is time-consuming. Flemmig et al. reported that the sensitivity and specificity of culture were 58% and 79%, respectively.[39] Thus, molecular methods have solved the limitations of the classical methods (culture method, biochem method, and immnuoassay).

The molecular methods to identify *A. actinomycetemcomitans* currently include DNA probe hybridization; for example, the checkerboard technique and gene amplification via PCR methodology (multiplex, nested multiplex and quantitative PCR, loop-mediated isothermal

amplification, and cloning and sequencing of 16S rRNA libraries). PCR, a molecular technique, has been recently used to identify *A. actinomycetemcomitans* because it shows great potential in the microbiological diagnosis of periodontal diseases, being more specific and more sensitive.[40] PCR uses species-specific primers to identify target microorganisms, which have been developed, commonly used, and demonstrated to have a high degree of efficiency and accuracy in the detection of target microorganisms and in the evaluation of bacterial colonization in saliva and dental plaque samples. However, several studies showed low frequency of *A. actinomycetemcomitans* in samples using PCR[41,42] because there were healthy subjects or a high socioeconomic populations, or because of the low amount of DNA of *A. actinomycetemcomitans* in the samples. For example, multiplex PCR could detecte *A. actinomycetemcomitans* in 16% of patients with the disease.[19] Other molecular techniques have been proposed for identification of *A. actinomycetemcomitans* such as Arbitrarily-Primed Polymerase Chain Reaction (AP-PCR),[43] Restriction Fragment Length Polymorphism (PCR-RFLP),[44] quantitative real-time PCR (qPCR),[45] and DNA hybridization.[13, 46] For instance, *A. actinomycetemcomitans* was detected in 85.1% by qPCR and 44.0% by PCR of the subgingival samples but 67.3% by qPCR and 59.2% by PCR in supragingival plaque samples.[45] Therefore, the detection method for *A. actinomycetemcomitans* identification depends on the requirements of the study and the equipment in each laboratory.[24]

2.3 The prevalence of periodontal diseases caused by *A. actinomycetemcomitans* and reservoirs

The prevalence of *A. actinomycetemcomitans* is highly variable depending upon the population, age, disease severity, detection method, sampling strategy, etc. The prevalence and distribution of *A. actinomycetemcomitans* in healthy periodontium and disease are still unclear.[47] *A. actinomyceterncornitans* is an important pathogen in severe chronic periodontitis. *A. actinomycetemcomitans* is found to be associated with aggressive periodontitis such as in localized juvenile periodontitis, periodontal inflammation and refractory periodontitis.[48-50] *A. actinomycetemcomitans* has been isolated in high numbers from young patients with rapidly progressing alveolar bone loss and is detected in higher numbers and frequency in aggressive periodontitis than in chronic periodontitis.[51,52] Aggressive periodontitis is modified by rapid progression result in tooth loosening and is characteristically present with significant periodontal

attachment loss at an early age, with a tendency for familial clustering of cases.[24] The frequencies of aggressive periodontitis and chronic periodontitis (caused by *A. actinomycetemcomitans*) worldwide were 82% (164/200 subjects) and 39% (501/1273 subjects), respectively.[2] The frequency of localized aggressive periodontitis in the US's adolescent population was revealed to be approximately 0.5%, but this differed in relation to the subjects' racial group (African-American, Asian-American, and Caucasian population).[53] Kenyans showed a prevalence of only 0.3%,[54] suggesting that not all African populations show an increased prevalence of this disease. The highest prevalence of aggressive periodontitis is seen in subjects from North-Africa.[24]

The habitat of *A. actinomycetemcomitans* showed that 47% of the subgingival samples, 17% of the tongue samples, 40% of the cheek mucosa samples, and 29% of the saliva samples contained *A. actinomycetemcomitans.*[55] In addition, periodontal pockets often contained 100-fold or more cells of *A. actinomycetemcomitans* than other oral sites studied.[55] These data suggested that dental plaque and the periodontal pocket area were a main oral reservoir for *A. actinomycetemcomitans*.

2.4 Serotyping and detection methods of *A. actinomycetemcomitans*

A. actinomycetemcomitans is divided into seven serotypes; a through g, based on O-polysaccharides. Each serotype represents a distinct clonal lineage.[5-7] Different serotypes have been shown to be associated with periodontal health, aggressive or chronic periodontitis, and also with non-oral infections. The predominant serotypes of *A. actinomycetemcomitans* were serotypes a through c and a minor frequency of four serotypes (d through g) among *A. actinomycetemcomitans* oral isolates. [15, 56, 57]

The frequent distribution of *A. actinomycetemcomitans* serotypes differs among populations from geographically distant regions, suggesting that serotype-specific strain differences may be responsible for host specificity and virulence. Serotype b is an important serotype because it was found more frequently in periodontitis subjects under the age of 18 years (60.9%) in comparison to subjects older than 35 years (29%) and also was associated with aggressive periodontitis.[58] African, Asian, Europeans, and North and South American populations show differences in serotype distribution.[59] For instance, in the US, serotype b is detected more frequent than serotypes a and c in patients with localized juvenile periodontitis, whereas serotype a is more

commonly detected in samples from adult subjects with chronic periodontitis.[16] In Finland, serotype b is predominant in periodontitis patients and serotype c is frequently isolated in periodontally healthy individuals.[4] Kim et al. found that in German patients the predominant serotypes are serotypes b, c, and a.[15] In a Greek population serotype c was more predominant within the periodontally diseased groups.[41] Roman-Torres et al. studied serotypes of *A. actinomycetemcomitans* in a subgingival sample, and found that the *A. actinomycetemcomitans* serotype a and c are prevalent in slight/moderate periodontitis whilst serotype c is predominant in severe periodontitis.[42] The prevalence of serotype c in severe periodontitis is greater than that of serotypes a and b in Asian and Eurasian populations.[55] Dahlen et al.[13] and Bandhaya et al.[12] found serotypes a and c in a southern Thai population; however, they are not particularly associated with periodontal disease.[13] However, non-serotypeable strains have been previously demonstrated in Asian populations (8.9%, Mombelli et al.[60]; 12%, Yamamoto et al.[61]; 14.7%, Yoshida et al.[17]; 54.5%, Pahumunto et al.,[38]) and these strains appear to be more common in Asian populations than in American and African populations (1.2%, Chen et al.[16]; 3%, Saarela et al.[62]; 9.5%, Fine et al.[63]). Therefore, *A. actinomycetemcomitans* has more than seven serotypes and shows a diversity of serotypes depending on age, oral health, behaviors and nations. The global distribution of the different serotypes of *A.actinomycetemcomitans* is heterogeneous, which implies that the association between the serotype and periodontal status may depend on the geographical location and/or ethnic status of the study population.[58]

Serotypes of *A. actinomycetemcomitans* can be distinguished by using several methods such as an immunoassay, the biochemical method, and the molecular method. In the past, serological studies have exhibited higher serum antibody levels against *A. actinomycetemcomitans.*[64, 65] This technique is not stable and requires a high quantity of bacterial cells. The PCR technique was later developed to distinguish different serotypes of *A. actinomycetemcomitans.*[5, 6] PCR appears to have great potential in distinguishing serotypes of *A. actinomycetemcomitans* (Fig. 3);[5, 6] it uses multiple sets of oligonucleotide primers to identify the six serotypes of *A. actinomycetemcomitans* but it cannot distinguish subtyping of *A. actinomycetemcomitans*. PCR-DGGE was developed to solve PCR limitations. PCR-DGGE is a molecular method that separates PCR-generated DNA products. PCR products from a given reaction are of similar size (bp) and conventional separation by agarose gel electrophoresis results

only in a single DNA band that is largely non-descriptive. PCR-DGGE can overcome this limitation by separating PCR products based on sequence differences that results in differential denaturing characteristics of the DNA. Differing sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands. For example, 16S rDNA PCR-DGGE distinguished serotype b and serotype c of *A. actinomycetemcomitans*.[18] Also, three different groups of *A. actinomycetemcomitans* serotypes could be distinguished by DGGE (1. serotypes a, d, e, and f; 2. serotype b; 3. serotype c),[20] or five different serotypes (1. serotypes a, d, and f; 2. serotype b; 3. serotype c; 4. serotype e; 5. non-serotypeable).[38] Moreover, the DGGE technique is suitable to distinguish subtyping of *A.actinomycetemcomitans*, serotypeable and non-serotypeable, in to 19 subgroups.[38] Thus, DGGE has great potential method to distinguish serotypes and subtyping of *A.actinomycetemcomitans*.

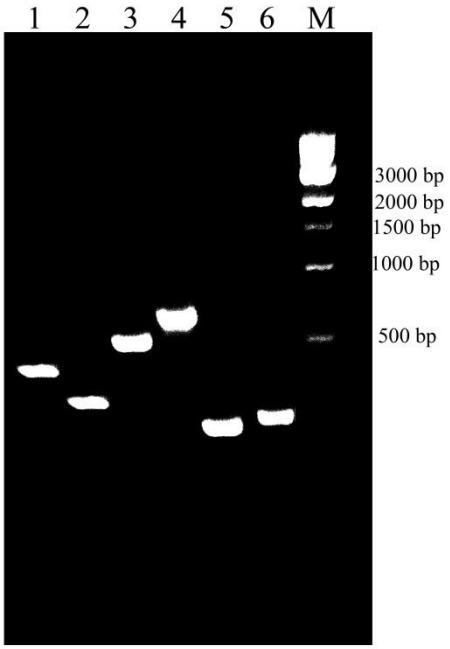


Fig. 3 Serotyping of *A.actinomycetemcomitans* was distinguished using PCR with specific primer lane 1: serotype a (CCUG 37004), lane 2: serotype b (CCUG 37002), lane 3: serotype c (CCUG 13227), lane 4: serotype d (CCUG 38565), lane 5: serotype e (CCUG 37399), lane 6: serotype f (3816), and lane M: Marker [38]

3. Virulence factors of *A. actinomycetemcomitans*

Virulence factors are characteristics of a microorganism that capacitate it to colonize a particular niche in its host, overcome the host defenses and initiate a disease process. These factors are frequently associated with the ability to be transmitted to susceptible hosts causing them to become greater pathogenicity. Virulence factors of *A. actinomycetemcomitans* enhance its survival in the oral cavity and enable it able to circumvent the host's protective strategies. These virulence factors including adherence ability,[23, 24] a chemotactic inhibitor,[23] a leukotoxin,[21] a cytolethal distending toxin,[66] inhibitors of fibroblast proliferation,[26] and the ability to invade epithelial cells. [23, 24] All of these factors may be involved in the pathogenesis of periodontitis.

3.1 The leukotoxin of *A. actinomycetemcomitans*

Leukotoxins are exotoxins that affect leukocytes, especially polymorphonuclear cells (PMNs). Leukotoxin contains a variety of chemicals ranging from 9,10-epoxy to 12-octadecenoate, a fatty acid derivative produced by leukocytes and proteins, such as RTX which repeats in toxin. Leukotoxin is produced during the early stages of growth of *A. actinomycetemcomitans.*[67] Leukotoxin of *A. actinomycetemcomitans* is a 116 kDa water-soluble protein that is expressed by an operon on the surface of the bacteria consisting of four genes, designated as *ltxC*, *ltxA*, *ltxB* and *ltxD* (Fig. 4).[68]

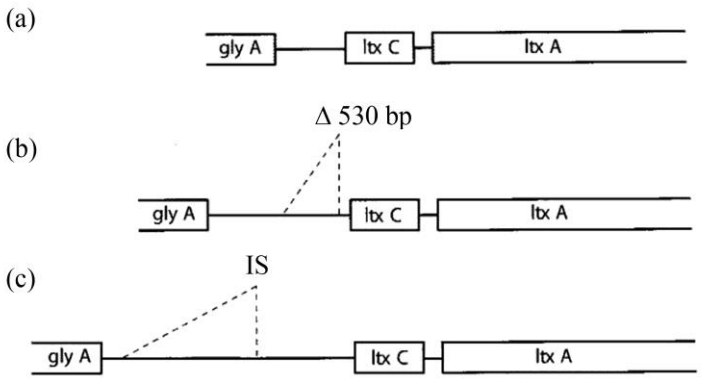


Fig. 4 The various types of *ltx* structure of *A. actinomycetemcomitans,* (a) Strains with the Δ530 deletion or JP2 clone; (b) Strains without the Δ530 deletion or normal strain; (c) Strains with the 886-bp insertion sequence (IS) or insertion strain [69]

3.1.1 Mechanism of leukotoxin of *A. actinomycetemcomitans*

A. actinomycetemcomitans leukotoxin can kill lymphocyte cells and monomyelocytic lineage of human and some non-human primates by forming pores on the lipid bilayer.[70] Non-specific adsorption of this toxin onto the cell membrane of several toxin sensitive and toxin resistant cells occurs. However, leukotoxin has a very narrow host range, suggesting that there is a specific cell surface receptor for this toxin.

Effects of leukotoxin on target cells are divided into three categories depending on the dose dependent and activation–inhibition paradox. At very low concentrations, the release of the lysosomal content enhances oxidative burst activity leading to surrounding host tissue damage. The strains that produce leukotoxin are less efficiently phagocytosed by PMNs.[71] Low doses of leukotoxin (less than 0.5 leukotoxin units) kill pro-myelocytic HL-60 cells via an apoptotic mechanism. HL-60 cells treated with leukotoxin show a decrease in size, increase in granularity, and flocculation the nucleus in early apoptosis.[72] High concentrations of leukotoxin induce necrosis subsequent to pore formation in target cells. The pore formation and membranolysis of human neutrophils are evidenced by rapid efflux of extracellular calcium, collapse of membrane channels, release of lactic dehydrogenase (LDH), and disintegration of the plasma membrane. Leukotoxin destroys the cell membrane which induces pores in target cells (such as PMNs, macrophages, NK cells and lymphocytes) with a functional diameter of approximately 0.96 nm.[73] This activation–inhibition paradox of the phagocytic and immune-mediating cells brought about by *ltx*, enables *A. actinomycetemcomitans* to evade the host immune system and help establish a local infection. Leukotoxin can also act as a bridge that physically bonds the bacterium to receptors on the surface of various peripheral blood leukocytes, thereby helping its movement through various organs of the host disregarded by the host immune system. Leukotoxin may support *A. actinomycetemcomitans* to establish secondary endogenous infections such as endocarditis, osteomyelitis, and pneumonia.[67]

3.1.2 Regulation and secretion of leukotoxin gene

The *A. actinomycetemcomitans ltx* operon consists of four coding genes and an upstream promoter.[74] Operon creates leukotoxin, which is a *ltxA* gene that encodes toxic activity, and the *ltx*C gene product is used for activation of the leukotoxin protein. The *ltx*B and *ltx*D genes

are associated with the *sec*-independent secretion of the toxin polypeptide.[76] The *ltxA* protein of *A. actinomycetemcomitans* has 40–50% amino acid, which is similar to *Escherichia coli* hemolysin (*HlyA*) and *Mannheimia haemolytica* leukotoxin (*lktA*).[75]

The expressed leukotoxin is transported to the bacterial outer membrane by a type I secretion system.[24] Whether the expressed and secreted leukotoxin remains associated with the bacterial outer membrane or whether it is secreted into the environment is still a topic of controversy. The mechanisms of leukotoxin secretion are still not fully understood either.[21] Leukotoxin destroyed host cells through a unique receptor on the target cells (LFA-1).[76, 77] The LFA-1 molecule, identified as the *ltxA* target cell receptor, is a heterodimer consisting of the αL (CD11a) and β2 (CD18) subunits. The residues 1-128 on human CD11a have been shown to be important for the human specificity of leukotoxin induced cell lysis.[77] In addition, the extracellular region of human CD18 (residues 500-600) has been shown to be critical for conferring susceptibility to leukotoxin induced cell lysis.[76, 78]

The production of the toxin varies among *A. actinomycetemcomitans* strains. The JP2 clone, a virulent strain of *A. actinomycetemcomitans*, is strongly associated with aggressive periodontitis producing higher levels of leukotoxin, and it has shown to have a 530-bp deletion in the promoter region of the leukotoxin gene operon. *A. actinomycetemcomitans* with 530-bp deletion on its *ltx* promoter produces over 10 to 20 fold higher levels of toxin than does the non-JP2 genotypes of *A. actinomycetemcomitans.*[14, 22, 79] Some researchers report that the JP2 clone is found to belong to serotype b; however, serotype b is not always associated with a 530 bp deletion. Haubek et al. reported strains of serotype b recovered from aggressive periodontitis patients, and all strains contained a normal leukotoxin gene.[80] *A. actinomycetemcomitans* with 530-bp deletion on its *ltx* promoter, which produces high levels of leukotoxin, and may be associated with severity and progression of disease in some ethnic populations.[79]

3.1.3 Prevalence of *A. actinomycetemcomitans* strains with 530–bp deletion on the leukotoxin promoter

A previous study has reported prevalence of *A. actinomycetemcomitans* strains with 530–bp deletion of the leukotoxin promoter among patients with localized juvenile periodontitis in all racial origins of 0.53%, which made it a reasonably common disease.[53]

Teenagers of African-American descent with aggressive periodontitis were found to have a 15-fold higher incidence of the disease than Caucasian Americans. Notably, this clone (a 530-bp deletion strain) demonstrated racial tropism and could be isolated from subjects originating from Africa, although the subjects did not reside in Africa.[9, 80, 81] In Brazil, one research of localized juvenile periodontitis in 15–16-year-old teenagers reported that it existed in 3.7% of the population.[82] In Nigeria, a prevalence of 0.8% was found.[83] The 530 bp deletion strains could be endemic in North African populations and strongly related to the particularly high prevalence and progression of aggressive periodontitis in Moroccan and US school children.[4, 80] Nonetheless, this specific clone was not detected in Asian subjects.[38]

3.2. Cytolethal distending toxin

The cytolethal distending toxin (Cdt) of *A. actinomycetemcomitans* is a cytotoxin that affects mammalian cells by inhibiting cell division and causing apoptosis.[84] Cdt is encoded by three genes, designated as *cdtA*, *cdtB* and *cdtC*, which are organized in an apparent operon (Fig. 5).[66] *cdtA* and *cdtC* are necessary for the secretion of the toxin, while *cdtB* is responsible for the biological activity.[85] There is a sequence homology between *cdtB* and mammalian DNase I, showing a critical role for nuclease activity in host parasite interactions.[86] The *cdt* gene has been reported in *Haemophilus ducrey.*[87] One study revealed that Cdt has the ability to induce G2 cell cycle arrests of human T-cells *in vitro*, which means that it has also been shown to have lethal effects on mammalian cells in culture.[88] However, not all strains of *A. actinomycetemcomitans* possessed the *cdt* gene, in other words, not all *A. actinomycetemcomitans* strains presented cytotoxic CDT activity.[87, 89] While the roles and the molecular mechanism of the action of the toxin are being elucidated, the association between this cytotoxin of *A. actinomycetemcomitans* and periodontal disease remains unknown and it has not been possible so far to demonstrate such a role for Cdt.[21, 84]

Fig. 5 The *cdt* gene structure of *A. actinomycetemcomitans*.[66]

3.3 Adhesion ability of *A. actinomycetemcomitans*

Adhesion is an important property for bacterial attachment on host surfaces. Adhesion is therefore a powerfully evolved survival mechanism and thus a virulence mechanism for pathogens. *A. actinomycetemcomitans* can adhere to oral epithelial cells better than other cells.[90] It was found that the adhesion of *A. actinomycetemcomitans* to KB cells occurred rapidly and was detected within ten minutes after the addition of the bacteria. *A. actinomycetemcomitans* adhered using fimbria, adhesin, non-fimbriated *A. actinomycetemcomitans*,[91] extracellular matrix (ECM) adhesin A (EmaA), and an oligomeric coiled-coil adhesin bind collagen.[92] Occasionally, the adherence of *A. actinomycetemcomitans* to epithelial cells is mediated by multiple adhesins.[93] Bacterial colonization depends on both bacteria and host cells, and specificity of adhesion to a host related to adhesin expression of the individual bacterium.[93] Besides, a previous study has reported that adhesion ability depends on the conditions of cultivation. *A. actinomycetemcomitans* can adhere better when it is cultured in broth in anaerobic conditions than when cultured on agar in aerobic conditions.[94] Adhesion ability at exponential growth phase is better than at the stationary phase of bacterial growth. Moreover, ionic interactions play an essential role in adhesion of *A. actinomycetemcomitans* to KB cells.[94]

Fujise et al. suggested that polysaccharides on the surface of *A. actinomycetemcomitans* are related to adhesion to host cells.[95] Specific polysaccharide antigen-a (*A. actinomycetemcomitans* serotype a) in the lipopolysaccharide molecule assisted the adherence to abiotic surfaces.[95] Thus, adhesion ability of different individual serotype *A. actinomycetemcomitans* may depend on the structure of the cells enveloped in a self-produced matrix, such as protein, polysaccharide, etc. The relationship of serotypes and adhesion ability is reported in some bacteria. For example, *Pseudomonas aeruginosa* serotypes I, G and E are more adhesive to contact lenses than other serotypes (A, C, D, and F).[96] *Escherichia coli* serotype 0113:H21 has also been found to adhere to HEp-2 cells with a distinct pattern of adhesion, which differs from the enteropathogenic *E. coli* and *E.coli* serotype 0157:H7.[97] This variation in adhesion can therefore indicate differences in the molecule(s) expressed on the outer membrane of the bacteria, which affect adhesion of individual strains of bacteria. Therefore, it was expected that adhesion ability of *A. actinomycetemcomitans* may be related to certain serotypes.

3.4. Invasion ability of *A. actinomycetemcomitans*

It is established that many bacteria can invade host cells, and invasiveness is a requisite for the pathology of certain bacterial pathogens. This ability is an important factor in the disease process because invasion of bacteria will protect bacterial cells from immune defenses and antibiotics.[98] In the oral cavity, bacteria can enter the mucosal epithelial cell layer and reach the underlying connective tissue. Colombo et al. found *A. actinomycetemcomitans* within gingival and buccal epithelial cells from both healthy individuals and patients with periodontitis.[99] The cytoplasm of buccal epithelial cells may act as the role of a reservoir for periodontal bacteria, and the mechanical periodontal therapy provided by antimicrobial agents would not affect the intracellular levels of bacteria.[100]

The invasion process is initiated when *A. actinomycetemcomitans* contacts with the microvilli of the KB cells as cell attachment is necessary for invasion to proceed.[101] The degree of invasion by *A. actinomycetemcomitans* is greater in KB cells and human primary gingival cells than in cells of non-oral origin.[25] *A. actinomycetemcomitans* invades KB cells through the mechanism of a cytochalasin D-sensitive inhibitor of actin filament network dynamics, whereby cytochalasin D can promote the invasion of certain *A. actinomycetemcomitans* strains (SUNY 523 and 465). Nonetheless, Meyer et al. revealed that *A. actinomycetemcomitans* internalization of epithelial cells is a multistep process which involves entry, escape from the vacuole, rapid multiplication, and intracellular spread.[102] Most bacteria use the actin microfilaments for uptake and intracellular movement, and there is increasing evidence that microtubules are also important. Microtubules are strongly involved in the intracellular and intercellular spread of *A. actinomycetemcomitans*. *A. actinomycetemcomitans* rapidly exits from cells after invasion. It has the ability to move from one cell to another and the capacity to divide rapidly within host cells.[103] The process of intracellular movement and cell spreading is inhibited by agents that interfere with microtubule dynamics, suggesting that this bacterium interacts closely with the microtubules of the host cell.[104]

Bacterial invasion is a complex process depending on both the bacterium and the host cell. Meyer et al. found that a smooth colony of *A. actinomycetemcomitans* could invade more efficiently than could a rough colony, although some smooth isolates did not invade.[25] Henderson et al. analyzed invasion of ten clinical *A. actinomycetemcomitans* isolates, in which they found five isolates that were invasive and five isolates that were clearly non-invasive.[24] It has been found that

there are limitations with the bacterial strains and human cells used. Henderson and co-worker revealed 25% of *A. actinomycetemcomitans* isolates were invasive.[24] These data suggest that invasion of *A. actinomycetemcomitans* depends on several factors from both the host and the bacteria including the character of different serotypes of *A. actinomycetemcomitans*. Furthermore, no study has ever examined the association between the invasion ability and different serotypes of *A. actinomycetemcomitans*. Thus, it is interesting to further study the different seorotypes and their invasion abilities.

3.5 Chemotactic inhibitor

Host defense mechanisms play a major role in controlling quantities and types of bacterial communities in the oral cavity. Gingival epithelial cells are a barrier to prevent microorganisms from invading periodontal tissues. They can produce some chemokines (chemotactic cytokines) that are considered the most important mediators for accumulation of neutrophils and T cells.[105] Interleukin-8 (IL-8) is a chemoattractant for neutrophils and monocytes.[106] The levels and the mRNA of IL-8 are increased depending on the concentration and incubation times with *A. actinomycetemcomitans*.[107] Nevertheless, it is not known whether *A. actinomycetemcomitans* induces IL-8 in gingival epithelial cells (GECs) and little is known about the IL-8 expression profile in GECs.[107] However, some bacteria, *Porphyromonas gingivalis* and *A. actinomycetemcomitans*, have been shown to elaborate many factors capable of suppressing these host defense mechanisms.[88, 108, 109] It is also suggested that they can evade host recognition, resulting in minimal activation of immune responses in a proposed strategy, known as “chemokine paralysis”, reinforcing the emerging view that these bacteria do not demonstrate characteristics normally associated with bacterium contributing to an inflammatory disease.[108] The organisms can produce proteins that stop synthesis of DNA, RNA and protein in mitogen-activated human T-cells.[110] *A. actinomycetemcomitans* produces a 60-kDa protein to inhibit IgG and IgM synthesis by human lymphocytes.[110] This means that the 60-kDa protein secreted by *A. actinomycetemcomitans* affects immunoglobulin production by down regulating the ability of the B and T cells to respond to mitogens. Cells expressing chemotactic inhibitors (such as IL-8) after being infected with bacteria may be the potentially important for effective host protection against infection and for the pathogenesis of periodontal diseases.

3.6 Growth inhibition of fibroblasts

Fibroblasts are a major source of collagen production and confer structural integrity to the tissue. All strains of *A. actinomycetemcomitans* can inhibit fibroblast proliferation including human and murine cells.[26] Thus, this is considered a virulence factor due to its impact on fibroblast viability. One toxin (heat labile toxin) is recognized: as a 50-kDa protein.[113] This toxin suppresses DNA and RNA synthesis in the fibroblast.[26, 111] However, the mechanism of this toxin is unclear. It may result from several different factors including supernatants (after being cultured in broth) that contain protein are capable of stimulating or suppressing growth of fibroblast cells, substances produced by PMNs or leukocytes or macrophages after being stimulated *A. actinomycetemcomitans*, etc.[26] Therefore, the inhibition of fibroblast proliferation can possibly play an important role in collagen loss associated with periodontal disease.

Objectives

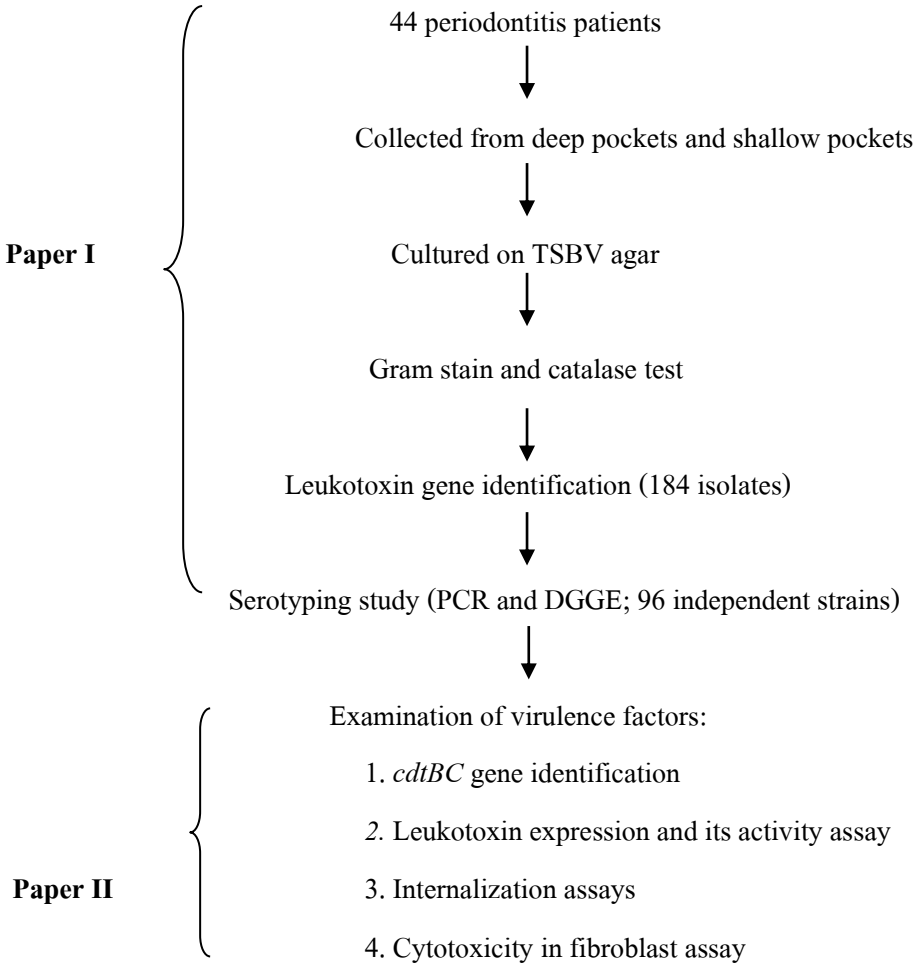
The specific objectives of this study were:

1. To investigate the prevalence and relationship of serotypes of *A. actinomycetemcomitans* in Thai patients with chronic periodontitis
2. To examine the relationship between different serotypes of *A. actinomycetemcomitans* and their virulence factors, including the *ltx* gene, *cdt* genes, leukotoxin expression, internalization, and cytotoxicity in fibroblasts

2. MATERIALS AND METHODS

Study design

This thesis is based on a microbiological study (Paper I), and virulence factors of *A. actinomycetemcomitans* (Paper II)



Study population

Forty-four subjects from the patient recall list (during June 2012-December 2013) of the Periodontology Clinic, Faculty of Dentistry, Prince of Songkla University, Thailand, were sequentially recruited for this study. They were all be diagnosed as having generalized moderate to severe chronic periodontitis according to the American Academy of Periodontology in 1999.[34] They were then selected based on the following inclusion criteria: (i) $\leq$ 60 years of age with $\geq$ 15 remaining teeth, (ii) showing clinical attachment level of $\geq$ 4 mm on 30% of sites, (iii) having a pocket depth of $\geq$ 5 mm and bleeding on probing, (iv) presentation of bone loss on a radiograph, and (v) having good general medical health. Any patients with the following conditions were excluded: (i) having systemic diseases, (ii) taking any antibiotic medication within the previous six months, and (iii) receiving periodontal treatment or preventive prophylaxis within six months prior to this study.

The study was approved by the Ethics Committee of the Faculty of Dentistry at Prince of Songkla University, Thailand (EC550936P). All patients gave their informed consent and were offered dental treatment after the study. Demographic data of these patients on general health, age, gender and smoking use were collected.

Clinical examination

A full mouth recording of the patient's clinical attachment loss (CAL), probing pocket depth (PPD), and bleeding on probing (BOP) were assessed. All three parameters were recorded at four sites per tooth; the mesio-buccal, mid-buccal, disto-buccal, and mid-lingual aspect of all teeth except third molars. Probing pocket depth was measured to the nearest whole mm as the distance between the gingival margin and the bottom of the probeable pocket. The clinical attachment level was measured as the distance in mm from the cement enamel junction to the bottom of the pocket.

Subgingival plaque sampling

Two subgingival samples were taken from each patient randomly. The first sample was taken from one deep pocket site, which was defined as having clinical attachment loss (CAL $\geq$ 4 mm), a probing pocket depth (PPD $\geq$ 5mm), and bleeding on probing (BOP). The second sample was also randomly taken from one shallow pocket sample, being a site with no CAL and BOP, and PPD $\leq$ 3 mm. All samples were obtained by introducing 2 sterile paper points subgingivally, which were kept in place for 15 s. Then they were transferred into a vial containing reduced transport medium (RTF), which was immediately transported to the laboratory.

Cultivation and identification

Samples were cultured on tryptic soy serum bacitracin vancomycin agar (TSBV agar), a selective medium for *A. actinomycetemcomitans*, under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2) at 37°C for 3-5 days.[36] Initial identification was based on showing a typical *A. actinomycetemcomitans* morphology as star-shaped or small smooth, adherent and catalase-positive colonies, and a micro-morphology including gram-negative coccobacilli. All isolates were further confirmed as *A. actinomycetemcomitans* by the presence of the leukotoxin gene (*ltx*) using the PCR with leukotoxin primer.[69] The selected isolates were purified and kept at -80°C for further use in serotyping and subtyping. The following reference strains of *A. actinomycetemcomitans* were included: CCUG (Culture Collection, University of Göteborg, http://www.ccug.se/) strain 37004 serotype a, CCUG 37002 serotype b, CCUG 13227 serotype c, ATCC 33384 serotype c, CCUG 38565 serotype d, CCUG 37399 serotype e, OMGS 3816 serotype f (received from Sirkka Asikainen, Umeå, Sweden) as well as a P48:2 JP2 clone strain (received from Gunnar Dahlen, Gothenburg, Sweden).

Determination of the leukotoxin (*ltx*) gene

This study aimed to determine the *ltx* gene and *ltx* gene variants of *A. actinomycetemcomitans.* Primers used included a forward primer ltx3 and a reverse primer ltx4

(Table 1).[69] All DNA samples of *A. actinomycetemcomitans* isolates were prepared according to the method of Owen and Borman.[113] The PCR mixture included 1 unit of *Taq* DNA polymerase, 10x PCR buffer, 600 μM dNTP, 2.5 mM $MgCl_2$, 5 pM of ltx3 and ltx4, and extracted DNA. The thermo cycling program was carried out at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, at 60°C for 1 min, at 72°C for 2 min, and a final extension at 72°C for 8 min. A PCR product of 1,216 bp and 2,117 bp represented the complete promoter and the insertion promoter, respectively, while 686 bp was indicated as a deletion promoter, being a characteristic of the JP2 clone. *A. actinomycetemcomitans* ATCC 33384 (non-JP2) and P48:2 (JP2) were used as the negative and positive control, respectively. The PCR products were analyzed by 1.5% agarose gel electrophoresis and photographed under UV light. The molecular weights of the PCR products were compared with standard molecular weight markers (TriDye™ 1 kb DNA Ladder, New England, Biolab, MA, USA).

Serotyping using the specific primers in PCR

Serotyping using the specific primers of the PCR method was performed according to Kaplan et al.[5] and Suzuki et al.[6] The sequences of the specific primers for each serotype are shown in Table 1. PCR mixture consisted of 200 μM dNTP, a 10x PCR buffer with 2.5 mM $MgCl_2$, 1 unit of *Taq* polymerase, 5 pM of each primer, and 10 ng of DNA template. The PCR condition included initial denaturation at 94°C for 30 s followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 54°C for 45 s, with extension at 72°C for 1 min, and a final extension at 72°C for 8 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The gel was stained with 0.5 μg/mL ethidium bromide. The gel was photographed under UV light and the molecular weights of the PCR products were compared with standard molecular weight markers (TriDye™ 1 kb DNA Ladder, New England, Biolab, MA, USA).

Subtyping using denaturing gradient gel electrophoresis (DGGE)

Not all isolates can be serotyped, and non-serotypeable *A. actinomycetemcomitans* still exists, thus this technique was used for detection of subtypes of *A. actinomycetemcomitans* and to study the distribution of *A. actinomycetemcomitans* subtypes.

Subtyping was performed using DGGE, the V2-V3 region of the 16S rRNA gene was amplified with primers of HDA1-GC and HDA-2 (Table 1) according to the method of Ledder et al.[19] The diversity in the DNA sequence of the V2-V3 region of *A. actinomycetemcomitans* is suitable for distinguishing subtypes of *A. actinomycetemcomitans*. The reaction mixture consisted of 1 unit of *Taq* DNA polymerase, a 10x PCR buffer with 1.5 mM $MgCl_2$, 5 pM of forward primer (HDA1-GC) and reverse primer (HDA-2), 200 µM dNTP, and 10 ng of DNA template. The PCR cycle was performed at 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 60 s. The final cycle was stationary at 68°C for 7 min to provide a chain elongation step. PCR products (238 bp) were determined using a D-Code universal mutation detection system (Bio-Rad, Hemel Hempstead, UK) with 8% polyacrylamide gel (16 x 16 cm, 1 mm deep). The gel was run with a final concentration of 1x TAE buffer (2 M Tris base, 2 M glacial acetic acid, 0.5 M EDTA pH 8.0). On this basis, a denaturation gradient for parallel DGGE analysis ranging from 35% to 60% (100% denaturant equals 7 M urea and 40% formamide) was selected for distinguishing the serotypes of *A. actinomycetemcomitans*. The PCR product of the clinical strains of *A. actinomycetemcomitans* isolates was compared with the reference serotypes. Reference serotypes of *A. actinomycetemcomitans* (a through f) were loaded into the gel to compare with clinical strains. Electrophoresis was carried out at 120 volts at 60°C for approximately 6 h. The gel was stained with SYBR® safe stain for 30 min and was viewed under UV light (Spectroline® UV Transilluminator Select™ Series). Images of the gels were analyzed using BioNumerics version 6.0 (Applied Maths). The resulting patterns were compared to one another using the dice similarity coefficient and the matrix generated was clustered by the UPGMA method (Ultra-Violet Products Ltd., UK).

Table 1 Primer sequences used in this study

Name	Sequence (5' - 3')	Target	Product size (bp)	Reference
lx3	GCCGACACCAAAGACAAAGTCT	*ltx* A - *gly*A gene	686 (deletion strain)	Poulsen et al.[69]
ltx4	GCCCATAACCAAGCCACATAC		1,216 (normal strain)	
			2,117 (insertion strain)	
HDA1 - GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGG	V2 - V3		
	GGGCACGGGGGGACTCCTACGGGAGGCA	(16s rRNA)	238	Ledder et al.[19]
HDA - 2	GCAGT GTATTACCGCGGCTGCTGGCAC			
SA - F	GCAATGATGTATTGTCTTCTTTTGGA	Putative	428	Suzuki et al.[6]
SA - R	CTTCAGTTGAATGGGGATTGACTAAAAC	mannosyltransferase		
SB - F	CGGAAATGGAATGCTTGC	dTDP-4-keto-6-deoxy-	298	Suzuki et al.[6]
SB - R	CTGAGGAAGCCTAGCAAT	D-glucose reductase		
SC - F	AATGACTGCTGTCGGAGT	Putative	559	Suzuki et al.[6]
SC - R	CGCTGAAGGTAATGTCAG	acetyltransferase		

Table 1 (continued)

Name	Sequence (5' - 3')	Target	Product size (bp)	References
SD - F	TTACCAGGTGTCTAGTCGGA	Putative	690	Suzuki et al.[6]
SD - R	GGCTCCTGACAACATTGGAT	mannosyltransferase		
SF - F	CCTTTATCAATCCAGACAGC	4813–5044[b]	232	Kaplan et al.[5]
SF - R	ARAAYTTYTCWTCGGGAATG[a]			
cdtBF	CAACAACACAATTCCAACCC	*cdtB-cdt*C	1,971	Tan et al.[114] ; Umeda et
cdtR	TTAGCTACCCTGATTTCTCC			al.[115]

[a] R =A or G; Y = C or T; W = A or T; [b] GenBank accession no. AF213680

A. actinomycetemcomitans strains were selected for virulence factor tests

A total of 96 independent strains included 36 serotypeable and 60 non-serotypeable strains isolated from 37 subjects with *A. actinomycetemcomitans* positive strains (79 strains from 37 deep pockets and 17 strains from 11 shallow pockets). Strains were serotyped using PCR with specific primers (as described above) including serotype a having 11 strains, serotype c having 11 strains (included 2 strains of JP-2 like), serotype e having 7 strains, and serotype f having 7 strains. Denaturing gel gradient electrophoresis was used for subtyping 60 non-serotypeable strains (as described above) including the non-serotypeable group 1 (NS1) of 11 strains, the non-serotypeable group 2 (NS2) of 9 strains and other groups of non-serotypeable (other NS) with 40 strains. The culture collection was kept at –80°C in the Department of Stomatology at the Faculty of Dentistry, Prince of Songkla University, Thailand. *A. actinomycetemcomitans* was cultured on brain heart infusion agar (BHI) under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2) at 37°C for 3-5 days before being used. Reference strains of *A. actinomycetemcomitans* were included in this study (as described above).

Determination of *cdtBC* genes

The presence of *cdtBC* genes was investigated as in a previous investigation.[114,115] The reaction mixture consisted of 10 ng of extracted bacterial DNA, 5 pM each of cdtBF and cdtR,[114,115] 600 µM dNTP, 10x PCR buffer, 2.5 mM $MgCl_2$, and one unit of *Taq* polymerase. The thermo cycling program was performed at 94°C for 5 min followed by 35 cycles at 94°C for 30s, at 57°C for 30s, at 72°C for 2 min, and a final extension at 72°C for 8 min. Amplicons were analysed using 1.5 % agarose gel electrophoresis and the molecular weights of the PCR products were compared with standard molecular weight markers (TriDye™ 1 kb DNA Ladder, New England Biolabs, Ipswich MA).

Determination of leukotoxin expression

The total of bacterial RNA was extracted using an innuPREP DNA/RNA Mini kit (Analytikjena, Berlin, Germany) and the total RNA concentration in each sample was calculated from the A260. Twelve µg/mL of the total RNA was added in a Sensi-FAST™SYBR One-step kit (Bioline Reagent Ltd.), and the DNA sequences of primers used were ltx3 and ltx4. The PCR thermal profile consisted of an initial cDNA step of 10 min at 45°C followed by 94°C for 5 min and then 40 cycles at 94°C for 1 min, at 60°C for 1 min, and at 72°C for 2 min. Amplification, detection, and data analysis were performed with the Rotor gene Q (Qiagen, Valencia, CA). *A. actinomycetemcomitans* ATCC 33384 was used as a positive control to compare with other samples. The mRNA expression level of leukotoxin was expressed as the $2^{-\Delta\Delta CT}$, of which the 16s rRNA gene was used as a housekeeping gene.

Determination of leukotoxin activity

The leukotoxicity of *A. actinomycetemcomitans* was determined using the trypan blue exclusion method with some modification.[14] Briefly, the promyelocytic leukemic cell line (HL-60 derived from the Faculty of Associated Medical Science, Chiang Mai University) was grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Gibco®, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and with 1% pen-strep in an atmosphere of 5% CO_2 at 37ºC. HL-60 was adjusted in RPMI 1640 at a density of 1 x 10^6 cells/ml and bacterial inoculum (10^8 CFU/ml) was added and incubated at 37ºC for 2 h. HL-60 cells without bacteria were used as a negative control. After incubation, 0.4% trypan blue was added to the mixtures to stop the reaction, and the mixture placed on a hemocytometer and observed under a light microscope. Leukotoxin activity was reported as a proportion according to the formula of death cells (%) = 100 – (surviving cells of the test/surviving cells of the control x 100).

Determination of internalization

A. actinomycetemcomitans cells were resuspended in DMEM (Dulbecco's Modified Eagle's Medium; Gibco®, Grand Island, NY, USA) at a density of 10^8 CFU/ml. Fibroblast cells (Mouse fibroblast cell lines (L929) Balb/c 3T3 ATCC CCL 163 obtained from Dr. Carl T. Hanks, University of Michigan, School of Dentistry, Ann Arbor, Michigan) were cultured in fibroblast growth medium (FGM; DMEM supplemented with 10% fetal bovine serum, 1% pen-strep, and 1% fungizone) and incubated with 5% CO_2 at 37°C for 48-72 h or until the cells propagated a full flask. Cells (7 x 10^4 cells/well) were seeded onto 24-well plates and incubated with 5% CO_2 at 37°C for 48-72 h. *A. actinomycetemcomitans* cells (10^8 CFU/ml) were incubated with a monolayer, having a 95-100% confluence, at 37°C for 1 h in anaerobic conditions. Then non-adherent bacteria were washed off with PBS pH 7.0. Gentamycin (100 µg/ml) was added to the 24-well plates, which were then incubated at 37°C for 2 h to kill external cells.[25] After washing twice with PBS pH 7.0, cells were disrupted using 0.05% trypsin-EDTA and incubated for 5 min at 37°C. A 10-fold dilution of samples was made in PBS pH 7.0 and then plated on BHI agar. The plates were incubated at 37°C for 24-48 h for counting the number of *A. actinomycetemcomitans*. The internalized percentages were calculated from the number of internalizations divided by the total number of bacterial cells at the start x 100.

Determination of cytotoxicity to fibroblasts

Cytotoxicity to fibroblasts was determined using the trypan blue exclusion method with some modification.[14] Briefly, *A. actinomycetemcomitans* was harvested and resuspended in DMEM at a density of 1 x 10^7 CFU/ml. L929 was cultured in FGM in an atmosphere with 5% CO_2 at 37°C for three days. The cells were adjusted in DMEM to a density of 1 x 10^6 cells/ml. Bacterial inoculum (50 µl) and fibroblasts (50 µl) were incubated at 37°C for 2 h. Fibroblasts without bacteria were used as a negative control. After incubation, 100 µl of 0.4% trypan blue was added and viable cells were observed under a light microscope. Cytotoxicity to fibroblasts was reported as a percentages according to the formula of death fibroblasts (%) = 100 – (surviving cells of the test/surviving cells of the control x 100).

Data analysis

The prevalence of *A. actinomycetemcomitans* serotypes between deep and shallow pocket sites and the number of *A. actinomycetemcomitans* serotypes with positive *cdtBC* genes between deep pockets and shallow pockets were analyzed by the Chi-square test. The differences of cytotoxicity in fibroblasts, internalization into fibroblasts, leukotoxin activity, and leukotoxin expression of *A. actinomycetemcomitans* serotypes and subtypes were evaluated using the Kruskall-Wallis test, and the Mann-Whitney U test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. RESULTS

Subjects and clinical characteristics

A total of 44 chronic periodontitis patients fulfilled the inclusion criteria. The mean age was 46.4 ± 9.5 years with a range of 24-60 years. They were all systemically healthy and had not taken any antibiotics during the last 6 months. Twenty subjects (45.5%) were males and 23.3% of all subjects smoked, while the mean percentage (±SD) of sites with gingival bleeding was 72.3 ± 29.3%. The mean percentage of sites with CAL $\geq$ 4 mm and CAL $\geq$ 7 mm was 35.3 ± 21.6% and 18.0 ± 22.3%, respectively (Table 2), and the mean percentage of sites with PPD $\leq$ 3 mm, PPD $\geq$ 5 mm, and PPD $\geq$7 mm was 58.8 ± 30.2%, 19.3 ± 17.6%, and 8.3 ± 11.6%, respectively.

The deep pocket sampling sites exhibited a mean CAL of 8.8 ± 1.9 mm, PPD of 8.9 ± 2.0 mm and all showed bleeding on probing. The shallow pocket sampling sites had no CAL and no BOP, and PPD was 2.64 ± 0.65 mm.

Study of the leukotoxin (*ltx*) gene

A total of 184 *A. actinomycetemcomitans* isolates (145 from deep pockets and 39 from shallow pockets) were positive for the *ltx* promoter gene. Two strains (2 isolates) isolated from 2 different subjects exhibited 530-bp deletion of the *ltx* promoter region (or JP2 clone like) (Figure 1). These two patients, one man (57 years of age) and one woman (24 years of age), were diagnosed as having generalized moderate to severe chronic periodontitis with 21 and 15 teeth remaining, respectively. They showed percentage of sites with PPD $\leq$ 3 mm, PPD $\geq$ 5 mm, and PPD $\geq$ 7 mm of 39.0%, 41.9% and 19.1%, respectively, whereas the percentage of sites with CAL $\geq$ 4 mm and $\geq$ 7 mm was 36.3% and 18.8 %, respectively. Two other subjects harbored one strain each (from 10 isolates) with an 886-bp insertion on the *ltx* promoter gene (Fig. 6). They were diagnosed as having generalized moderate to severe chronic periodontitis with 21 and 25 teeth remaining. They showed that the percentages of sites with

PPD $\leq$ 3 mm, PPD $\geq$ 5 mm, and PPD $\geq$ 7 mm were 70.9%, 21.5%, and 7.6%, respectively, whereas the percentage of sites with CAL $\geq$ 4 mm and $\geq$ 7 mm were 18.5% and 3.9%, respectively.

Table 2 Characteristics of the study population

Subjects parameters	**Subjects** **n = 44** **Mean ± SD**
Age (years)	46.4 ± 9.5
Males (%)	45.5
Smokers (%)	23.3
No. of teeth present	25.5 ± 4.7
% sites with BOP	72.3 ± 29.3
% sites with CAL $\geq$ 4 mm	35.3 ± 21.6
% sites with CAL $\geq$ 7 mm	18.0 ± 22.3
% sites with PPD $\leq$ 3 mm	58.8 ± 30.2
% sites with PPD $\geq$ 5 mm	19.3 ± 17.6
% sites with PPD $\geq$ 7 mm	8.3 ± 11.6

Analysis of the prevalence and subtypes of *A. actinomycetemcomitans*

The prevalence of *A. actinomycetemcomitans* was 84.1% (37/44 subjects) from 37 deep pocket sites and 11 shallow pocket sites. A total of 96 independent strains, 79 from deep pocket and 17 from shallow pocket sites, were used for analysis. Initially, 145 isolates from deep pocket samples and 39 from shallow pocket samples were pure cultured and tested for the *ltx*-gene (see above) and then serotyped. If the same serotype occurred for two or more isolates from the same sample, they were considered the same strain. Thus, 24 independent serotypeable strains were isolated from deep pockets and 12 independent strains from shallow pockets ($P < 0.05$). Sixty strains (55 from deep and 5 from shallow pockets) were non-serotypeable. The

prevalence and serotype distribution is shown in Table 3 and Figure 7a. The prevalence of serotypeable and non-serotypeable (NS) strains was equal at subject levels (54.5%). Among the serotypeable strains, serotypes a and c were most prevalent (18.2% and 15.9%, respectively), while serotypes b and d were not found. Notably, the 2 JP2 like strains and the 2 strains with the insertion gene as identified above were all classified as serotype c. *A. actinomycetemcomitans* serotypes and non-serotypeable (NS) strains showed a similar distribution pattern in deep pockets, while the frequency of serotypeable isolates was significantly higher in shallow compared to deep pockets ($P < 0.01$).

Additionally, all 184 isolates were subtyped with DGGE. Similarly as for the serotypeable isolates, when the same DGGE subtype occurred for two or more isolates from the same sample they were considered the same strain. The 24 serotypeable strains fell into 3 DGGE subtypes (S1-3). The DGGE subtyping could not distinguish between serotype a, d, and f (Fig. 7b), and this subtype was designated as DGGE subtype S1 (S for serotypeable). Serotype c isolates formed a separate DGGE subtype (S2) and serotype e formed the DGGE subtype S3 (Table 4). The 60 non-serotypeable isolates (55 from deep pockets and 5 from shallow pockets, Table 3) fell into 16 different DGGE subtypes (NS1-16), and the distribution is shown in Table 4. The NS1 and NS2 subtypes were more frequent (12.7% and 10.1% in deep pockets, respectively) than the others. A dendrogram showing the similarity between the subtypes as presented in Figure 7c. The UPGMA method could not distinguish between serotype b and e, between a and c, or between d and f (Fig. 7c), although the differences between serotype b and e and serotype a and c were quite visually clear under UV-light in the gels (Fig. 8).

The same pocket/sample could harbor more than one subtype, in fact up to four different subtypes (serotypes and DGGE subtypes) were found in 3 deep pocket samples from three different patients (Table 5).

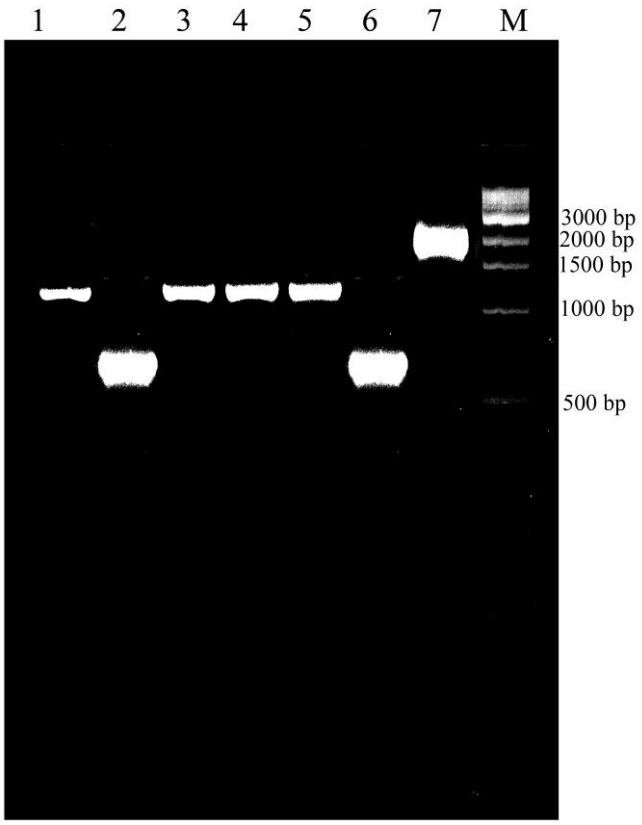


Fig. 6 The *ltx* gene of different strains of *A. actinomycetemcomitans* was amplified using ltx3 and ltx4 primers: lane 1 contained *A. actinomycetemcomitans* ATCC 33384 (serotype c and non-JP2 clone); lane 2 contained P48:2 (JP2 clone); lanes 3-5 were clinical isolates with a normal *ltx* gene; lane 6 was a clinical isolate with 530 bp deletion gene; lane 7 was a clinical isolate with an 886 bp insertion gene; and lane M was the marker.

Table 3 Prevalence of *A. actinomycetemcomitans* at subject and site level, and serotypes/ subtypes distribution in deep and shallow pockets

Serotypes[a]/Subtypes	No. (%) subjects n = 44	No. of (%) sites		No. (%) of strains isolated	
		Aa-positive deep pockets n = 37	Aa-positive shallow pockets n = 11	Aa-positive deep pockets n = 79	Aa-positive shallow pockets n = 17
a	8 (18.2)	8 (21.6)	3 (27.3)	8 (10.1)	3 (17.6)
b	ND	ND	ND	ND	ND
c	7 (15.9)	7 (18.9)	4 (36.4)	7 (8.9)	4 (23.5)
d	ND	ND	ND	ND	ND
e	4 (9.1)	4 (10.8)	3 (27.3)	4 (5.1)	3 (17.6)
f	5 (11.4)	5 (13.5)	2 (18.2)	5 (6.3)	2 (11.7)
Total serotypeable	24 (54.5)	24 (64.9)	11 (100.0)	24 (30.3)[c]	12 (70.5)[b]
Non-serotypeable	24 (54.5)	24 (64.9)	4 (36.4)	55 (69.2)[b,c]	5 (29.4)

ND: not detected,

[a,] serotypeable,

[b,] The difference between the number of serotypeable and non-serotypeable strains isolated from deep and shallow pockets was significant ($P < 0.01$),

[c,] The difference between the number of strains totally isolated from deep pockets and shallow pockets was significant ($P < 0.05$)

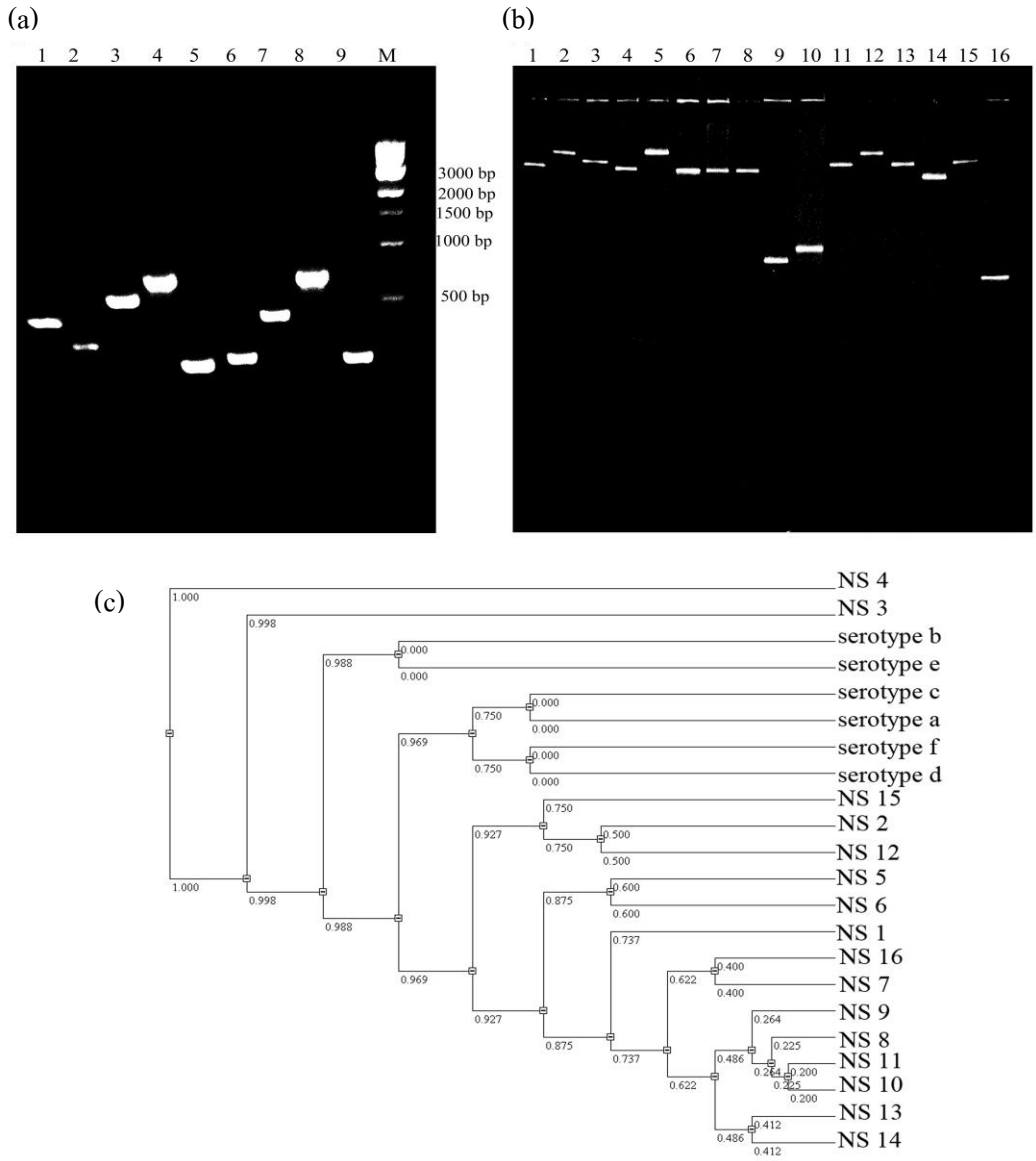


Fig. 7 (a) Serotyping of *A.actinomycetemcomitans* by the PCR with specific primers: lane 1was serotype a (CCUG 37004), lane 2 was serotype b (CCUG 37002), lane 3 was serotype c (CCUG 13227), lane 4 was serotype d (CCUG 38565), lane 5 was serotype e (CCUG 37399), lane 6 was serotype f (OMGS 3816), lanes 7–9 were clinical isolates of serotype a, d, and f, respectively, and lane M was the marker.

(b) Subtyping of *A.actinomycetemcomitans* by DGGE: lane 1 was serotype a (CCUG 37004), lane 2 was serotype b (CCUG 37002), lane 3 was serotype c (CCUG 13227), lane 4 was serotype d (CCUG 38565), lane 5 was serotype e (CCUG 37399), lane 6 was serotype f (OMGS 3816), and lanes 7-16 were clinical isolates (lanes 7, 8, 11, 13, and 15

were serotype a, d, f, lane 12 was serotype e, lanes 9 and 16 were NS 2 and NS 1, respectively, and lanes 10 and 14 were other NS)

(c) Dendrogram illustrating the similarities among the *A.actinomycetemcomitans* strains for different subtypes, which were calculated based on the intensity and the position of each band in the gel. The scale bar indicates the value of similarity, which if closer to zero (0) are very similar but if closer to 1 are very dissimilar.

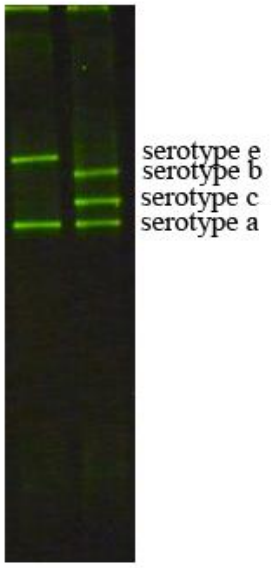


Fig. 8 Subtyping of *A.actinomycetemcomitans* serotype a (CCUG 37004) and c (ATCC 33384) as well as serotype b (CCUG 37002) and e (CCUG 37399) clearly distinguished by DGGE.

Table 4 Prevalence and distribution of DGGE subtypes of *A. actinomycetemcomitans* strains

DGGE subtypes	No. (%) of strains isolated from	
	Deep pockets	Shallow pockets
	n = 79	n = 17
S 1 (serotype a,d,f)	13 (16.5)	5 (29.4)
S 2 (serotype c)	7 (8.9)	4 (23.5)
S 3 (serotype e)	4 (5.1)	3 (17.6)
NS 1	10 (12.7)	1 (5.9)
NS 2	8 (10.1)	1 (5.9)
NS 3, 7, 9, 10	3 (3.8)	ND
NS 4, 5	1 (1.3)	ND
NS 6, 11, 14	2 (2.5)	ND
NS 8, 12	4 (5.1)	ND
NS 13	1 (1.3)	1 (5.9)
NS 15, 16	4 (5.1)	1 (5.9)

Table 5 Distribution of serotypes and DGGE-subtypes of *A. actinomycetemcomitans* in subjects and sites (deep and shallow pockets)

Subtypes distribution	Subject level	Site level	
		Deep pocket	Shallow pocket
	n = 37	n = 37	n = 11
Single type	12 (32.4)	12 (32.4)	5 (45.5)
Two types	11 (29.7)	11 (29.7)	6 (54.4)
Three types	11 (29.7)	11 (29.7)	ND
Four types	3 (8.1)	3 (8.1)	ND

ND: not detected

Virulence factors of *A. actinomycetemcomitans* serotypes

Presence of *cdtBC* genes

The *cdtBC* gene was detected in 84.4% of all strains (81 of 96 strains: 66 strains from deep pockets and 15 strains from shallow pockets), and distribution of *cdtBC* genes between serotypeable and non-serotypeable strains in the deep pocket was significantly different (Table 6).

Virulence factors of serotypeable or non-serotypeable strains from deep and shallow pockets

Considering the abilities of leukotoxin expression, leukotoxin activity, internalization of fibroblast cells and cytotoxicity to fibroblasts between the strains isolated from deep and shallow pockets of individual serotypeable or non-serotypeable strains, the strains from deep pockets, especially non-serotypeable strains, showed significantly higher abilities in all means ($P < 0.01$, Table 7). When comparing the strains from deep pockets between serotypeable and non-serotypeable, non-serotypeable strains exhibited significance higher means for leukotoxin expression, leukotoxin activity, internalization of fibroblast cells and cytotoxicity to fibroblasts, compared to serotypeable strains ($P < 0.001$, Table 7).

Generally, mean values for leukotoxin expression for non-serotypeable and serotypeable strains showed significantly higher ($P < 0.001$) means for strains isolated from deep pockets compared with those isolated from shallow pockets (Table 7). The non-serotypeable strains isolated from deep pockets showed significantly ($P < 0.01$) higher means of leukotoxin expression, leuktoxicity, internalization and cytotoxicity to fibroblasts than the serotypeable strains (Table 7). The non-serotypeable subtypes, NS1 and NS2, isolated from deep pockets, showed significantly ($P < 0.05$) higher means of cytotoxicity to fibroblasts compared to the serotypeable and other NS strains. However, no significant differences were found between these groups if the strains were isolated from shallow pockets.

Table 6 Presence of *A. actinomycetemcomitans cdtBC* genes in serotypeable and non-serotypeable subtypes isolated from deep and shallow pocket sites

Serotypes/subtypes[a]	**No. (%) of strains with *cdtBC* genes**	
	Deep pocket	**Shallow pocket**
	n = 66	**n = 15**
Serotypeable:	22 (33.3)	11 (73.3)
a	7 (10.6)	3 (20.0)
c	7 (10.6)	3 (20.0)
e	4 (6.1)	3 (20.0)
f	4 (6.1)	2 (13.3)
Non-serotypeable:	44 (66.7)[*]	4 (26.7)
NS1	10 (15.2)	1 (6.7)
NS2	8 (12.1)	1 (6.7)
other NS	26 (39.4)	2 (13.3)

[a,] no serotype b and d strains were found,

[*,] significant difference of *cdtBC* gene positive between serotypeables and non-serotypeable strains ($P < 0.001$)

Table 7 Mean ± SD of leukotoxin expression, leukotoxin activity, internalization, and cytotoxicity to fibroblasts for serotypeable and non-serotypeable strains of *A. actinomycetemcomitans* isolated from deep and shallow pocket sites, respectively

Virulence factors	Serotypeable		Non-serotypeable	
	Deep pockets n = 24	Shallow pockets n = 12	Deep pockets n = 55	Shallow pockets n = 5
Leukotoxin expression (multiple of control)	11.0 ± 7.6[*]	2.1 ± 1.7	42.9 ± 39.6[*,‡]	0.3 ± 0.3
Leukotoxin activity (%)	40.7 ± 13.7[*]	29.2 ± 18.2	47.5 ± 11.8[*,‡]	32.0 ± 14.9
Internalization (%)	48.3 ± 2.7	39.7 ± 2.7	57.9 ± 13.2[*,‡]	44.5 ± 9.9
Cytotoxicity to fibroblasts (%)	51.3 ± 6.8	43.0 ± 6.0	61.7 ± 14.4[*,‡]	38.4 ± 5.5

[*,] significant difference of individual characteristics of *A. actinomycetemcomitans* strains between deep pockets and shallow pockets ($P < 0.01$),

[‡,] significant difference of individual characteristics of *A. actinomycetemcomitans* strains of deep pockets between serotypeable and non-serotypeable ($P < 0.001$)

3.3. Leukotoxin expression, leukotoxin activity, internalization, and cytotoxicity in fibroblasts of *A. actinomycetemcomitans* between serotypeable and non-serotypeable strains

The results of Table 8 demonstrate that subtype NS1 exhibited highly significant leukotoxin expression ($P < 0.001$) compared to others (serotypeable, NS2, and other NS) with mean of 89.1 ± 37.1. Also, subtype NS2 showed highly significant leukotoxin expression ($P < 0.001$) compared to serotypeable and other NS with mean of 36.3 ± 6.8.

In addition, subtype NS1 exhibited highly significant leukotoxin activity ($P < 0.001$) compared to serotypeable strains, NS2 and other NS. Moreover, NS1 showed correlation

between leukotoxin expression and leukotoxin activity in the same way ($P < 0.001$; $r_s = 0.936$). NS2 showed significant difference from serotype f ($P < 0.05$).

All strains showed various degrees of internalization of bacterial cells into fibroblasts compared to the number at the start. The non-serotypeable (NS1, NS2, and other NS) showed a significantly higher degree of internalization of fibroblast cells (62.8 ± 12.1%, 64.4 ± 11.8%, and 55.9 ± 13.0%, respectively) compared to serotypeable strains (48.6 ± 8.3%).

The cytotoxicity to fibroblasts of NS1 (66.3 ± 10.3%) and NS2 (75.1 ± 13.1%) was significantly higher than that serotypeable strains (50.5 ± 9.7%) and other NS (58.1 ± 12.6%) ($P < 0.001$).

Two strains (JP2-like) with a 530 bp leukotoxin gene deletion showed high levels of leukotoxin expression (596.0 ± 291.3) and leukotoxin activity (54.7 ± 9.3%), similar to what was found for the JP2 reference strain (761.0 ± 34.6) and significantly higher than all other strains tested. Internalization and cytotoxicity to fibroblasts were of the same magnitude or even lower than that of the non-serotypeable NS1 and NS2.

Table 8 Mean ± SD of leukotoxin expression, leukotoxin activity, internalization, and cytotoxicity to fibroblasts for serotypeable and non-serotypeable strains of *A. actinomycetemcomitans*

Virulence factors / Serotypes/ subtypes[a]	Leukotoxin expression (fold of control)	Leukotoxin activity (%)	Internalization (%)	Cytotoxicity to fibroblasts (%)
a	8.0 ± 3.9	41.4 ± 15.9	44.4 ± 10.2	57.5 ± 4.9
c[b]	8.1 ± 3.2	37.8 ± 18.5	50.3 ± 12.6	56.0 ± 12.3
e	11.9 ± 7.7	36.9 ± 14.0	50.1 ± 14.8	48.4 ± 9.2
f	3.7 ± 2.4	32.1 ± 16.8	48.5 ± 12.3	43.1 ± 11.8
NS1	89.1 ± 37.1[*]	50.6 ± 9.9[*]	62.8 ± 12.1[†]	66.3 ± 10.3[¶]
NS2	36.3 ± 6.8[**]	44.7 ± 11.5[‡]	64.4 ± 11.8[†]	75.1 ± 13.1[**]
Other NS	8.1 ± 13.0	34.7 ± 12.4	55.9 ± 13.0[†]	58.1 ± 12.6
JP2-like (serotype c)	596.0 ± 291.3	54.7 ± 9.3	48.4 ± 0.5	66.4 ± 0.5
JP2	761.0 ± 34.6	57.3 ± 8.4	53.9 ± 0.6	57.8 ± 0.6

Statistical analysis was considered within the same column,

[a,] no serotype b and d strains were found; [b], serotype c excluded 2 strains of JP2-like,

[*,] significant difference between NS1and others (serotypeable, NS2, and other NS) $P < 0.001$,

[**,] significant difference between NS2 and serotypeable strains and other NS $P < 0.001$,

[‡,] significant difference between NS2 and serotype f $P < 0.05$,

[†,] significant difference of NS1, NS2 and other NS compared to serotypeable strains $P < 0.001$, $P < 0.001$, $P < 0.05$, respectively,

[¶,] significant difference between NS1and others (serotypeable and other NS) $P < 0.001$

4. DISCUSSION

This study was carried out with the aim to investigate the prevalence and relationship of *A. actinomycetemcomitans* serotypes with chronic periodontitis patients in Thailand and its virulence factor such as *cdtBC* genes, leukotoxin expression and its activity, internalization, amd cytotoxicity in fibroblasts. The main finding are that there was a high prevalence (84.1%) of *A. actinomycetemcomitans* was found in deep pocket samples that was significantly higher than the prevalence in shallow pockets (11.0%), and that there was a similar prevalence of non-serotypable strains (54.5%) and serotypable strains (54.5%) among the patients. Serotypes a and c were the most prevalent serotypes (18.2 and 15.9% respectively), while serotype b and d were not found. Two strains with a JP2- like deletion of 530 bp in the promoter region of the leukotoxin gene and two strains with a 886 bp insertion of the *ltx* promoter were found and identified as serotype c. The DGGE subtyping disclosed 16 subtypes among the non-serotypable strains, among which two subtypes, NS1 and NS2, were more common than the others, and up to four different subtypes could be found in the same individual and in the same sample/pocket. This means that subtype NS1 and NS2 associated with chronic periodontitis and strains with deletion of 530 bp in *ltx* promoter did not belong to only serotype b. The virulence factors of 96 independent strains of *A. actinomycetemcomitans* were 84.4% of the *cdtBC* gene. A higher virulence was found in the two non-serotypeable DGGE subtypes, NS1 and NS2, than in the serotypeable strains (serotype a-f, serotypes b and d were absent). Furthermore, two strains with a JP2-like *ltx* gene with a deletion of 530 bp in the promoter region (serotype c) showed a magnitude of cytotoxicity similar to that of JP2 clone reference strains (serotype b). The strains obtained from deep periodontal pockets had generally higher virulence than those isolated from shallow pockets.

The proportion of the population that harbors *A. actionomycetemcomitans* differs dramatically between various geographical areas and different clinical presentations of periodontitis.[47] Most studies have focused on aggressive forms of periodontitis in juveniles, while only a few studies reported on periodontitis in adults. Similarly, available prevalence data of *A. actionomycetemcomitans* are comparatively few from Asian populations compared with data from Caucasians, Hispanics or Afro-americans.[112] The prevalence of *A. actionomycetemcomitans* in general adult Asian populations was low in Japanese and Koreans

(19.5% and 22.2%).[15,17] However, in some countries such as Indonesia, China, and Thailand the prevalence of *A. actionomycetemcomitans* is quite high (44.5%, 63.0%, and 88.0-92.7%, respectively).[13,45,58,60] This may depend on the periodontitis and socioeconomic status of the population. The present study showed a similar high prevalence (84.1%) of *A. actionomycetemcomitans* in Thai chronic periodontitis cases. A higher prevalence is thus expected from diseased cases compared to healthy subjects as also has been reported, and a higher prevalence has also been found in deep pockets compared to healthy shallow pockets in several studies.[4, 46, 116] This is supported by the present study showing 84.1% prevalence in deep pockets compared to 11% prevalence in shallow healthy pockets. It should be emphasized that the prevalence of *A. actionomycetemcomitans* is highly dependent on the population characteristic and the number of samples used for analysis. An increasing number of samples will certainly increase the prevalence in accordance with Meng et al. (2009), who collected 2 sites with the deepest pockets, which showed significantly higher prevalence of *A. actinomycetemcomitans* than that of healthy sites.[116] In fact, in the present study we sampled 2-3 deep sites in each case, which increased the prevalence from 37 to 42 out of the 44 subjects (unpublished observations). It needs to be pointed out that we, for statistical and computational reasons, randomly selected only one single deep pocket to be included in this study. Another strategy could have been to use pooled samples, which also would have increased the prevalence on the subject level, although at the cost of limiting the calculation of site prevalence.[117] It is tempting to argue, similarly to Dahlen et al. (2002), that if the number of samples increased, we would have found 100% prevalence leading to the conclusion that Thai adults are generally colonized with *A. actionomycetemcomitans* and that these bacterium is members of the resident oral microbiota.[13]

A critical step in prevalence studies is the detection method of choice. Culture on selective media, the most commonly used strategy, is reasonably sensitive and has the advantage of strain isolation for further genetic and phenotypic subtyping. In this study, we selected up to five colonies from each sample with the purpose of isolating different subtypes; altogether 96 independent strains were isolated. While culture-independent methods, such as PCR, real-time PCR, etc, give a high prevalence of *A. actinomycetemcomitans*,[46] nevertheless, they are problems for studying bacterial phenotypic characteristics such as sugar fermentation, immune response, etc.

The diversity of *A. actionomycetemcomitans* was investigated using serotyping and DGGE subtyping. Traditionally, serotyping used an immunodiffusion assay, later serotype specific primers were identified. Nowadays, serotyping is carried out with PCR methodology.[4-6] Thus 6 serotypes (a-f) can be identified, with a, b, and c, being found to be the most common. [5, 6, 13, 60, 62, 118] A seventh serotype (serotype g) has been proposed; however, the non-serotype was still present in some populations.[7,17] It has previously been suggested that serotype c is more common in the Asian population, while serotype b is detected more frequently in Caucasian or African populations. [12, 13, 15, 17, 60, 118,119] Both serotypes have been associated with periodontitis, especially aggressive and chronic periodontitis.[2,4, 8, 9,13,58] The highly toxic genotype (JP2) was found in children of North and West Africa[9-11]; however, this strain can also be found in adults (33 and 62 years) in Sweden. [9, 120] All of them are serotyped as serotype b. However, serotype b was not found in the present study, supporting the observation that serotype b is not a very common serotype in the Asian population. The absence of serotype b may be due to the small sample size (44 periodontitis cases), which is too small to find uncommon bacterial subtypes in a particular population. Also, it may be due to other factors such as patient age, disease severity and an Asian subpopulation such as adults of southern Thailand.

It was surprising to find two JP2-like strains (strains with 530 bp deletion on the *ltx* promoter gene) from Thai chronic periodontitis that were serotyped as c. The JP2 strain has not been found in an Asian population and this strain has only been reported in African populations and were serotyped as b.[4,8,9] The rate of non-serotypeable *A. actionomycetemcomitans* strains in chronic periodontitis cases in this study was remarkable (54.5%) being as common as the serotypeable strains (54.5%). Non-serotypeable strains have been previously found in Asian populations (8.9-14.7%)[17, 60, 61] and seem to be more common than in American and African populations (1.2-9.5%).[16, 18, 63]

The high rate of non-serotypeable strains led us to use some other subtyping methods such as DGGE. The DGGE method has been recommended to use for studying the competitive ability of *A.actinomycetemcomitans* strains. Although there has been only one report on the use of DGGE for subtyping between serotype b and c,[18] DGGE disclosed 16 subtypes among the non-serotypeable strains and showed much higher subtype diversity than that found for serotypeable strains. The findings from non-serotype groups in this study indicate that new serotypes beyond serotypes a-f may be possible as serotype g is the newest serotype

that has been found in 2014.[7] However, to further analyze other serotypes was beyond the scope of this investigation. The non-serotypeable strains were significantly ($P < 0.05$) more common among the strains isolated from the deep pockets than those from the shallow pockets, which indicates an association between some DGGE subtypes, deep pockets and periodontitis. Two subtypes (NS1 and NS2) were more common, but whether they are more related to periodontitis or are more toxic than other strains is a matter for further study. The subtype diversity among the isolates needs explanation in view of the fact that up to four different subtypes could be found in the same sample – indicating that the diversity is much more illustrated when using culture methodology and DGGE subtyping. This diversity may be population related but it could also be due to a change in disease pattern and/or age of the individuals. It is suggested that the competitive process that governs those subtypes that are best fitted to the changing environment in slowly progressing sites is when the individual grows older and that the non-serotypeable strains have such abilities allowing them to colonize and increase in deep periodontal pockets of adult patients with chronic periodontitis.

Also, the present study focused on the relation of serotypes/subtypes of *A. actinomycetemcomitans* and their virulence. Variation in the virulence factors, such as leukotoxin expression and its activity, internalization, cytotoxicity to fibroblasts, and *cdtBC* genes, among *A. actinomycetemcomitans* strains was found and this may play a role in the pathogenesis of periodontal diseases.

This investigation showed a high prevalence of *cdtBC* genes (84.4 %) in contrast to the lower prevalence (27%) that was observed in Thai patients by Bandhaya et al.[12] This is explained by the fact that *A. actinomycetemcomitan*s may be positive for the *cdtBC,* while still being *cdtABC* negative.[121] It is possible that mutation had occurred in the *cdtA* gene, which results in unsuccessful annealing of the primers. It may also be due to different methods leading to discrepancies in detection frequency of *cdtABC* genes in *A. actinomycetemcomitans*.[12,13,17,60] Also, the difference of the sample (healthy or diseased sites) may affect the detection rate of the *cdt* gene. However, the distribution of *cdt* genes among the analyzed isolates did not show any serotype-dependent pattern and it was not possible to demonstrate a specific pattern for *cdt* genes among the *A. actinomycetemcomitans* subtypes in the present study.

Leukotoxin of *A. actinomycetemcomitans* is known as a crucial virulence factor, especially the highly leukotoxin producing JP2 clone, but the leukotoxin expression has

not yet been studied among the Asian population. The *ltx* gene was found in all *A. actinomycetemcomitans* strains in this study; however, leukotoxin expression varied among different strains. It was found that a high leukotoxin expression was associated with a deletion of 530 bp in the *ltx* promoter gene. [4,8,9] Additionally, we examined leukotoxin expression and its activity to indicate that leukotoxin produced from *A. actinomycetemcomitans* strains could be synthesized and secreted to kill target cells (THP-1, HL-60).[14,27] Our results confirmed that the leukotoxin gene with 530-bp deletion enhanced leukotoxin expression approximately 17-fold compared to the normal *ltx* gene, resulting in higher leukotoxin activity by killing more HL-60 cells in this study.

It has been previously reported that the highly leukotoxic strains (JP2 clone) usually belong to serotype b,[11, 14, 21, 22] thus it is believed that there is an association between serotype b and aggressive periodontitis.[21] Our study has shown that such a claim is not always true because the strains with a deletion of 530 bp found in this study were identified as serotype c. In addition, serotype b was not detected in this study although the periodontal severities among the patients with chronic periodontitis in this study were relatively obvious. Both JP2-like strains found in this study originated from deep periodontal pockets (twelve mm) from two different patients with severe periodontitis.

Strains with inserted sequence elements were found among isolated *A. actinomycetemcomitans* strains in this study. He et al.[14] demonstrated that highly toxic strains of *A. actinomycetemcomitans* contained a novel insert sequence element. However, such an insertion did not seem to influence leukotoxin expression in this study (data not shown).

The factors that influence the expression of various toxins in bacterial cells of subgingival biofilm *in vivo* in the presence of inflammation and complex polymicrobial microbiota are matters of conjecture. Most strains, including the non-serotypeable ones, isolated from deep periodontal pockets showed a higher virulence compared with the virulence of those that originated from shallow pockets. It is tempting to argue that the higher leukotoxin expression in the non-serotypeable and the NS1 and NS2 subtypes in particular (although they were not in the magnitude of the genetically based highly toxic JP2 clone) indicates a higher leukotoxin expression of these subtypes. On the other hand, the expression of virulence factors and the toxicity are probably due to an environmentally driven adaptation to the pathological periodontal pocket. We have limited knowledge of how the genes in detail are regulated and

about the role of environmental conditions for leukotoxin expression *in vivo*. The results of the present study should primarily be regarded as showing the virulence capacity and the comparative toxicity between strains under similar conditions *in vitro*.

The internalization ability of *A. actinomycetemcomitans* has been considered an important factor in the disease process because *A. actinomycetemcomitans* can escape from host defense and antibiotics by internalization into host cells such as epithelial cells and fibroblasts.[21,23,25] The present study showed highly significant internalization of non-serotypeable strains, particularly NS1 and NS2. It is likely that this is part of the subtype selection that takes place in the periodontal pocket due to a more competitive survival ability of these subtypes. Also, NS1 and NS2 strains showed a significantly higher degree of cytotoxicity to fibroblasts, which may be a consequence of their internalization in cell lines. It was reported that cytotoxicity to fibroblasts after exposure to *A. actinomycetemcomitans* followed inhibition of DNA synthesis, decreased RNA synthesis, and a decrease in fibroblast cell growth.[26]

The fact that multiple subtypes are commonly isolated from a patient or even from one pocket warrants comment. It is commonly found that the resident flora, as the age of the host increases, is successively colonized by new genotypes of the same bacterial species, climaxing with a community of multiple subtypes.[122,123] Several genotypes of the same species increase the survival rate. At the same time, there are competition and internal control between the subtypes favoring those that have certain abilities to adapt in a dynamic and changing environment. The production of leukotoxin and the ability to internalize host cells are ways to escape the host defense as discussed above. It is therefore tempting to speculate whether the presence of toxic subtypes such as NS1 and NS2 are due to a competitive process between genotypes of the same species favoring those with a higher toxicity or due to a significantly higher ability for cytotoxicity and internalization than other *A. actinomycetemcomitans* subtypes. Such abilities of NS1 and NS2 strains may express more in chronic periodontitis in adults.

5. CONCLUSIONS

The present study was performed to characterize oral *A. actinomycetemcomitans* serotypes/subtypes in relation to periodontitis status in Thai chronic periodontitis. There was a strong relationship between deep pockets and the *A. actinomycetemcomitans* level. The level of *A. actinomycetemcomitans* in deep pockets was significantly higher than that in shallow pockets.

The assessment of chronic periodontitis *A. actinomycetemcomitans* was achieved in this thesis using a molecular method and inclusion of numerous periodontitis patients. Furthermore, the classifications of *A. actinomycetemcomitans* serotypes/subtypes were examined. The serotypes/subtypes frequency analysis of *A. actinomycetemcomitans* was performed on 96 strains from 37 subjects. Five serotypes and nineteen subtypes were detected: non-serotypeable (54.4% of subjects) strains were the most frequent followed by serotype a (18.2%), serotype c (15.9%), serotype e (9.1%), and serotype f (11.4%). Serotype b and serotype d were not detected in this study. Furthermore, this is the first time that isolates with a 530 bp deletion or 886 bp insertion of the *ltx* promoter have been described in an Asian population. Some unique strains were found, of which two strains had a 530 bp deletion in the *ltx* promoter similar to the previously demonstrated JP2 genotype but were identified as serotype c. Non-serotypeable subtype NS1 and NS2 (12.7 and 10.1%) showed a higher detection rate than other subtypes. An association between non-serotypeable *A. actinomycetemcomitans*, especially subtype NS1 and NS2, and chronic periodontitis in Thai adult was found.

By clonal analysis, a genetic heterogeneity among subjects was found and up to four different clonal serotypes/subtypes of *A. actinomycetemcomitans* were detected in a single deep pocket. The genetic heterogeneity of serotypes/subtypes of *A. actinomycetemcomitans* found in deep pockets suggests that changing disease patterns or age of the individual or competition process or changing environment may promote various *A. actinomycetemcomitans* serotypes/subtypes to colonize and persist in the subgingival tissue.

Virulence factors, including *cdtBC* genes, leukotoxin expression, leukotoxin activity, internalization, and cytotoxicity in fibroblasts were determined. Eighty-four percent of *A. actinomycetemcomitans* strains presented *cdtBC* genes. No association was found between *A.*

actinomycetemcomitans serotypes/subtypes and *cdtBC* genes. Leukotoxin expression varied among the different of *A. actinomycetemcomitans* strains. Highly virulence was found in two strains with a JP2-like 530 bp deletion of the promoter gene of the *ltx* gene but were serotyped as c. The non-serotypeable subtype NS1 and NS2 showed significantly higher leukotoxin expression, leukotoxin activity, cytotoxicity to fibroblasts, and internalization than serotypeable and other NS. JP2-like, NS1, and NS2 showed high virulence. Thus the severity of periodontitis may be related with these strains. The ability of virulence factors of *A. actinomycetemcomitans* was higher in strains from deep periodontal pockets than from shallow pockets. *A. actinomycetemcomitans* serotypes/subtypes isolated from Thai adults with chronic periodontitis showed a highly variable virulence, leukotoxin expression, leukotoxin activity, internalization, and cytotoxicity to fibroblasts, and are regulated both genetically and environmentally.

More information about the pattern and virulence of *A. actinomycetemcomitans* and their relationship to chronic periodontitis in Thai adults could help increase our understanding about the roles of these microorganisms in periodontitis development and lead to a definition of a risk group of bacteria. The finding of marked virulence strains of *A. actinomycetemcomitans* (JP2-like, NS1, and NS2) present in severe chronic periodontitis reported here raises the possibility that these strains may be related to severe chronic periodontitis.

REFERENCES

1. Mı´nguez M, Pousa X, Herrera D, Blasi A, Sa´nchez MC, Leo´n R, Sanz M. Characterization and serotype distribution of *Aggregatibacter actinomycetemcomitans* isolated from a population of periodontitis patients in Spain. ***Arch Oral Biol*** 2014; 59: 1359-67
2. Mombelli A, Casagni F, Madianos P. Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? A systematic review. ***J Clin Periodontol*** 2002; 29: 10-21.
3. van Winkelhoff A, Slots J. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in nonoral infections. ***Periodontol 2000*** 1999; 20: 122-35.
4. Asikainen S, Lai C, Alaluusua S, Slot J. Distribution of *Actinobacillus ctinomycetemcomitans* serotypes in periodontal health and disease. ***Oral Microbiol Immunol*** 1991; 6: 115-8.
5. Kaplan J, Perry M, Maclean L, Furgang D, Wilson M, Fine D. Structural and Genetic Analyses of O Polysaccharide from *Actinobacillus actinomycetemcomitans* Serotype f. ***Infect Immun*** 2001; 69: 5375–84
6. Suzuki N, Nakano Y, Yoshida Y, Ikeda D, Koga T. Identification of *Actinobacillus actinomycetemcomitans* serotypes by multiplex PCR. ***J Clin Microbiol*** 2001; 39: 2002-5.
7. Tsuzukibashi O, Saito M, Kobayashi T, Koji U, Fumio N, Nagahama F, Hiroi T, Hirasawa M, Takada K. A gene cluster for the synthesis of serotype g-specific polysaccharide antigen in *Aggregatibacter actinomycetemcomitans*. ***Arch Microbiol*** 2014; 196: 261-5.
8. Zambon JJ, Slots J, Genco RJ. Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. ***Infect Immun*** 1983; 41: 19-27.
9. Haubek D, Dirienzo JM, Tinoco EM, Westergaard J, Lopez NJ, Chung CP, Poulsen K, Kilian M. Racial tropism of a highly toxic clone of *Actinobacillus actinomycetemcomitans* associated with juvenile periodontitis. ***J Clin Microbiol*** 1997; 35: 3037-42.
10. Haubek D. The highly leukotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans*: evolutionary aspects, epidemiology and etiological role in aggressive periodontitis. ***APMIS*** 2010; 118: 1-53.

11. Haubek D, Poulsen K, Westergaard J, Dahlen G, Kilian M. Highly toxic clone of *Actinobacillus actinomycetemcomitans* in geographically widespread cases of juvenile periodontitis in adolescents of African origin. ***J Clin Microbiol*** 1996; 34: 1576-8.

12. Bandhaya P, Saraithong P, Likittanasombat K, Hengprasith B, Torrungruang K. *Aggregatibacter actinomycetemcomitans* serotypes, the JP2 clone and cytolethal distending toxin genes in a Thai population. ***J Clin Periodontol*** 2012; 39: 519-25.

13. Dahlen G, Widar F, Teanpaisan R, Papapanou PN, Baelum V, Fejerskov O. *Actinobacillus actinomycetemcomitans* in a rural adult population in southern Thailand. ***Oral Microbiol Immunol*** 2002; 17: 137-42.

14. He T, Nishihara T, Demuth DR, Ishikawa I. A novel insertion sequence increases the expression of leukotoxicity in *Actinobacillus actinomycetemcomitans* clinical isolates. ***J Periodontol*** 1999; 70: 1261-8.

15. Kim TS, Frank P, Eickholz P, Eick S, Kim CK. Serotypes of *Aggregatibacter actinomycetemcomitans* in patients with different ethnic backgrounds. ***J Periodontol*** 2009; 80: 2020-7.

16. Chen C, Wang T, Chen W. Occurrence of *Aggregatibacter actinomycetemcomitans* serotypes in subgingival plaque from United States subjects. ***Mol Oral Microbiol*** 2010; 25: 207-14.

17. Yoshida Y, Suzuki N, Nakano Y, Shibuyu K, Ogawa Y, Koga T. Distribution of *Actinobacillus actinomycetemcomitans* serotypes and *Porphyromonas gingivalis* in Japanese adults. ***Oral Microbiol Immunol*** 2003; 18: 135-9.

18. Ihalin R, Asikainen S. 16S rDNA PCR-denaturing gradient gel electrophoresis in determining proportions of coexisting *Actinobacillus actinomycetemcomitans* strains. ***J Microbiol Meth*** 2006; 65: 417-24.

19. Ledder RG, Gilbert P, Huws SA, Aarons L, Ashley MP, Hull PS, McBain AJ. Molecular analysis of the subgingival microbiota in health and disease. ***Appl Environ Microbiol*** 2007; 73: 516-23.

20. Zijnge V, Welling G, Degener J, van Winkelhoff A, Abbas F, Harmsen H. Denaturing gradient gel electrophoresis as a diagnostic tool in periodontal microbiology. ***J Clin Microbiol*** 2006; 44: 3628-33.

21. Haubek D, Johansson A. Pathogenicity of the highly leukotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans* and its geographic dissemination and role in aggressive periodontitis. ***J Oral Microbiol*** 2014; 6: 1-22.

22. Brogan J, Lally E, Poulsen K, Kilian M, Demuth D. Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. ***Infect Dis*** 1994; 62: 501-8.

23. Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C. Virulence factors of *Actinobacillus actinomycetemcomitans*. ***Periodontol 2000*** 1999; 20: 136-67.

24. Henderson B, Ward J, Ready D. *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*: a triple A* periodontopathogen? ***Periodontol 2000*** 2010; 54: 78-105.

25. Meyer DH, Sreenivasan PK, Fives-Taylor PM. Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*. ***Infect Immun*** 1991; 59: 2719-26.

26. Shenker BJ, Kushner ME, Tsai CC. Inhibition of Fibroblast Proliferation by *Actinobacillus actinomycetemcomitans*. ***Infect Immun*** 1982; 38: 986-92.

27. Åberg C, Haubek D, Kwamin F, Johansson A, Claesson R. Leukotoxin activity of *Aggregatibacter actinomycetemcomitans* and periodontal attachment loss. ***Plos One*** 2014; 9: 1-11.

28. Lally ET, Golub EE, Kieba IR, Taichman NS, Rosenbloom J, Rosenbloom JC, Gibson CW, Demuth DR. Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Delineation of unique features and comparison to homologous toxins. ***J Biol Chem*** 1989; 264: 15451-6.

29. Claesson R, Gudmundson J, Aberg C, Haubek D, Johansson A. Detection of a 640-bp deletion in the *Aggregatibacter actinomycetemcomitans* leukotoxin promoter region in isolates from an adolescent of Ethiopian origin. ***J Oral Microbiol*** 2015; 7: 1-4.

30. Haffajee AD, Dibart S, Kent RL, Jr., Socransky SS. Clinical and microbiological changes associated with the use of 4 adjunctive systemically administered agents in the treatment of periodontal infections. ***J Clin Periodontol*** 1995; 22: 618-27.

31. Moore WE, Moore LV. The bacteria of periodontal diseases. ***Periodontol 2000*** 1994; 5: 66-77.

32. Slots J, Rams T. Microbiology of periodontai disease. In: SLOTS J TAUBM\N M *Coniemporary oral microbiology and imtmmology* St Louis MS: Mosby 1992: 425-43.

33. American Academy of Periodontology. Parameters of Care. ***J Periodontal*** 2000; 71: 847-83.

34. Armitage GC. Development of a classification system for periodontal diseases and conditions. ***Ann Periodontol*** 1999; 4: 1-6.

35. Bascones Martínez A, Figuero Ruiz E. Periodontal diseases as bacterial infection. ***Av Periodon Implantol*** 2005; 17: 111-8.

36. Slots J. Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. ***J Clin Microbiol*** 1982: 606-11.

37. Holtta P, Alaluusua S, Saarela M, Asikainen S. Isolation frequency and serotype distribution of mutans streptococci and *Actinobacillus actinomycetemcomitans*, and clinical periodontal status in Finnish and Vietnamese children. ***Scand J Dent Res*** 1994; 102: 113-9.

38. Pahumunto N, Ruangsri P, Wongsuwanlert M, Piwat S, Dahlen G, Teanpaisan R. *Aggregatibacter actinomycetemcomitans* serotypes and DGGE Subtypes in Thai Adult Chronic Periodontitis. ***Arch Oral Biol*** 2015; 60: 1789-96.

39. Flemmig TF, Rudiger S, Hofmann U, Schmidt H, Plaschke B, Stratz A, Klaiber B, Karch H. Identification of *Actinobacillus actinomycetemcomitans* in subgingival plaque by PCR. ***J Clin Microbiol*** 1995; 33: 3102-5.

40. Tonjum T, Haas R. Identification of *Actinobacillus actinomycetemcomitans* by leukotoxin gene-specific hybridization and polymerase chain reaction assays. ***J Clin Microbiol*** 1993; 31: 1856-9.

41. Sakellari D, Katsikari A, Slini T, Ioannidis I, Konstantinidis A, Arsenakis M. Prevalence and distribution of *Aggregatibacter actinomycetemcomitans* serotypes and the JP2 clone in a Greek population. ***J Clin Periodontol*** 2011; 38: 108-14.

42. Roman-Torres CV, Aquino DR, Cortelli SC, Franco GC, Dos Santos JG, Corraini P, Holzhausen M, Diniz MG, Gomez RS, Cortelli JR. Prevalence and distribution of serotype-specific genotypes of *Aggregatibacter actinomycetemcomitans* in chronic periodontitis Brazilian subjects. ***Arch Oral Biol*** 2010; 55: 242-8.

43. Avila-Campos MJ, Sacchi CT, Whitney AM, Steigerwalt AG, Mayer LW. Specific primer for AP-PCR identification of *Actinobacillus actinomycetemcomitans*. ***J Clin Periodontol*** 1999; 26: 699-704.

44. Dogan B, Saarela MH, Jousimies-Somer H, Alaluusua S, Asikainen S. *Actinobacillus actinomycetemcomitans* serotype e--biotypes, genetic diversity and distribution in relation to periodontal status. ***Oral Microbiol Immunol*** 1999; 14: 98-103.

45. Walter J, Yao G, Stefanie L, Zhou C, Ralph V, Germain J, Li Y. PCR detection of *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* in dental plaque samples from Haitian adolescents. ***Clin Oral Investig*** 2010.

46. Papapanou P, Teanpaisan R, Obiechina N, Pithpornchaiyakul W, Pongpaisal S, Pisuithanakan S, Baelum V, Fejerskov O, Dahlen G. Periodontal microbiota and clinical periodontal status in a rural sample in southern Thailand. ***Eur J Oral Sci*** 2002; 110: 345-52.

47. Rylev M, Kilian M. Prevalence and distribution of principal periodontal pathogens worldwide. ***J Clin Periodontol*** 2008; 35: 346-61.

48. Rodenburg JP, van Winkelhoff AJ, Winkel EG, Goene RJ, Abbas F, de Graff J. Occurrence of *Bacteroides gingivalis, Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in severe periodontitis in relation to age and treatment history. ***J Clin Periodontol*** 1990; 17: 392-9.

49. Slots J, Evans R, Lobbins P, Genco R. In Vitro Antimicrobial Susceptibility of *Actinobacillus actinomycetemcomitans*. ***Antimicrob Agents Chemother*** 1980; 18: 9-12.

50. Slots J, Ting M. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. ***Periodontol 2000*** 1999; 20: 82-121.

51. Schacher B, Baron F, Rossberg M, Wohlfeil M, Arndt R, Eickholz P. *Aggregatibacter actinomycetemcomitans* as indicator for aggressive periodontitis by two analysing strategies. ***J Clin Periodontol*** 2007; 34: 566-73.

52. Slots J. The predominant cultivable organisms in juvenile periodontitis. ***Scand J Dent Res*** 1976; 84: 1-10.

53. Loe H, Brown L. Early onset periodontitis in the United States of America. ***J Periodontol*** 1991; 62: 608-16.

54. Wagaiyu EG, Wagaiyu CK. Prevalence of juvenile periodontitis in national youth service trainees. ***East Afr Med J*** 1992; 69: 31-3.

55. Mandell R. A longitudinal microbiological investigation of *Actinobacillus actinomyce-temcomitans* and *Eikenella corrodens* in juvenile periodontitis. ***Infect Immun*** 1984; 45: 778-80.

56. Teixeira RE, Mendes EN, Roque de Carvalho MA, Nicoli JR, Farias Lde M, Magalhaes PP. *Actinobacillus actinomycetemcomitans* serotype-specific genotypes and periodontal status in Brazilian subjects. ***Can J Microbiol*** 2006; 52: 182-8.

57. Tinoco EM, Sivakumar M, Preus HR. The distribution and transmission of *Actinobacillus actinomycetemcomitans* in families with localized juvenile periodontitis. ***J Clin Periodontol*** 1998; 25: 99-105.

58. van der Reijden WA, Bosch-Tijhof CJ, van der Velden U, van Winkelhoff AJ. Java project on periodontal diseases: serotype distribution of *Aggregatibacter actinomycetemcomitans* and serotype dynamics over an 8-year period. ***J Clin Periodontol*** 2008; 35: 487-92.

59. Yang HW, Huang YF, Chan Y, Chou MY. Relationship of *Actinobacillus actinomycetemcomitans* serotypes to periodontal condition: prevalence and proportions in subgingival plaque. ***Eur J Oral Sci*** 2005; 113: 28-33.

60. Mombelli A, Gmur R, Lang NP, Corbert E, Frey J. *Actinobacillus actinomycetemcomitans* in Chinese adults. Serotype distribution and analysis of the leukotoxin gene promoter locus. ***J Clin Periodontol*** 1999; 26: 505-10.

61. Yamamoto M, Nishihara T, Koseki T, He T, Yamato K, Zhang YJ, Nakashima K, Oda S, Ishikawa I. Prevalence of *Actinobacillus actinomycetemcomitans* serotypes in Japanese patients with periodontitis. ***J Periodont Res*** 1997; 32: 676-81.

62. Saarela M, Asikainen S, Alaluusua S, Pyhala L, Lai CH, Jousimies-Somer H. Frequency and stability of mono- or poly-infection by *Actinobacillus actinomycetemcomitans* serotypes a, b, c, d or e. ***Oral Microbiol Immunol*** 1992; 7: 277-9.

63. Fine DH, Markowitz K, Furgang D, Fairlie K, Ferrandiz J, Nasri C, McKiernan M, Gunsolley J. *Aggregatibacter actinomycetemcomitans* and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. ***J Clin Microbiol*** 2007; 45: 3859-69.

64. Ebersole JL, Taubman MA, Smith DJ, Genco RJ, Frey DE. Human immune responses to oral micro-organisms. I. Association of localized juvenile periodontitis (LJP) with serum antibody responses to *Actinobacillus actinomycetemcomitans*. ***Clin Exp Immunol*** 1982; 47: 43-52.

65. Genco R, Taichma, Sadowski C. Precipitating antibodies to *Actinobacillus actinomycetem-comitans* in localized juvenile periodontitis. ***J Dent Res*** 1980; 59: 329.

66. Sugai M, Kawamoto Y, Peres S, Ueno Y, Komatsuzawa H, Fujiwara T, Kurihara H, Suginaka H, Oswald E. The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. ***Infect Immun*** 1998; 66: 5008-19.

67. Narayanan SK, Nagaraja TG, Chengappa MM, Stewart GC. Leukotoxins of gram-negative bacteria. ***Vet Microbiol*** 2002; 84: 337-56.

68. DiRienzo J, McKay T. Identification and characterization of genetic cluster groups of *Actinobacillus actinomycetemcomitans* isolated from the human oral cavity. ***J Clin Microbiol Rev*** 1994; 32: 75-81.

69. Poulsen K, Ennibi OK, Haubek D. Improved PCR for detection of the highly leukotoxic JP2 clone of *Actinobacillus actinomycetemcomitans* in subgingival plaque samples. ***J Clin Microbiol*** 2003; 41: 4829-32.

70. Lally E, Hill R, Kieba I, Korostoff J, . The interaction between RTX toxins and target cells. ***Trends Microbio*** 1999; 7: 356-61.

71. Eick S, Pfister W, Sigusch B, Straube E. Phagocytosis of periodontopathogenic bacteria by crevicular granulocytes is depressed in progressive periodontitis. ***Infection*** 2000; 28: 301-4.

72. Korostoff J, Wang JF, Kieba I, Miller M, Shenker BJ, Lally ET. *Actinobacillus actinomycetemcomitans* Leukotoxin Induces Apoptosis in HL-60 Cells. ***Infect Immun*** 1998; 66: 4474-83.

73. Iwase M, Lally ET, Berthold P, Korchak HM, Taichman NS. Effects of cations and osmotic protectants on cytolytic activity of *Actinobacillus actinomycetemcomitans* leukotoxin. ***Infect Immun*** 1990; 58: 1782-8.

74. Lally E, Golub E, Kieba T, Taichman N, Decker S, Berthold P, Gibson C, Demuth D, Rosenbloom J. Structure and function of the ltxB and ltxD genes of the *Actinobacillus actinomycetemcomitans* leukotoxin complex. ***Microbiol Pathog*** 1991; 11: 111-21.

75. Roger D, Lourdes A, Jigna P, Nataliya V, Anna C, Irene L, Scott C. Characterization of leukotoxin from a clinical strain of *Actinobacillus actinomycetemcomitans*. ***Microbial Pathogenesis*** 2006; 40: 48-55.

76. Dileepan T, Kachlany SC, Balashova NV, Patel J, Maheswaran SK. Human CD18 is the functional receptor for *Aggregatibacter actinomycetemcomitans* leukotoxin. ***Infect Immun*** 2007; 75: 4851-6.

77. Kieba IR, Fong KP, Tang H-Y, Hoffman KE, Speicher DW, Klickstein LB, Lally ET. *Aggregatibacter actinomycetemcomitans* leukotoxin requires β-sheets 1 and 2 of the human CD11a β-propeller for cytotoxicity. ***Cell Microbiol*** 2007; 9: 2689–99.

78. Reinholdt J, Poulsen K, Brinkmann CR, Hoffmann SV, Stapulionis R, Enghild JJ, et al. Monodisperse and LPS-free *Aggregatibacter actinomycetemcomitans* leukotoxin: interactions with human β-2 integrins and erythrocytes. ***Biochim Biophys Acta*** 2013; 1834: 546-58.

79. Pahumunto N, Ruangsri P, Wongsuwanlert M, Piwat S, Dahlen G, Teanpaisan R. Virulence of *Aggregatibacter actinomycetemcomitans* Serotypes and DGGE Subtypes isolated from Chronic Adult Periodontitis in Thailand. ***Anaerobe*** 2015; 36: 60-4.

80. Haubek D, Ennibi OK, Poulsen K, Poulsen S, Benzarti N, Kilian M. Early-onset periodontitis in Morocco is associated with the highly leukotoxic clone of *Actinobacillus actinomycetemcomitans.* ***J Dent Res*** 2001; 80: 1580-3.

81. Haubek D, Ennibi OK, Vaeth M, Poulsen S, Poulsen K. Stability of the JP2 clone of *Aggregatibacter actinomycetemcomitans*. ***J Dent Res*** 2009; 88: 856-60.

82. Gjermo P, Bellini H, Pereira Santos V, Martins J, Ferracyoli J. revalence of bone loss in a group of Brazilian teenagers assessed on bite-wing radiographs. ***J Clin Periodontol*** 1984; 11: 104-13.

83. Henderson B, Wilson M, Sharp L, Ward JM. *Actinobacillus actinomycetemcomitans.* ***J Med Microbiol*** 2002; 51: 1013-20.

84. Aberg C, Antonoglou G, Haubek D, Kwamin F, Claesson R, Johansson A. Cytolethal Distending Toxin in Isolates of *Aggregatibacter actinomycetemcomitans* from Ghanaian Adolescents and Association with Serotype and Disease Progression. ***Plos One*** 2013; 8: 1-8.

85. Lara-Tejero M, Galan J. *CdtA, CdtB,* and *CdtC* form a tripartite complex that is required for cytolethal distending toxin activity. ***Infect Immun*** 2001; 69: 4358-65.

86. Elwell C, Dreyfus L. DNase I homologous residues in *CdtB* are critical for cytolethal distending toxin-mediated cell cycle arrest. ***Mol Microbiol*** 2000; 37: 952-63.

87. Ahmed HJ, Svensson LA, Cope LD, Latimer JL, Hansen EJ, Ahlman K, Bayat-Turk J, Klamer D, Lagergard T. Prevalence of *cdtABC* genes encoding cytolethal distending toxin among *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* strains. ***J Med Microbiol*** 2001; 50: 860-4.

88. Shenker BJ, Laura AV, Deborah AW. Immune suppression induced by *Actinobacillus actinomycetemcomitans*: effects on immunoglobulin production by human B cells. ***Infect Immun*** 1990; 58: 3856-62.

89. Ando ES, De-Gennaro LA, Faveri M, Feres M, DiRienzo JM, Mayer MP. Immune response to cytolethal distending toxin of *Aggregatibacter actinomycetemcomitans* in periodontitis patients. ***J Periodontal Res*** 2010; 45: 471-80.

90. Mintz KP, Fives-Taylor PM. Adhesion of *Actinobacillus actinomycetemcomitans* to a human oral cell line. ***Infect Immun*** 1994; 62: 3672-8.

91. Inoue T, Shingaki R, Sogawa N, Sogawa CA, Asaumi J, Kokeguchi S, Fukui K. Biofilm formation by a fimbriae-deficient mutant of *Actinobacillus actinomycetemcomitans*. ***Microbiol Immunol*** 2003; 47: 877-81.

92. Tang G, Kitten T, Munro CL, Wellman GC, Mintz KP. EmaA, a potential virulence determinant of *Aggregatibacter actinomycetemcomitans* in infective endocarditis. ***Infect Immun*** 2008; 76: 2316-24.

93. Finlay BB, Falkow S. Common themes in microbial pathogenicity. ***Microbiol Rev*** 1989; 53: 210-30.

94. Meyer D, Fives-Taylor P. Characteristics of adherence of *Actinobacillus actinomycetem-comitans* to epithelial cells. ***Infect Immun*** 1994; 62: 928-35.

95. Fujise O, Wang Y, Chen W, Chen C. Adherence of *Aggregatibacter actinomycetemcomitans* via serotype-specific polysaccharide antigens in lipopolysaccharides. ***Oral Microbiol Immunol*** 2008; 23: 226-33.

96. Thuruthyil SJ, Zhu H, Willcox MD. Serotype and adhesion of *Pseudomonas aeruginosa* isolated from contact lens wearers. ***Clin Experiment Ophthalmol*** 2001; 29: 147-9.

97. Marlene T, Ismailoi A, Philott D, Soni R, Brunton J, Sherman A. Distinct binding properties of eaeA-negative verocytotoxin-producing *Escherichia coli* of serotype O113:H21. ***Infect Immun*** 1994; 8: 3494-505.

98. Wilson M, McNab R, Henderson B. Bacterial Disease Mechanisms: An Introduction to Cellular Microbiology. Cambridge, UK: Cambridge University Press, 2002.

99. Colombo A, da Silva C, Haffajee A, Colombo A. Identification of intracellular oral species within human crevicular epithelial cells from subjects with chronic periodontitis by fluorescence in situ hybridization. ***J Periodontal Res*** 2007; 42: 236-43.

100. Rudney JD, Chen R, Sedgewick GJ. Intracellular *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in buccal epithelial cells collected from human subjects. ***Infect Immun*** 2001; 69: 2700-7.

101. Sreenivasan PK, Meyer DH, Fives-Taylor PM. Factors influencing the growth and viability of *Actinobacillus actinomycetemcomitans*. ***Oral Microbiol Immunol*** 1993; 8: 361-9.

102. Meyer D, Lippmann J, Fives-Taylor P. Invasion of Epithelial Cells by *Actinobacillus actinomycetemcomitans*: a Dynamic, Multistep Process. ***Infect Immun*** 1996; 64: 2988-97.

103. Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. ***Cell*** 2006; 124: 823-35.

104. Kato S, Kowashi Y, Demuth D. Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. ***Microb Pathog*** 2002; 32: 1-13.

105. Huang GT, Haake SK, Park NH. Gingival epithelial cells increase interleukin-8 secretion in response to *Actinobacillus actinomycetemcomitans* challenge. ***J Periodontol*** 1998; 69: 1105-10.

106. Jiang Y, Russell TR, Graves DT, Cheng H, Nong SH, Levitz SM. Monocyte chemoattractant protein 1 and interleukin-8 production in mononuclear cells stimulated by oral microorganisms. ***Infect Immun*** 1996; 64: 4450-5.

107.Sfakianakis A, Barr CH, Hreutzer D. Mechanisms of *Actinobacillus actinomycetem-comitans* induced expression of Interleukin-8 in gingival epithelial cells. ***J Periodontol*** 2001; 73: 1413-9.

108. Darveau RP, Belton CM, Reife RA, Lamont RJ. Local Chemokine Paralysis, a Novel Pathogenic Mechanism for *Porphyromonas gingivalis*. ***Infect Immun*** 1998; 66: 1660-5.

109. Kurita-Ochiai T, Ochiai K. Immunosuppressive factor from *Actinobacillus actinomycetem-comitans* down regulates cytokine production. ***Infect Immun*** 1996; 64: 50-4.

110. Shenker BJ, Vitale LA, Welham DA. Immune Suppression Induced by *Actinobacillus actinomycetemcomitans*: Effects on Immunoglobulin Production by Human B Cells. ***Infect Immun*** 1990; 58: 3856-62.

111. Helgeland K, Nordby O. Cell cycle-specific growth inhibitory effect on human gingival fibroblasts of a toxin isolated from the culture medium of *Actinobacillus actinomycetemcomitans*. ***J Periodont Res*** 1993; 28: 161-5.

112. Contreras A, Rusitanonta T, Chen C, Wagner WG, Michalowicz BS, Slots J. Frequency of 530-bp deletion in *Actinobacillus actinomycetemcomitans* leukotoxin promoter region. ***Oral Microbiol Immunol*** 2000; 15: 338-40.

113. Owen RJ, Borman P. A rapid biochemical method for purifying high molecular weight bacterial chromosomal DNA for restriction enzyme analysis. ***Nucleic Acids Res*** 1987; 15: 3631.

114. Tan KS, Song KP, Ong G. Cytolethal distending toxin of *Actinobacillus actinomycetemco-mitans.* Occurrence and association with periodontal disease. ***J Periodontal Res*** 2002; 37: 268-72.

115. Umeda J, Longo P, Simionato M, Mayer M. Differential transcription of virulence genes in *Aggregatibacter actinomycetemcomitans* serotypes. ***J Oral Microbiol*** 2013; 5: 1-8.

116. Meng S, Zhao L, Yang H, Wu Y, Ouyang Y. Prevalence of *Actinobacillus actino-mycetemcomitans* in Chinese chronic periodontitis patients and periodontally healthy adults. ***Quintessence Int*** 2009; 40: 53-60.

117. Psoter W, Ge Y, Russell S, Chen Z, Katz R, Jean-Charles G, Li Y. PCR detection of *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* in dental plaque samples from Haitian adolescents. ***Clin Oral Investig*** 2011; 15: 461-9.

118. Chung H, Chung C, Son S, Nisengard R. *Actinobacillus actinomycetemcomitans* serotypes and leukotoxicity in Korean localized juvenile periodontitis. ***J Periodontol*** 1989; 60: 506-11.

119. Leung WK, Ngai VK, Yau JY, Cheung BP, Tsang PW, Corbet EF. Characterization of *Actinobacillus actinomycetemcomitans* isolated from young Chinese aggressive periodontitis patients. ***J Periodontal Res*** 2005; 40: 258-68.

120. Claesson R, Lagervall M, Höglund-Aberg C, Johansson A, Haubek D. Detection of the highly leucotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans* in members of a Caucasian family living in Sweden. ***J Clin Periodontol*** 2011; 38: 115-21.

121. Kawamoto D, Ando ES, Longo PL, Nunes AC, Wikstrom M, Mayer MP. Genetic diversity and toxic activity of *Aggregatibacter actinomycetemcomitans* isolates. ***Oral Microbiol Immunol*** 2009; 24: 493-501.

122. Percival RS, Challacombe SJ, Marsh PD. Age-related microbiological changes in the salivary and plaque microflora of healthy adults. ***J Med Microbiol*** 1991; 35: 5-11.

123. Percival S. Ageing Theories, Diseases and Microorganisms. Microbiology and Aging: Humana Press, 2009: 1-13.

APPENDICES

APPENDIX A

Nuntiya Pahumunto, Praphansri Ruangsri, Mutita Wongsuwanlert, Supatcharin Piwat, Gunnar Dahlen and Rawee Teanpaisan. *Aggregatibacter actinomycetemcomitans* serotypes and DGGE subtypes in Thai adults with chronic periodontitis. ***Arch Oral Biol*** 2015; 60: 1789-96.

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Aggregatibacter actinomycetemcomitans serotypes and DGGE subtypes in Thai adults with chronic periodontitis

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ABSTRACT

Objective: To investigate the distribution of *Aggregatibacter actinomycetemcomitans* serotypes and DGGE subtypes among isolates from Thai chronic periodontitis patients.
Design: Forty-four adult Thai periodontitis patients were assessed by a full mouth recording for CAL, PPD, and BOP. Seventy-nine strains of *A. actinomycetemcomitans* were isolated from deep pockets on selective TSBV agar and 17 strains were isolated from shallow pockets. The strains were serotyped using PCR and subtyped using DGGE.
Results: The prevalence of *A. actinomycetemcomitans* was 84.1%. Non-serotypeable *A. actinomycetemcomitans* strains occurred equally frequent as serotypeable (54.5%); serotype a 18.2%, serotype c 15.9%, serotype e 9.1%, and serotype f 11.4%. Serotype b and d were not detected. A JP2 like strain but serotyped as c was isolated from two patients, and another two strains showed an 886 bp insertion on the *ltx* promoter of their *A. actinomycetemcomitans* isolates. DGGE typing disclosed 16 different subtypes among the non-serotypeable strains. Two of them (NS1 and NS2) were more common (12.7 and 10.1%) among the strains than the other 14 subtypes (<5.1%). Most patients showed only one subtype (32.4%) but 29.7% had 2 and 3 different subtypes while 8.1% revealed 4 subtypes in one and the same deep pocket.
Conclusion: This study showed a greater subtype diversity of *A. actinomycetemcomitans* predominated by non-serotypeable strains than previously reported in an adult Thai population. It was also revealed for the first time that isolates with a 530 bp deletion or 886 bp insertion of the *ltx* promoter were serotyped as serotype c.

1. Introduction

Aggregatibacter actinomycetemcomitans, a gram-negative, capnophilic coccobacillus, has been associated with several forms of periodontitis including aggressive and chronic periodontitis in juveniles and adults (Mínguez et al., 2014; Mombelli, Casagni, & Madianos, 2002). *A. actinomycetemcomitans* has been traditionally classified in at least 6 serotypes (a–f) based on a surface polysaccharide antigen and are disclosed phenotypically by an immunodiffusion assay or genotypically by PCR (Asikainen, Lai, Alaluusua, & Slot, 1991; Kaplan et al., 2001; Suzuki, Nakano, Yoshida, Ikeda, & Koga, 2001). It has been reported that there was an increased proportion of serotype b among patients with aggressive periodontitis (formerly juvenile periodontitis), whereas serotypes a and c have been associated with periodontal health (Zambon, Slots, & Genco, 1983). In addition, a highly virulent subtype of *A. actinomycetemcomitans* (the JP2 genotype) has been classified as serotype b (Haubek et al., 1997). This particular strain has been claimed for a racial tropism due to oral colonization exclusively in individuals with an African origin (Haubek et al., 1997; Haubek, 2010; Haubek, Poulsen, Westergaard, Dahlen, & Kilian, 1996). This particular JP2 clone has not yet been found in the Asian population (Bandhaya, Saraithong, Likittanasombat, Hengprasith, & Torrungruang, 2012; Dahlen et al., 2002; He, Nishihara, Demuth, & Ishikawa, 1999; Kim, Frank, Eickholz, Eick, & Kim, 2009). Not all isolates can be serotyped and non-serotypeable *A.*

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actinomycetemcomitans isolates have been previously reported in a frequency rate of approximately 1.2-11.4% (Chen, Wang, & Chen, 2010; Mínguez et al., 2014). A higher rate of non-serotypeable strains was reported to occur in Asian populations (14.7%) (Yoshida et al., 2003). Thus, it was hypothesized for the present study that we might face yet another example of racial tissue tropism involving non-serotypeable *A. actinomycetemcomitans* strains that call for another subtyping strategy of *A. actinomycetemcomitans* rather than the traditional serotyping. Denaturing Gradient Gel Electrophoresis (DGGE) has been used as a diagnostic tool in periodontal microbiology to detect putative periodontal pathogens including *A. actinomycetemcomitans* in dental plaque samples (Ihalin & Asikainen, 2006; Ledder et al., 2007; Zijnge et al., 2006). Genotyping using DGGE has been used to disclose various serotypes of *A. actinomycetemcomitans* and was found to detect only 3 out of the 6 serotypes, however, DGGE has not so far been used for subtyping non-serotypeable *A. actinomycetemcomitans* isolates.

The aim of this study was to investigate the prevalence and distribution of serotypes and DGGE subtypes of *A. actinomycetemcomitans* derived from deep and shallow pocket sites of chronic periodontitis cases in Thai adults.

2. Materials and methods

2.1. Study population

Forty-four subjects were consequentially recruited during 18 months (June 2012–December 2013) for this study from the patient recall list of the specialist Clinic for Periodontology, at the Faculty of Dentistry, Prince of Songkla University, Thailand. They were should all be diagnosed as having generalized moderate to severe chronic periodontitis according to the American Academy of Periodontology in 1999 (Armitage, 1999), and were then selected based on the following inclusion criteria: (i) ≤60 years of age with remaining teeth ≥15, (ii) showing clinical attachment level ≥4 mm on 30% of sites, (iii) showing pocket with a depth ≥5 mm and bleeding on probing, (iv) presenting of bone loss on radiograph, and (v) showing in good general medical health. Any patients with the following condition: (i) having systemic disease, (ii) taking any antibiotic medication within the previous six months, and (iii) receiving periodontal treatment or preventive prophylaxis within six months prior to this study were excluded.

The study was approved by the Ethics Committee of the Faculty of Dentistry at Prince of Songkla University, Thailand (EC550936P). All patients gave their informed consent and were offered dental treatment after the study. Patients were also registered for general health, age, gender and smoking.

2.2. Clinical examination

A full mouth recording of the patient's clinical attachment level (CAL), probing pocket depth (PPD) and bleeding on probing (BOP) was assessed. All three parameters were recorded at four sites per tooth; the mesio-buccal, mid buccal, disto-buccal and mid lingual aspect of all teeth except third molars. Probing pocket depth was measured to the nearest whole mm as the distance between the gingival margin and the bottom of the probeable pocket, and the clinical attachment level was measured as the distance in mm from the enamel-cement junction to the bottom of the pocket.

2.3. Subgingival plaque sampling

Two subgingival samples were taken from each patient randomly. The first sample was taken from one deep pocket site, which was defined as having clinical attachment loss (CAL ≥ 4 mm), a probing pocket depth (PPD ≥ 5 mm), and bleeding on probing (BOP). The second sample was also randomly taken from one shallow pocket sample, being a site with no CAL and BOP, and PPD ≤ 3 mm. All samples were obtained by introducing 2 sterile paper points subgingivally, which were kept in place for 15 s. Then they were transferred into a vial containing reduced transport medium (RTF), which was immediately transported to the laboratory.

2.4. Cultivation and identification

Samples were cultured on the tryptic soy serum bacitracin vancomycin agar (TSBV agar), a selective medium for *A. actinomycetemcomitans*, under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2) at 37 °C for 3–5 days (Slots, 1982). The initial identifying based on showing a typical *A. actinomycetemcomitans* morphology as star-shaped or small smooth, adherent and catalase-positive colonies, a micro-morphology being gram-negative coccobacilli. All isolates were further confirmed being *A. actinomycetemcomitans* as the presence of the leukotoxin gene (*ltx*) using the PCR with

Table 1
Primer sequence used in this study.

Name	Sequence (5′–3′)	Target	Product size (bp)	Reference
lx3	GCCGACACCAAAGACAAAGTCT	*ltx A-glyA* gene	686 (Deletion strain	Poulsen et al., 2003
ltx4	GCCCATAACCAAGCCACATAC		1216 (Normal strain)	
			2117 (Insertion strain)	
HDA1-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGGACTCCTACGGGAGGCAGC	V2–V3 (16s rRNA)	238	Ledder et al., 2007
HDA-2	AGT GTATTACCGCGGCTGCTGGCAC			
SA-F	GCAATGATGTATTGTCTTCTTTTGGA	Putative mannosyltransferase	428	Suzuki et al., 2001
SA-R	CTTCAGTTGAATGGGGATTGACTAAAAC			
SB-F	CGGAAATGGAATGCTTGC			
	CTGAGGAAGCCTAGCAAT	dTDP-4-keto-6-deoxy-D-glucose reductase	298	Suzuki et al., 2001
SC-F	AATGACTGCTGTCGGAGT			
SC-R	CGCTGAAGGTAATGTCAG	Putative acetyltransferase	559	Suzuki et al., 2001
SD-F	TTACCAGGTGTCTAGTCGGA	Putative mannosyltransferase	690	Suzuki et al., 2001
SD-R	GGCTCCTGACAACATTGGAT			
SE-F	CGTAAGCAGAAGAATAGTAAACGT	Unknown	211	Suzuki et al., 2001
SE-R	AATAACGATGGCACATCAGACTTT			
SF-F	CCTTTATCAATCCAGACAGC	4813–5044[b]	232	Kaplan et al., 2001
SF-R	ARAAYTTYTCWTCGGGAATG[a]			

[a] R = A or G; Y = C or T; W = A or T.
[b] GenBank accession no. AF213680.

leukotoxin gene (*ltx*) (Pouslen, Ennibi, & Haubek, 2003). The selected isolates were purified, and were kept at −80 °C for a further use in serotyping and subtyping. The following reference strains of *A. actinomycetemcomitans* were included: CCUG (Culture Collection, University of Göteborg, http://www.ccug.se/) strain 37004 serotype a, CCUG 37002 serotype b, CCUG 13227 serotype c, ATCC 33384 serotype c, CCUG 38565 serotype d, CCUG 37399 serotype e, OMGS 3816 serotype f (received from Sirkka Asikainen, Umeå, Sweden) as well as a P48:2 JP2 clone strain (received from Gunnar Dahlen, Gothenburg, Sweden).

2.5. Determination of the leukotoxin (ltx) gene

This study aimed to determine the *ltx* gene and *ltx* gene variants of *A. actinomycetemcomitans*. Primers used included a forward primer ltx3 and a reverse primer ltx4 (Table 1) (Poulsen et al., 2003). PCR mixture included 1 unit of *Taq* DNA polymerase, 10x PCR buffer, 600 μM dNTP, 2.5 mM $MgCl_2$, 5 pM of ltx3 and ltx4, and extracted DNA. The thermo cycling program was carried out at 94 °C for 5 min and followed by 30 cycles at 94 °C for 1 min, at 60 °C for 1 min, at 72 °C for 2 min, and a final extension at 72 °C for 8 min. A PCR product of 1216 bp and 2117 bp represented the complete promoter and the insertion promoter, respectively, while 686 bp was indicated as a deletion promoter, being a characteristic of the JP2 clone. A. actinomycetemcomitans ATCC 33384 (non-JP2) and P48:2 (JP2) were used as negative and positive controls, respectively. The PCR products were analyzed by 1.5% agarose gel electrophoresis and photographed under UV light. The molecular weights of the PCR products were compared with standard molecular weight markers (TriDye™ 1 kb DNA Ladder, Biolab, New England).

2.6. Serotyping using the specific primers in PCR

All DNA samples of *A. actinomycetemcomitans* isolates were prepared according to the method of Owen and Borman (1987). Serotyping using the specific primers of the PCR method was performed according to Kaplan et al. (2001) and Suzuki et al. (2001) and the sequences of the specific primers for each serotype are shown in Table 1 (Kaplan et al., 2001; Suzuki et al., 2001). PCR mixture consisted of 200 μM dNTP, a 10x PCR buffer with 2.5 mM $MgCl_2$, 1 unit of *Taq* polymerase, 5 pM of each primer, and 10 ng of DNA template. The PCR condition included initial denaturation at 94 °C for 30 s, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 54 °C for 45 s, with extension at 72 °C for 1 min, and a final extension at 72 °C for 8 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The gel was stained with 0.5 μg/mL ethidium bromide. The gel was photographed under UV light and the molecular weights of the PCR products were compared with standard molecular weight markers (TriDye™ 1 kb DNA Ladder, Biolab, New England).

2.7. Subtyping using denaturing gradient gel electrophoresis (DGGE)

Subtyping using DGGE, the V2-V3 region of the 16S rRNA gene was amplified with primers of HDA1-GC and HDA-2 (Table 1) according to the method of Ledder et al. (2007). The reaction mixture consisted of 1 unit of *Taq* DNA polymerase, a 10x PCR buffer with 1.5 mM $MgCl_2$, 5 pM of forward primer (HDA1-GC) and reverse primer (HDA-2), 200 μM dNTP, and 10 ng of DNA template. The PCR cycle was performed at 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 68 °C for 60 s. The final cycle was stationary at 68 °C for 7 min to be a chain elongation step.

PCR products (238 bp) were determined using a D-Code universal mutation detection system (Bio-Rad, Hemel Hempstead, UK) with 8% polyacrylamide gel (16 × 16 cm, 1 mm deep) that the gel was run with a final concentration of 1× TAE buffer (2 M Tris base, 2 M glacial acetic acid, 0.5 M EDTA pH 8.0). On this basis, a denaturation gradient for parallel DGGE analysis ranging from 35% to 60% (100% denaturant equals 7 M urea and 40% formamide) was selected for distinguishing the serotypes of *A. actinomycetemcomitans*. Reference serotypes of *A. actinomycetemcomitans* (a through f) were loaded into the gel to compare with clinical strains. Electrophoresis was carried out at 120 volts at 60 °C for approximately 6 h. The gel was stained with SYBR® safe stain for 30 min and was viewed under a UV light (Spectroline® UV Transilluminator Select™ Series). The PCR product of the clinical strains of *A. actinomycetemcomitans* isolates were compared with the reference serotypes. Images of the gels were analyzed using the BioNumerics version 6.0 (Applied Maths). The resulting patterns were compared to one another using the Dice similarity coefficient, and the matrix generated was clustered by the UPGMA method (Ultra-Violet Products Ltd., UK).

2.8. Statistical analysis

The frequency of *A. actinomycetemcomitans* serotypes between deep and shallow pocket sites was analyzed by Chi-square test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Subjects and clinical characteristics

A total number of 44 chronic periodontitis patients fulfilled the inclusion criteria. The mean age was 46.4 ± 9.5 years with a range of 24–60 years. They were all systemically healthy and had not taken any antibiotics during the last 6 months. Twenty subjects (45.5%) were males and 23.3% of all subjects smoked, while the mean percentage of sites with gingival bleeding was 72.3 ± 29.3%. The mean percentage of sites with CAL ≥ 4 mm and CAL ≥ 7 mm was 35.3 ± 21.6% and 18.0 ± 22.3%, respectively (Table 2), and the mean percentage of sites with PPD ≤ 3 mm, PPD ≥ 5 mm, and PPD ≥ 7 mm was 58.8 ± 30.2%, 19.3 ± 17.6%, and 8.3 ± 11.6%, respectively.

The deep pocket sampling sites exhibited a mean CAL of 8.8 ± 1.9 mm, PPD of 8.9 ± 2.0 mm and all showed bleeding on probing. The shallow pocket sampling sites had no CAL and no BOP, and PPD was 2.64 ± 0.65 mm.

3.2. Study of the leukotoxin (ltx) gene

Totally 184 *A. actinomycetemcomitans* isolates (145 from deep pockets and 39 from shallow pockets) were positive for the *ltx* promoter gene. Two strains (2 isolates) isolated from 2 different subjects exhibited the 530-bp deletion of the *ltx* promoter region (or JP2 clone like) (Fig. 1). These two patients, one man (57 years of

Table 2
Characteristics of the study population.

Subjects parameters	Subjects $n = 44$ Mean ± SD
Age (year olds)	46.4 ± 9.5
Males (%)	45.5
Smokers (%)	23.3
No. of teeth present	25.5 ± 4.7
% Sites with BOP	72.3 ± 29.3
% Sites with CAL ≥4 mm	35.3 ± 21.6
% Sites with CAL ≥7 mm	18.0 ± 22.3
% Sites with PPD ≤3 mm	58.8 ± 30.2
% Sites with PPD ≥5 mm	19.3 ± 17.6
% Sites with PPD ≥7 mm	8.3 ± 11.6

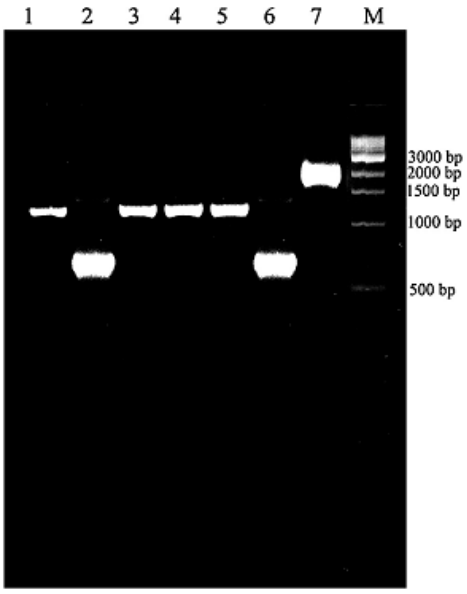

Fig. 1. The *ltx* gene of different strains of *A. actinomycetemcomitans* was amplified using ltx3 and ltx4 primers: lane 1, *A. actinomycetemcomitans* ATCC 33384 (serotype c and non-JP2 clone); lane 2, P48:2 (JP2 clone); lanes 3–5, clinical isolates with normal *ltx* gene; lane 6, clinical isolate with 530 bp deletion gene; lane 7, clinical isolate with 886 bp insertion gene; and lane M, the marker.

age) and one woman (24 years of age), were diagnosed to have a generalized moderate to severe chronic periodontitis with 21 and 15 teeth remaining, respectively. They showed the percentage of sites with PPD ≤ 3 mm, PPD ≥ 5 mm, and PPD ≥ 7 mm of 39.0%, 41.9% and 19.1%, respectively, whereas the percentage of sites with CAL ≥ 4 mm and ≥7 mm was 36.3% and 18.8%, respectively. Two other subjects harbored one strain each (from 10 isolates) with an 886-bp insertion on the *ltx* promoter gene (Fig. 1). They were diagnosed to have a generalized moderate to severe chronic periodontitis with 21 and 25 teeth remaining. They showed the percentage of sites with PPD ≤ 3 mm, PPD ≥ 5 mm, and PPD ≥ 7 mm of 70.9%, 21.5%, and 7.6%, respectively, whereas the percentage of sites with CAL ≥ 4 mm and ≥7 mm was 18.5% and 3.9%, respectively.

3.3. Analysis of the prevalence and subtypes of actinomycetemcomitans

The prevalence of *A. actinomycetemcomitans* was found 84.1% in 37 subjects; from 37 deep pockets sites and 11 shallow pockets sites. A total of 96 independent strains, 79 from deep pocket and 17 from shallow pocket sites, were used for analysis. Initially 145 isolates from deep pocket samples and 39 from shallow pocket samples were pure cultured and tested for the *ltx*-gene (see above) and then serotyped. If the same serotype occurred for two or more isolates from the same sample, they were considered the same strain. Thus, 24 independent serotypeable strains were isolated from deep pockets and 12 independent strains from shallow pockets ($P < 0.05$). Sixty strains (55 from deep and 5 from shallow pockets) were non-serotypeable. The prevalence and serotype distribution is shown in Table 3, Fig. 2a. The prevalence of serotypeable and non-serotypeable (NS) strains was equal on subject level (54.5%). Among the serotypeable strains, serotypes a and c were most prevalent (18.2% and 15.9%, respectively), while serotype b and d were not found. Notably, the 2 JP2 like strains and the 2 strains with the insertion gene as identified above were all classified as serotype c. *A. actinomycetemcomitans* serotypes and non-serotypeable (NS) strains showed a similar distribution pattern in deep pockets, while the frequency of serotypeable isolates was significantly higher in shallow compared to deep pockets ($P < 0.01$).

Additionally, all 184 isolates were subtyped with DGGE. Similarly as for the serotypeable isolates, when the same DGGE subtype occurred for two or more isolates from the same sample they were considered the same strain. The 24 serotypeable strains fell into 3 DGGE subtypes (S1–3). The DGGE subtyping could not distinguish between serotype a, d, and f (Fig. 2b) and this subtype was designated DGGE subtype S1 (S for serotypeable). Serotype c isolates formed a separate DGGE subtype (S2) and serotype e formed the DGGE subtype S3 (Table 4). The 60 non-serotypeable isolates (55 from deep pockets and 5 from shallow pockets, Table 3) fell into 16 different DGGE subtypes (NS1-16) and the distribution is shown in Table 4. The NS1 and NS2 subtype were more frequent (12.7% and 10.1% in deep pockets, respectively) than the others. A dendrogram showing the similarity between the subtypes is presented in Fig. 2c.

The UPGMA method could not distinguish the difference between serotype b and e, between a and c, and between d and f (Fig. 2c), although the differences between serotype b and e and serotype a and c were quite clear visually under UV-light in the gels (Fig. 2b).

Table 3
Prevalence of *A. actinomycetemcomitans* at subject and site level, and serotypes/subtypes distribution in deep and shallow pockets.

Serotypes[a]/subtypes	No. (%) subjects $n = 44$	No. of (%) sites		No. (%) of strains isolated from	
		Aa positive deep pockets $n = 37$	Aa positive shallow pockets $n = 11$	Aa positive deep pockets $n = 79$	Aa positive shallow pockets $n = 17$
a	8 (18.2)	8 (21.6)	3 (27.3)	8 (10.1)	3 (17.6)
b	ND	ND	ND	ND	ND
c	7 (15.9)	7 (18.9)	4 (36.4)	7 (8.9)	4 (23.5)
d	ND	ND	ND	ND	ND
e	4 (9.1)	4 (10.8)	3 (27.3)	4 (5.1)	3 (17.6)
f	5 (11.4)	5 (13.5)	2 (18.2)	5 (6.3)	2 (11.7)
Total serotypeable	24 (54.5)	24 (64.9)	11 (100.0)	24 (30.3)[b,c]	12 (70.5)[b,c]
Non-serotypeable	24 (54.5)	24 (64.9)	4 (36.4)	55 (69.2)[b,c]	5 (29.4)[b,c]

ND: not detected.
[a] Serotypeable.
[b] The difference between no. of serotypeable and non-serotypeable strains isolated from deep and shallow pockets was significant ($P < 0.01$).
[c] The difference between no. of strains totally isolated from deep pockets and shallow pockets was significant ($P < 0.05$).

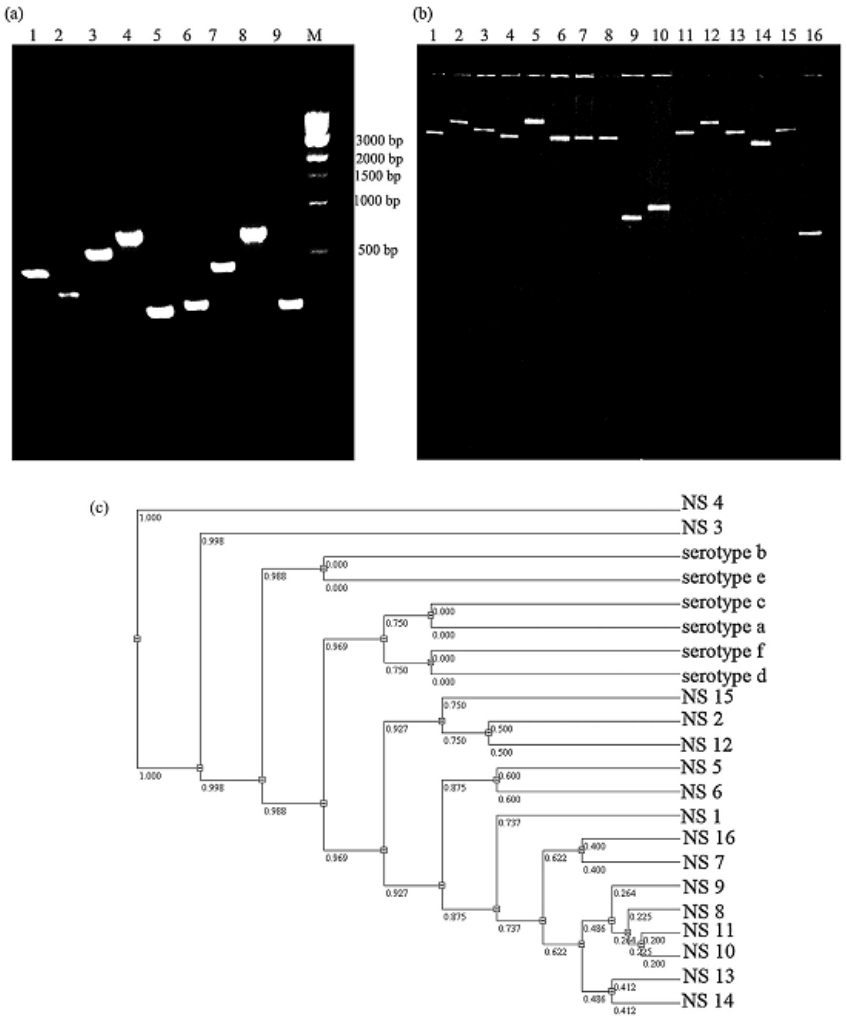

Fig. 2. (a) Serotyping of *A. actinomycetemcomitans* by the PCR with specific primer: lane 1, serotype a (CCUG 37004); lane 2, serotype b (CCUG 37002); lane 3, serotype c (CCUG 13227); lane 4, serotype d (CCUG 38565); lane 5, serotype e (CCUG 37399); lane 6, serotype f (OMGS 3816); lanes 7–9, clinical isolates of serotype a, d, and f, respectively; and lane M, the marker. (b) Serotyping of *A. actinomycetemcomitans* by DGGE: lane 1, serotype a (CCUG 37004); lane 2, serotype b (CCUG 37002); lane 3, serotype c (CCUG 13227); lane 4, serotype d (CCUG 38565); lane 5, serotype e (CCUG 37399); lane 6, serotype f (OMGS 3816); and lanes 7–16, clinical isolates (lanes 7, 8, 11, 13, and 15, serotype a, d, f; lane 12, serotype e; lanes 9 and 16, NS 2 and NS 1; lanes 10 and 14, other NS). (c) The dendrogram illustrating the similarities among the *A. actinomycetemcomitans* strains of different subtypes and were calculated based on the intensity and the position of each band in the gel. Scale bar indicates value of similarity which closer zero (0) are very similarity but closer 1 are very difference.

The same pocket/sample could harbor more than one subtype, in fact up to four different subtypes (serotypes and DGGE subtypes) were found in 3 deep pocket samples from three different patients (Table 5).

4. Discussion

The proportion of the population that harbors *A. actionomycetemcomitans* differs dramatically between various geographical areas and different clinical presentations of periodontitis (Rylev & Kilian, 2008). Most studies have been focused on aggressive forms of periodontitis in juveniles, while less has been reported on periodontitis in adults. Similarly, available prevalence data of *A. actionomycetemcomitans* are comparatively few from Asian populations than from Caucasians, Hispanics or Afro-americans (Contreras et al., 2000). Using various methods, the prevalence in general adult Asian populations has been reported to be 19.5% (328 Japanese adults by culture) (Yoshida et al., 2003), 63% (185Chinese young and older adults by culture) (Mombelli, Gmur, Lang, Corbert, & Frey, 1999), 27 and 22.2% (96 German and 98 Korean cases by real-time PCR) (Kim et al., 2009), 19.0% (453 Thai adults by PCR) (Bandhaya et al., 2012; Torrungruang, Bandhaya,

Table 4
Prevalence and distribution of DGGE subtypes of *A. actinomycetemcomitans* strains.

DGGE subtypes	No. (%) of strains isolated from	
	Deep pockets $n = 79$	Shallow pockets $n = 17$
S 1 (serotype a,d,f)	13 (16.5)	5 (29.4)
S 2 (serotype c)	7 (8.9)	4 (23.5)
S 3 (serotype e)	4 (5.1)	3 (17.6)
NS 1	10 (12.7)	1 (5.9)
NS 2	8 (10.1)	1 (5.9)
NS 3, 7, 9, 10	3 (3.8)	ND
NS 4, 5	1 (1.3)	ND
NS 6, 11, 14	2 (2.5)	ND
NS 8, 12	4 (5.1)	ND
NS 13	1 (1.3)	1 (5.9)
NS 15, 16	4 (5.1)	1 (5.9)

ND: not detected; S: serotypeable DGGE subtypes; NS: non-serotypeable DGGE subtypes.

Likittanasombat, & Grittayaphong, 2009), 88% (60 rural Thai adults by culture) (Dahlen et al., 2002), 92.7% (356 rural Thai adults by checkerboard DNA–DNA hybridization method) (Papapanou et al., 2002), and 44.5% (265 Indonesian adults by culture) (van der Reijden, Bosch-Tijhof, van der Velden, & van Winkelhoff, 2008). The present study, using culture, showed a similar high prevalence (84.1%) of *A. actionomycetemcomitans* in Thai chronic periodontitis cases. A higher prevalence is thus expected from diseased cases compared to the general population as also reported, and a higher prevalence has also been found in deep pockets compared to healthy shallow pockets in other studies (Asikainen et al., 1991; Meng, Zhao, Yang, Wu, & Ouyang, 2009; Papapanou et al., 2002). This is supported by the present study showing 84.1% prevalence in deep pockets compared to shallow healthy pockets of 11%. It should be emphasized that a prevalence study is highly dependent on the sampling strategy and the number of samples used for analysis. An increasing number of samples will certainly increase the prevalence in accordance with Meng et al. (2009) who collected 2 sites with the deepest pockets, which showed significantly higher prevalence than that of healthy sites (Meng et al., 2009). In fact, in the present study we sampled 2–3 deep sites in the cases, which increased the prevalence from 37 to 42 out of the 44 subjects (unpublished observations). It needs to be pointed out that we, for statistical and computational reasons, randomly selected only one single deep pocket to be included in this study. Another strategy would have been to use pooled samples, which also would have increased the prevalence on the subject level, although at the price of limiting calculating the site prevalence (Psoter et al., 2011). It is tempting to argue, similarly to Dahlen et al. (2002) that if the number of samples increased, we would have found 100% prevalence leading to the conclusion that Thai adults are generally colonized with *A. actionomycetemcomitans* and is a member of resident oral microbiota (Dahlen et al., 2002).

A critical step in prevalence studies is the detection method of choice. Culture on selective media is the most common used strategy, is reasonably sensitive and has the advantage of strain isolation for further genetic and phenotypic subtyping. In this study we selected up to 5 colonies from each sample with the purpose of isolating different subtypes; altogether 96 strains were isolated (see below). PCR using primers for 16S rDNA seems to give a lower prevalence although gene-specific methodology can be used for direct detection of certain serotypes, *cdt* genotypes and specific virulent genotypes such as JP2 (Bandhaya et al., 2012; Contreras et al., 2000; Haubek et al., 1997; Haubek et al., 2001; Haubek, Poulsen, Asikainen, & Kilian, 1995; Roman-Torres et al., 2010; Sakellari et al., 2011). The checkerboard DNA–DNA hybridization method was used in some epidemiological studies in Asia (China and Thailand) (Papapanou et al., 1997; Papapanou et al., 2002) showing a high prevalence both at the subject and site level, although it is claimed that the method is not very sensitive (Dahlen, Preus, & Baelum, 2015). Most studies show a lower detection rate of *A. actionomycetemcomitans* compared to other putative periodontopathogens, and it may well be so that this method also has a lower degree of specificity (by cross-hybridization) against the target bacteria, making prevalence studies for *A. actionomycetemcomitans* less reliable using the checkerboard DNA–DNA hybridization method (Dahlen et al., 2015; Papapanou et al., 1997).

Table 5
Distribution of serotypes and DGGE-subtypes of *A. actinomycetemcomitans* in subjects and sites (deep and shallow pockets).

Subtypes distribution	Subject level $n = 37$	Site level	
		Deep pocket $n = 37$	Shallow pocket $n = 11$
Single type	12 (32.4)	12 (32.4)	5 (45.5)
Two types	11 (29.7)	11 (29.7)	6 (54.4)
Three types	11 (29.7)	11 (29.7)	ND
Four types	3 (8.1)	3 (8.1)	ND

ND: not detected.

The diversity of *A. actionomycetemcomitans* was investigated using serotyping and DGGE subtyping. Traditionally, serotyping first used an immunodiffusion assay and later when serotype specific primers were identified, serotyping was carried out with PCR methodology (Asikainen et al., 1991; Kaplan et al., 2001; Suzuki et al., 2001). Thus 6 serotypes (a–f) can be identified, with a, b, and c, being found to be the most common (Chung, Chung, Son, & Nisengard, 1989; Dahlen et al., 2002; Kaplan et al., 2001; Mombelli et al., 1999; Saarela et al., 1992; Suzuki et al., 2001). It has also previously been suggested that serotype c is more common in the Asian population than in populations with Caucasian or African origin (Bandhaya et al., 2012; Dahlen et al., 2002; Kim et al., 2009; Leung et al., 2005; Mombelli et al., 1999; Yoshida et al., 2003). In those studies serotype b has been associated with periodontitis, especially aggressive periodontitis in young individuals (localized juvenile periodontitis) (Asikainen et al., 1991; Haubek et al., 1997; Zambon et al., 1983). The highly toxic genotype (JP2) found in children of North and West Africa is genotype b; it was also detected in samples from two adults (33 and 62 years) in Sweden (Claesson, Lagervall, Höglund-Aberg, Johansson, & Haubek, 2011; Haubek et al., 1997). However, serotype b was not found in the present study—supporting that serotype b is not a very common serotype in the Asian population. This may in part be due to the small sample size (44 periodontitis cases), which is too small for prevalence studies in general and to find uncommon bacterial subtypes in a population in particular. The absence of serotype b could also be due to other factors such as patient age, disease severity and an Asian subpopulation such as adults of Southern Thailand.

It was surprising to find 2 JP2-like strains that were serotyped as c. The patient records did not reveal that these two patients were clinically unique, nor were they clinically unique for the 2 strains of a genotype with insertion in the *ltx* gene. We are not able at this point to say that these 4 strains are equally toxic or virulent as the JP2 genotype but this will be subjected for further investigation. The rate of non-serotypeable *A. actionomycetemcomitans* strains in chronic periodontitis cases in this study was remarkable (54.5%) and was as common as the serotypeable strains (54.5%). Non-serotypeable strains have been previously found in Asian populations (8.9–14.7%) (Mombelli et al., 1999; Yamamoto et al., 1997; Yoshida et al., 2003) and seems to be more common than in American and African populations (1.2–9.5%) (Chen et al., 2010; Fine et al., 2007; Ihalin & Asikainen, 2006).

The high rate of non-serotypeable strains led us to use some other subtyping method such as DGGE. The DGGE method was suggested to be useful in investigations for the competitive ability of strains belonging to different lineages of *A. actinomycetemcomitans*, although there have been no reports on the use of DGGE for subtyping (Ihalin & Asikainen, 2006). DGGE disclosed 16 subtypes among the non-serotypeable strains and showed much higher subtype diversity than that found for the serotypeable strains. The findings from non-serotype groups in this study indicate that new serotypes beyond serotypes a–f may be possible; a serotype g has been proposed (Tsuzukibashi et al., 2014). However, to further analyze for other serotypes was beyond the scope of this investigation. The non-serotypeable strains were significantly ($P < 0.05$) more common among the strains isolated from the deep pockets than from the shallow pockets and indicate that some DGGE subtypes are associated with the deep pockets and periodontitis. Two subtypes (NS1 and NS2) were more common, but whether they are more related to periodontitis or are more toxic than other strains is a matter for further studies. The subtype diversity among the isolates needs explanation in view of the fact that up to 4 different subtypes could be found in the same sample—illustrating the diversity is much more expressed when using culture methodology and DGGE subtyping. This diversity may be population related but it could also be due to a changing disease pattern and/or age of the individual. It is suggested that the competitive process that governs those subtypes that are best fitted to the changing environment in slowly progressing sites is when the individual grows older and that the non-serotypeable strains have such abilities allowing them to colonize and increase in deep periodontal pockets of adult patients with chronic periodontitis.

In conclusion, the prevalence, distribution and diversity of *A. actionomycetemcomitans* subtypes in this adult periodontitis population were found to be much higher than earlier reported in other Asian populations or elsewhere. Some unique strains were found, of which 2 strains had a 530 bp deletion in the leukotoxin gene similar to the JP2 genotype, but was identified as serotype c.

Conflict of interests

None.

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References

Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology*, *4*(1), 1–6.

Asikainen, S., Lai, C., Alaluusua, S., & Slot, J. (1991). Distribution of *Actinobacillus actinomycetem comitans* serotypes in periodontal health and disease. *Oral Microbiology and Immunology*, *6*(2), 115–118.

Bandhaya, P., Saraithong, P., Likittanasombat, K., Hengprasith, B., & Torrungruang, K. (2012). *Aggregatibacter actinomycetemcomitans* serotypes, the JP2 clone and cytolethal distending toxin genes in a Thai population. *Journal of Clinical Periodontology*, *39*(6), 519–525.

Chen, C., Wang, T., & Chen, W. (2010). Occurrence of *Aggregatibacter actinomycetemcomitans* serotypes in subgingival plaque from United States subjects. *Molecular Oral Microbiology*, *25*(3), 207–214.

Chung, H., Chung, C., Son, S., & Nisengard, R. (1989). Actinobacillus actinomycetemcomitans serotypes and leukotoxicity in Korean localized juvenile periodontitis. *Journal of Periodontology*, *60*(9), 506–511.

Claesson, R., Lagervall, M., Höglund-Aberg, C., Johansson, A., & Haubek, D. (2011). Detection of the highly leucotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans* in members of a Caucasian family living in Sweden. *Journal of Clinical Periodontology*, *38*(2), 115–121.

Contreras, A., Rusitanonta, T., Chen, C., Wagner, W. G., Michalowicz, B. S., & Slots, J. (2000). Frequency of 530-bp deletion in *Actinobacillus actinomycetemcomitans* leukotoxin promoter region. *Oral Microbiology and Immunology*, *15*(5), 338–340.

Dahlen, G., Preus, H. R., & Baelum, V. (2015). Methodological issues in the quantification of subgingival mircroorganisms using the checkerboard technique. *Journal of Microbiological Methods*, *110*(0), 68–77.

Dahlen, G., Widar, F., Teanpaisan, R., Papapanou, P. N., Baelum, V., & Fejerskov, O. (2002). *Actinobacillus actinomycetemcomitans* in a rural adult population in southern Thailand. *Oral Microbiology and Immunology*, *17*(3), 137–142.

Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., et al. (2007). *Aggregatibacter actinomycetemcomitans* and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. *Journal of Clinical Microbiology*, *45*(12), 3859–3869.

Haubek, D. (2010). The highly leukotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans*: evolutionary aspects, epidemiology and etiological role in aggressive periodontitis. *APMIS*, *118*(130), 1–53.

Haubek, D., Dirienzo, J. M., Tinoco, E. M., Westergaard, J., Lopez, N. J., Chung, C. P., et al. (1997). Racial tropism of a highly toxic clone of *Actinobacillus actinomycetemcomitans* associated with juvenile periodontitis. *Journal of Clinical Microbiology*, *35*(12), 3037–3042.

Haubek, D., Ennibi, O. K., Poulsen, K., Poulsen, S., Benzarti, N., & Kilian, M. (2001). Early-onset periodontitis in Morocco is associated with the highly leukotoxic clone of *Actinobacillus actinomycetemcomitans*. *Journal of Dental Research*, *80*(6), 1580–1583.

Haubek, D., Poulsen, K., Asikainen, S., & Kilian, M. (1995). Evidence for absence in northern Europe of especially virulent clonal types of *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Microbiology*, *33*(2), 395–401.

Haubek, D., Poulsen, K., Westergaard, J., Dahlen, G., & Kilian, M. (1996). Highly toxic clone of *Actinobacillus actinomycetemcomitans* in geographically widespread cases of juvenile periodontitis in adolescents of African origin. *Journal of Clinical Microbiology*, *34*(6), 1576–1578.

He, T., Nishihara, T., Demuth, D. R., & Ishikawa, I. (1999). A novel insertion sequence increases the expression of leukotoxicity in *Actinobacillus actinomycetemcomitans* clinical isolates. *Journal of Periodontology*, *70*(11), 1261–1268.

Ihalin, R., & Asikainen, S. (2006). 16S rDNA PCR-denaturing gradient gel electrophoresis in determining proportions of coexisting *Actinobacillus actinomycetemcomitans* strains. *Journal of Microbiological Methods*, *65*(3), 417–424.

Kaplan, J., Perry, M., Maclean, L., Furgang, D., Wilson, M., & Fine, D. (2001). Structural and genetic analyses of O polysaccharide from *Actinobacillus actinomycetemcomitans* serotype f. *Infection and Immunity*, *69*(9), 5375–5384.

Kim, T. S., Frank, P., Eickholz, P., Eick, S., & Kim, C. K. (2009). Serotypes of *Aggregatibacter actinomyce temcomitans* in patients with different ethnic backgrounds. *Journal of Periodontology*, *80*(12), 2020–2027.

Ledder, R. G., Gilbert, P., Huws, S. A., Aarons, L., Ashley, M. P., Hull, P. S., et al. (2007). Molecular analysis of the subgingival microbiota in health and disease. *Applied and Environmental Microbiology*, *73*(2), 516–523.

Leung, W. K., Ngai, V. K., Yau, J. Y., Cheung, B. P., Tsang, P. W., & Corbet, E. F. (2005). Characterization of *Actinobacillus actinomycetemcomitans* isolated from young Chinese aggressive periodontitis patients. *Journal of Periodontal Research*, *40*(3), 258–268.

Mínguez, M., Pousa, X., Herrera, D., Blasi, A., Sánchez, M. C., León, R., et al. (2014). Characterization and serotype distribution of *Aggregatibacter actinomycetemcomitans* isolated from a population of periodontitis patients in Spain. *Archives of Oral Biology*, *59*(12), 1359–1367.

Meng, S., Zhao, L., Yang, H., Wu, Y., & Ouyang, Y. (2009). Prevalence of *Actinobacillus actinomycetemcomitans* in Chinese chronic periodontitis patients and periodontally healthy adults. *Quintessence International*, *40*(1), 53–60.

Mombelli, A., Casagni, F., & Madianos, P. (2002). Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? A systematic review. *Journal of Clinical Periodontology*, *29*(3), 10–21.

Mombelli, A., Gmur, R., Lang, N. P., Corbert, E., & Frey, J. (1999). *Actinobacillus actinomycetemcomitans* in Chinese adults: serotype distribution and analysis of the leukotoxin gene promoter locus. *Journal of Clinical Periodontology*, *26*(8), 505–510.

Owen, R. J., & Borman, P. (1987). A rapid biochemical method for purifying high molecular weight bacterial chromosomal DNA for restriction enzyme analysis. *Nucleic Acids Research*, *15*(8), 3631.

Papapanou, P., Teanpaisan, R., Obiechina, N., Pithpornchaiyakul, W., Pongpaisal, S., Pisuithanakan, S., et al. (2002). Periodontal microbiota and clinical periodontal status in a rural sample in southern Thailand. *European Journal of Oral Sciences*, *110*(5), 345–352.

Papapanou, P. N., Baelum, V., Luan, W. M., Medianos, P. N., Chen, X., Fejerskov, O., et al. (1997). Subgingival microbiota in adult chinese: prevalence and relation to periodontal disease progression. *Journal of Periodontology*, *68*(7), 651–666.

Poulsen, K., Ennibi, O. K., & Haubek, D. (2003). Improved PCR for detection of the highly leukotoxic JP2 clone of *Actinobacillus actinomycetemcomitans* in subgingival plaque samples. *Journal of Clinical Microbiology*, *41*(10), 4829–4832.

Psoter, W., Ge, Y., Russell, S., Chen, Z., Katz, R., Jean-Charles, G., et al. (2011). PCR detection of *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* in dental plaque samples from Haitian adolescents. *Clinical Oral Investigations*, *15*(4), 461–469.

Roman-Torres, C. V., Aquino, D. R., Cortelli, S. C., Franco, G. C., Dos Santos, J. G., Corraini, P., et al. (2010). Prevalence and distribution of serotype-specific genotypes of *Aggregatibacter actinomycetemcomitans* in chronic periodontitis Brazilian subjects. *Archives of Oral Biology*, 55(3), 242–248.

Rylev, M., & Kilian, M. (2008). Prevalence and distribution of principal periodontal pathogens worldwide. *Journal of Clinical Periodontology*, 35(8), 346–361.

Saarela, M., Asikainen, S., Alaluusua, S., Pyhala, L., Lai, C. H., & Jousimies-Somer, H. (1992). Frequency and stability of mono- or poly-infection by *Actinobacillus actinomycetemcomitans* serotypes a, b, c, d or e. *Oral Microbiology and Immunology*, *7*(5), 277–279.

Sakellari, D., Katsikari, A., Slini, T., Ioannidis, I., Konstantinidis, A., & Arsenakis, M. (2011). Prevalence and distribution of *Aggregatibacter actinomycetemcomitans* serotypes and the JP2 clone in a Greek population. *Journal of Clinical Periodontology*, *38*(2), 108–114.

Slots, J. (1982). Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Microbiology*, *15*(4), 606–611.

Suzuki, N., Nakano, Y., Yoshida, Y., Ikeda, D., & Koga, T. (2001). Identification of *Actinobacillus actinomycetemcomitans* serotypes by multiplex PCR. *Journal of Clinical Microbiology*, 39(5), 2002–2005.

Torrungruang, K., Bandhaya, P., Likittanasombat, K., & Grittayaphong, C. (2009). Relationship between the presence of certain bacterial pathogens and periodontal status of urban Thai adults. *Journal of Periodontology*, *80*(1), 122–129.

Tsuzukibashi, O., Saito, M., Kobayashi, T., Umezawa, K., Nagahama, F., Hiroi, T., et al. (2014). A gene cluster for the synthesis of serotype g-specific polysaccharide antigen in *Aggregatibacter actinomycetemcomitans*. *Archives of Microbiology*, *196*(4), 261–265.

van der Reijden, W. A., Bosch-Tijhof, C. J., van der Velden, U., & van Winkelhoff, A. J. (2008). Java project on periodontal diseases: serotype distribution of *Aggregatibacter actinomycetemcomitans* and serotype dynamics over an 8-year period. *Journal of Clinical Periodontology*, 35(6), 487–492.

Yamamoto, M., Nishihara, T., Koseki, T., He, T., Yamato, K., Zhang, Y. J., et al. (1997). Prevalence of *Actinobacillus actinomycetemcomitans* serotypes in Japanese patients with periodontitis. *Journal of Periodontal Research*, *32*(8), 676–681.

Yoshida, Y., Suzuki, N., Nakano, Y., Shibuyu, K., Ogawa, Y., & Koga, T. (2003). Distribution of *Actinobacillus actinomycetemcomitans* serotypes and *Porphyromonas gingivalis* in Japanese adults. *Oral Microbiology and Immunology*, *18*(3), 135–139.

Zambon, J. J., Slots, J., & Genco, R. J. (1983). Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. *Infection and Immunity*, *41*(1), 19–27.

Zijnge, V., Welling, G., Degener, J., van Winkelhoff, A., Abbas, F., & Harmsen, H. (2006). Denaturing gradient gel electrophoresis as a diagnostic tool in periodontal microbiology. *Journal of Clinical Microbiology*, *44*(10), 3628–3633.

APPENDIX B

Nuntiya Pahumunto, Praphansri Ruangsri, Mutita Wongsuwanlert, Supatcharin Piwat, Gunnar Dahlen and Rawee Teanpaisan. Virulence of *Aggregatibacter actinomycetemco- mitans* serotypes and DGGE subtypes isolated from chronic adult periodontitis in Thailand. ***Anaerobe*** 2015; 36: 60-4.

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Virulence of *Aggregatibacter actinomycetemcomitans* serotypes and DGGE subtypes isolated from chronic adult periodontitis in Thailand

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ABSTRACT

A high proportion of non-serotypeable isolates of *Aggregatibacter actinomycetemcomitans* among Thai periodontitis cases has been previously reported. The aim of this study was to investigate the expression of leukotoxin and toxicity, cytolethal distending toxin (Cdts), and internalization and the killing effect on fibroblasts by *A. actinomycetemcomitans* subtypes from Thai chronic periodontitis cases. A total of 96 *A. actinomycetemcomitans* strains from 37 periodontitis cases, previously serotyped with PCR and subtyped with DGGE, were examined for the presence of the *ltx* gene and *cdt* genes (*cdtBC*), and tested for leukotoxin expression, leukotoxicity, internalization, and apoptosis of fibroblast cells. The *ltx* gene was present in all isolates, while 84.4% showed the *cdtBC* gene. Two strains with a JP2-like *ltx* gene with a deletion of 530 bp in the promoter region, serotyped as c, showed virulence of similar magnitude to the JP2 strain. Furthermore, a higher virulence was found in the two non-serotypeable DGGE subtypes, NS1 and NS2, compared with the serotypeable strains (serotype a-f, serotype b and d were absent). Generally, the virulence of strains obtained from deep periodontal pockets was higher than those isolated from shallow non-bleeding pockets. *A. actinomycetemcomitans* subtypes isolated from adult Thais with chronic periodontitis showed a highly variable virulence, leukotoxin expression, leukotoxicity, internalization and apoptosis of fibroblast, and are regulated both genetically and environmentally.

1. Introduction

Aggregatibacter actinomycetemcomitans, a capnophilic gram negative bacterium, has been reported to be associated with several forms of periodontitis including chronic periodontitis [1,2]. This bacterium has also been reported to be involved in some systemic infectious diseases, such as endocarditis, meningitis, osteomyelitis, glomerulonephritis and arthritis [3]. *A. actinomycetemcomitans* has been traditionally classified into six serotypes (a-f), and a new serotype g has been recently proposed [4–6]. Serotype b is frequently associated with aggressive periodontitis in young individuals [7], however, the virulence and toxicity have not been found to strictly follow the serotype pattern [7,8]. The virulence of *A. actinomycetemcomitans* is thought to be associated to its production of leukotoxin and has been studied extensively [7,9,10]. Other virulence factors that may play a crucial role in the pathogenesis of periodontal diseases including cytolethal distending toxins (CDTs), internalization, and fibroblast apoptosis, have been studied less [7,9–13].

Leukotoxin of *A. actinomycetemcomitans*, encoded by *ltx* operon, typically causes cytotoxicity in neutrophils, macrophages, and endothelial cells [7,14]. All *A. actinomycetemcomitans* strains exhibit the *ltx* operon, however, differences in the *ltx* promoter region accounts for differences in leukotoxin expression among individual strains. The *ltx* operon strain with high leukotoxin production (JP2 clone) presents a deletion of 530 bp, resulting in 10–20 times higher leukotoxin expression than the non-deletion strains [7,9]. The JP2 clone of *A. actinomycetemcomitans* is identified as serotype

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b and is isolated most frequently in young individuals of African origin [7,15]. However, a number of non-JP2 genotype strains, mainly serotype b but also serotype c and a, have shown high leukotoxic activity similar to the JP2 genotype [8,16]. The relation between virulence and serotypes or other subtypes of *A. actinomycetemcomitans*, except for the JP2 clone, is unclear.

CDTs are multi-component bacterial toxins, which, by targeting and entering eukaryotic cells, cause a distention and cell cycle arrest. CDTs are produced by the *cdtABC* gene, in which *cdtB* is an active toxin, while *cdtA* and *cdtC* increase the toxicity significantly [10]. Moreover, *A. actinomycetemcomitans* could inhibit fibroblast proliferation and internalize in cell cultures [11,13].

In our previous study, a high (65%) prevalence of non-serotypeable strains of *A. actinomycetemcomitans* was reported from deep pockets with advanced periodontitis [2]. No serotype b strains were found. Two strains of a JP2-like subtype and two strains of an insertion gene, identified as serotype c, were isolated. In addition, 16 new subtypes were identified by the DGGE method, and up to four different subtypes were found in a single deep pocket. Such findings seem to differ from other populations and are more diverse than previously thought [2]. The virulence and toxicity of these subtypes are not known.

Therefore, the aim of this study was to investigate the virulence of *A. actinomycetemcomitans* strains derived from Thai chronic periodontitis cases with respect to the *ltx* gene, leukotoxin expression, *cdt* gene, internalization and killing activity on fibroblast cells and with special attention to *A. actinomycetemcomitans* subtypes.

2. Materials and methods

2.1. A. actinomycetemcomitans strains

All strains were obtained from a previous study of Pahumunto et al. [2]. A total of 96 independent strains included 36 serotypeable and 60 non-serotypeable strains isolated from 37 subjects (79 strains from 37 deep pockets and 17 strains from 11 shallow pockets). The 36 serotypeable strains included 11 serotype a, 11 serotype c (with 2 JP-2 like), 7 serotype e, 7 serotype f and the 60 non-serotypeable strains included 11 non-serotypeable group 1 (NS1), 9 non-serotypeable group 2 (NS2) and 40 other groups of non-serotypeable strains (other NS). The culture collection was kept at −80 °C in the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Thailand.

The type strains were also used as follows: CCUG 37004 serotype a, CCUG 37002 serotype b, CCUG 13227 serotype c, ATCC 33384 serotype c, CCUG 38565 serotype d, CCUG 37399 serotype e, OMGS 3816 serotype f (gift from Sirkka Asikainen) and OMGS P48:2 JP2 strain.

2.2. Determination of the leukotoxin (ltx) gene and cdtBC genes

To determine the *ltx* gene and *ltx* gene variants of *A. actinomycetemcomitans*, specific primers ltx3 (5′- GCCGACACCAAAGACAAAGTCT-3′) and ltx4 (5′-GCCCATAACCAAGCCACATAC-3′) were used [17]. The reaction mixture contained 1 unit of *Taq* polymerase, 600 μM dNTP, 2.5 mM $MgCl_2$, 5 pM of each primer and 10 ng of extracted DNA. PCR condition was performed at 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 8 min. PCR products of 686 bp, 1216 bp and 2117 bp represent the deletion, normal and insertion of the promoter gene, respectively, on 1.5% agarose gel electrophoresis. *A. actinomycetemcomitans* ATCC 33384 (non-JP2) and P48:2 (JP2) were used as the negative and positive controls, respectively.

The presence of the *cdtBC* genes was carried out using specific primers, cdtBF (5′-CAACAACACAATTCCAACCC-3′) and cdtR (5′-TTAGCTACCCTGATTTCTCC-3′) [18,19]. The reaction mixture consisted of 10 ng extracted DNA, 5 pM of each primer, 600 μM dNTP, 2.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase. The PCR condition was performed at 94 °C for 5 min followed by 35 cycles at 94 °C for 30s, 57 °C for 30s, 72 °C for 2 min, and a final extension at 72 °C for 8 min. The PCR product of *cdtBC* genes presented as 1917 bp on 1.5% agarose gel electrophoresis.

2.3. Determination of leukotoxin expression and its activity

Total RNA was extracted using an innuPREP DNA/RNA Mini kit (Analytikjena, Berlin, Germany) and RNA concentration was calculated from measuring at A260. The level of leukotoxin expression was evaluated by adding 12 μg/ml of RNA and 5 pM of each primer, ltx3 and ltx4, in a Sensi-FAST™SYBR One-step kit (Bioline Reagent Ltd.). The PCR condition was set for an initial cDNA step at 45 °C for 10 min followed by 94 °C for 5 min and 40 cycles at 94 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 2 min. Amplification, detection, and data analysis were performed with the Rotor gene Q (Qiagen, Valencia, CA). *A. actinomycetemcomitans* ATCC 33384 was used as a positive control and the 16s rRNA gene was used as a housekeeping gene. The leukotoxin expression level was expressed as fold of the control strain ATCC 33384.

Leukotoxin activity of *A. actinomycetemcomitans* was determined using the modified trypan blue exclusion method [20]. Briefly, an HL-60 cell, a promyelocytic leukemic cell line, was grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% pen-strep at 37 °C in 5% CO_2. An equal volume (50 μl) of HL-60 cell (10^6 cells/ml) and tested bacteria (10^8 CFU/ml) were mixed and incubated at 37 °C for 2 h. After incubation, 0.4% trypan blue was added and then observed under a light microscope. Leukotoxin activity (%) = 100 − (surviving cells of the test/surviving cells of the control × 100).

2.4. Determination of internalization and apoptosis of fibroblast cells

The mouse fibroblast cell line L929 Balb/c 3T3 ATCC CCL 163 (donated by Dr. Carl T. Hanks, University of Michigan, School of Dentistry, Ann Arbor, Michigan) was used for testing. The cell line was grown in the fibroblast growth medium supplemented with 10% fetal bovine serum, 1% pen-strep, and 1% fungizone at 37 °C under 5% CO_2 for 48–72 h. For the testing, L 929 cells, having a 95–100% confluence, were inoculated with the tested strain (10^8 CFU/ml), and incubated at 37 °C for 1 h. Then non-adherent bacteria were washed off, and gentamicin (100 μg/ml) was added and further incubated at 37 °C for 2 h to kill external bacterial cells [12]. After washing twice with PBS, cells were disrupted using 0.05% trypsin-EDTA, then the *A. actinomycetemcomitans* number was counted. The internalization (%) = the number of internalization/the total number of bacterial cells at start × 100.

Apoptosis of fibroblast cells was determined using the modified trypan blue exclusion method [20]. The details were mentioned as above; however, the mouse fibroblast cell line L929 was used instead of the HL-60 cell line in this experiment.

2.5. Statistical analysis

The data were expressed as means ± standard deviation (SD). The Chi-square test was used to analyze the presence of the *cdtBC* gene between serotypeables and non-serotypeable strains in deep pockets. The comparative differences of fibroblast apoptosis, internalization of fibroblast cells, leukotoxin activity, and

leukotoxin expression of *A. actinomycetemcomitans* serotypes and subtypes were evaluated using the Kruskall–Wallis test and the Mann–Whitney U test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Presence of ltx and cdtBC genes

All 96 *A. actinomycetemcomitans* strains (100%) were positive for the *ltx* operon gene; however, 2 strains isolated from different subjects exhibited the 530-bp deletion of the *ltx* promoter region (or JP2-like). Two other strains from two different subjects showed an 886-bp insertion on the *ltx* promoter gene.

The *cdtBC* gene was detected in 84.4% of all strains (81 of 96 strains; 66 strains from deep pockets and 15 strains from shallow pockets), and distribution of *cdtBC* genes between serotypeable and non-serotypeable strains in the deep pocket was significantly different (Table 1).

3.2. Virulence factors of serotypeable or non-serotypeable strains from deep and shallow pockets

Considering the abilities of leukotoxin expression, leukotoxin activity, internalization of fibroblast cells and fibroblast apoptosis between the strains isolated from deep and shallow pockets of individual serotypeable or non-serotypeable strains, the strains from deep pockets, especially non-serotypeable strains, showed significantly higher abilities in all means ($P < 0.01$, Table 2). When comparing the strains from deep pockets between serotypeable and non-serotypeable, non-serotypeable strains exhibited significant in all means for leukotoxin expression, leukotoxin activity, internalization of fibroblast cells and fibroblast apoptosis, compared to serotypeable strains ($P < 0.001$, Table 2).

Generally, mean values for leukotoxin expression for non-serotypeable and serotypeable strains showed significantly higher ($P < 0.001$) means for strains isolated from deep pockets compared with those isolated from shallow pockets (Table 2). The non-serotypeable strains isolated from deep pockets showed significantly ($P < 0.01$) higher means of leukotoxin expression, leukotoxicity, internalization and apoptosis than the serotypeable strains (Table 2). The non-serotypeable subtypes, NS1 and NS2, isolated from deep pockets, showed significantly ($P < 0.05$) higher means of apoptosis compared to the serotypeable and other NS strains. However, no significant differences were found between these groups if the strains were isolated from shallow pockets.

Table 1
Presence of *cdtBC* genes among serotypeable and non-serotypeable strains from deep and shallow pocket sites.

Serotypes[a]/subtypes	No. (%) of strains with *cdtBC* genes	
	Deep pocket n = 66	Shallow pocket n = 15
Serotypeable:	22 (33.3)	11 (73.3)
a	7 (10.6)	3 (20.0)
c	7 (10.6)	3 (20.0)
e	4 (6.1)	3 (20.0)
f	4 (6.1)	2 (13.3)
Non-serotypeable:	44 (66.7)[b]	4 (26.7)
NS1	10 (15.2)	1 (6.7)
NS2	8 (12.1)	1 (6.7)
other NS	26 (39.4)	2 (13.3)

[a] No serotype b and d strains were found.
[b] The significant presence of *cdtBC* gene between serotypeables and non-serotypeable strains in deep pockets ($P < 0.001$).

3.3. Leukotoxin expression, leukotoxin activity, internalization, and fibroblast apoptosis of A. actinomycetemcomitans between serotypeable and non-serotypeable strains

The results of Table 3 demonstrated that subtype NS1 exhibited highly significant leukotoxin expression ($P < 0.001$) compared to others (serotypeable, NS2, and other NS) with mean of 89.1 ± 37.1. Also, subtype NS2 showed highly significant leukotoxin expression ($P < 0.001$) compared to serotypeable and other NS with mean of 36.3 ± 6.8.

Also, subtype NS1 exhibited highly significant leukotoxin activity ($P < 0.001$) compared to serotypeable strains, NS2 and other NS. Moreover, NS1 showed correlation between leukotoxin expression and leukotoxin activity in the same way ($P < 0.001$; $r_s = 0.936$). NS2 showed significant difference from serotype f ($P < 0.05$).

All strains showed various degrees of internalization of bacterial cells into fibroblasts compared to the number at the start. The non-serotypeable (NS1, NS2, and other NS) showed a significantly higher degree of internalization of fibroblast cells (62.8 ± 12.1%, 64.4 ± 11.8%, and 55.9 ± 13.0%, respectively) compared to serotypeable strains (48.6 ± 8.3%).

The fibroblast apoptosis of NS1 (66.3 ± 10.3%) and NS2 (75.1 ± 13.1%) was significantly higher than serotypeable strains (50.5 ± 9.7%) and other NS (58.1 ± 12.6%) ($P < 0.001$).

Two strains (JP2-like) with a 530 bp leukotoxin gene deletion showed high levels of leukotoxin expression (596.0 ± 291.3) and leukotoxin activity (54.7 ± 9.3%), similar to what was found for the JP2 reference strain (761.0 ± 34.6), and significantly higher than all other strains tested. For internalization and apoptosis of fibroblasts, it was of the same magnitude or even lower than the non-serotypeable NS1 and NS2.

4. Discussion

The present study extends from our previous study focusing on the relation of serotypes/subtypes of *A. actinomycetemcomitans* and their virulence. They express several putative virulence factors *in vitro* that could play a role in the pathogenesis of periodontal diseases.

Leukotoxin of *A. actinomycetemcomitans* is known as a crucial virulence factor, especially the highly leukotoxin producing JP2 clone, but it has not yet been reported among the Asian population [21–24]. The *ltx* gene was found in all *A. actinomycetemcomitans* strains in this study, however, leukotoxin expression varied among different strains. Also a high leukotoxin expression associated with a deletion of 530 bp in the *ltx* promoter gene. In fact, it is quite surprising that 2 strains with a deletion of 530 bp were found in 2 different patients, which has not previously been reported in Asian populations [21–24]. Additionally, we examined leukotoxin expression and its activity to indicate that leukotoxin produced from *A. actinomycetemcomitans* strains could be expressed and secreted to kill target cells (THP-1, HL-60) [8,20]. The results confirmed that those which had a 530-bp deletion (JP2-like) enhanced leukotoxin expression approximately 17 fold compared to the normal *ltx* gene, and that positively correlated with the high leukotoxin activity by killing HL-60 in this study.

It was previously reported that highly leukotoxic strains (JP2 clone) usually belonged to serotype b [25], thus it was believed that there was an association between serotype b and aggressive periodontitis [7]. Our study has shown that it is not always true, because the strains with a deletion of 530 bp found in this study were identified as serotype c. In addition, serotype b was not detected in this study, although, the periodontal conditions among the subjects in this study were relatively severe. Both JP2-like

Table 2
Mean ± SD of leukotoxin expression, leukotoxin activity, internalization, and apoptosis of fibroblast cells for serotypeable and non-serotypeable strains of *A. actinomycetemcomitans* isolated from deep and shallow pocket sites, respectively.

Virulence factors	Serotypeable		Non-serotypeable	
	Deep pockets n = 24	Shallow pockets n = 12	Deep pockets n = 55	Shallow pockets n = 5
Leukotoxin expression (fold of control)	11.0 ± 7.6[a]	2.1 ± 1.7	42.9 ± 39.6[a,b]	0.3 ± 0.3
Leukotoxin activity (%)	40.7 ± 13.7[a]	29.2 ± 18.2	47.5 ± 11.8[a,b]	32.0 ± 14.9
Internalization (%)	48.3 ± 2.7	39.7 ± 2.7	57.9 ± 13.2[a,b]	44.5 ± 9.9
Apoptosis (%)	51.3 ± 6.8	43.0 ± 6.0	61.7 ± 14.4[a,b]	38.4 ± 5.5

[a] The significant difference of individual characteristics of *A. actinomycetemcomitans* strains between deep pockets and shallow pockets ($P < 0.01$).
[b] The significant difference of individual characteristics of *A. actinomycetemcomitans* strains of deep pockets between serotypeable and non-serotypeable ($P < 0.001$).

Table 3
Mean ± SD of leukotoxin expression, leukotoxin activity, internalization, and apoptosis of fibroblast cells for serotypeable and non-serotypeable strains of *A. actinomycetemcomitans*.

Serotypes/subtypes[a]	Virulence factors			
	Leukotoxin expression (fold of control)	Leukotoxin activity (%)	Internalization (%)	Apoptosis (%)
a	8.0 ± 3.9	41.4 ± 15.9	44.4 ± 10.2	57.5 ± 4.9
c[b]	8.1 ± 3.2	37.8 ± 18.5	50.3 ± 12.6	56.0 ± 12.3
e	11.9 ± 7.7	36.9 ± 14.0	50.1 ± 14.8	48.4 ± 9.2
f	3.7 ± 2.4	32.1 ± 16.8	48.5 ± 12.3	43.1 ± 11.8
NS1	89.1 ± 37.1[c]	50.6 ± 9.9[e]	62.8 ± 12.1[f]	66.3 ± 10.3[g]
NS2	36.3 ± 6.8[d]	44.7 ± 11.5[e]	64.4 ± 11.8[f]	75.1 ± 13.1[d]
Other NS	8.1 ± 13.0	34.7 ± 12.4	55.9 ± 13.0[f]	58.1 ± 12.6
JP2-like (serotype c)	596.0 ± 291.3	54.7 ± 9.3	48.4 ± 0.5	66.4 ± 0.5
JP2	761.0 ± 34.6	57.3 ± 8.4	53.9 ± 0.6	57.8 ± 0.6

[a] No serotype b and d strains were found.
[b] Serotype c excluded 2 strains of JP2-like.
[c] The significant difference between NS1 and others (serotypeable, NS2, and other NS) $P < 0.001$.
[d] The significant difference between NS2 and serotypeable strains and other NS $P < 0.001$.
[e] The significant difference between NS2 and serotype f $P < 0.05$.
[f] The significant difference of NS1, NS2 and other NS compared to serotypeable strains $P < 0.001$, $P < 0.001$, $P < 0.05$, respectively.
[g] The significant difference between NS1 and others (serotypeable and other NS) $P < 0.001$; statistical analysis was considered within the same column.

strains in this study originated from deep pockets (12 mm pocket depth) of 2 different subjects with severe periodontitis.

Strains with inserted sequence elements were found among *A. actinomycetemcomitans* strains in this study. He et al. [20] demonstrated highly toxic strains of *A. actinomycetemcomitans* contained a novel insert sequence element, but such strains did not seem to influence leukotoxin expression in the present study (data not shown).

The factors that influence the expression of various toxins in bacterial cells of subgingival biofilm *in vivo* in the presence of inflammation and complex polymicrobial microbiota are matters of conjecture. Most strains, including the non-serotypeable ones, isolated from deep periodontal pockets showed a higher virulence compared with the virulence of those that originated from shallow pockets. It is tempting to argue that the higher leukotoxin expression in the non-serotypeable and the NS1 and NS2 subtypes in particular, (although they were not in the magnitude of the genetically based highly toxic JP2 clone) indicate a higher virulence of these subtypes. On the other hand, the expression of virulence factors and toxicity is probably due to an environmentally driven adaptation to the pathological periodontal pocket. We have limited knowledge how the genes in detail are regulated and about the role of environmental conditions for leukotoxin expression *in vivo*. The interpretation of the difference in toxicity (although significant) found between strains from deep and shallow pockets should therefore be done with caution. The results of the present study should primarily be regarded as showing the virulence capacity and the comparative toxicity between strains under similar conditions *in vitro*.

This investigation showed a high prevalence of the *cdtBC* genes (84.4%), in contrast to the lower prevalence (27%) that was observed in Thai subjects by Bandhaya et al. [22]. This is explained by the fact that *A. actinomycetemcomitans* may be positive for the *cdtBC*, while still being *cdtABC* negative [26]. It is possible that mutation had occurred in the *cdtA* gene, which results in unsuccessful annealing of the primers. It may also be due to variation in the methodology, leading to discrepancies in detection frequency of *cdtABC* genes in *A. actinomycetemcomitans* [26]. However, the distribution of *cdt* genes among the analyzed isolates did not show any serotype-dependent pattern and it was not possible to demonstrate a specific pattern for *cdt* genes among the *A. actinomycetemcomitans* subtypes in the present study.

The internalization ability of *A. actinomycetemcomitans* has been considered an important factor in the disease process, because *A. actinomycetemcomitans* could escape from host defense and antibiotics by internalization into host cells such as epithelial cells and fibroblasts [6,12,27]. The present study showed highly significant internalization of non-serotypeable strains, particularly NS1 and NS2. It is likely that this is part of the subtype selection that takes place in the periodontal pocket and it is due to a more competitive survival ability of these subtypes. Also, NS1 and NS2 strains showed a significantly higher degree of apoptosis, which may be a consequence of their internalization in cell lines. It was reported that apoptosis of fibroblast cells after exposure to *A. actinomycetemcomitans* followed inhibition of DNA synthesis, decreased RNA synthesis, and a decrease in fibroblast cell growth [13].

The fact that multiple subtypes were commonly isolated from a patient or even from one pocket warrants comment. It is commonly found that the resident flora, as the age of the host increases, is successively colonized by new genotypes of the same bacterial species, climaxing with a community with multiple subtypes

[28,29]. Several genotypes of the same species increase the survival rate. At the same time, there is a competition and internal control between the subtypes favoring those that have certain abilities to adapt in a dynamic and changing environment. The production of leukotoxin and the ability to internalize host cells are ways to escape the host defense, as discussed above. It is therefore tempting to speculate whether the presence of toxic subtypes such as the NS1 and NS2, is due to a competitive process between genotypes of the same species favoring those with a higher toxicity or to a significantly higher ability for apoptosis and internalization than other *A. actinomycetemcomitans* subtypes and that this ability may be more expressed in chronic periodontitis in adults.

In conclusion, non-serotypeable strains, especially the DGGE subtypes NS1 and NS2, showed significantly higher leukotoxin expression, leukotoxicity, fibroblast apoptosis, and internalization of *A. actinomycetemcomitans*. High toxicity was also found for two strains with a JP2-like 530 bp deletion of the promoter gene in the deletion *ltx* gene but serotyped as c. No association was found between *A. actinomycetemcomitans* subtypes and *cdtBC* genes. The ability of leukotoxin expression, leukotoxicity, internalization and fibroblast apoptosis of *A. actinomycetemcomitans* was higher in strains from deep periodontal pockets than from shallow pockets. The toxicity is independent of serotypes and several genetic linkages of *A. actinomycetemcomitans* exist with a high toxicity, and the toxin expression is likely to be driven by environmental conditions.

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References

[1] A. Mombelli, F. Casagni, P. Madianos, Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? a systematic review, J. Clin. Periodontol. 29 (2002) 10–21.
[2] N. Pahumunto, P. Ruangsri, M. Wongsuwanlert, S. Piwat, G. Dahlen, R. Teanpaisan, Aggregatibacter actinomycetemcomitans serotypes and DGGE subtypes in Thai adult chronic periodontitis, Arch. Oral Biol. 60 (2015), 1798–1796.
[3] A. van Winkelhoff, J. Slots, *Actinobacillus actinomycetemcomitans* and Porphyromonas gingivalis in nonoral infections, Periodontology 2000 (20) (1999) 122–135.
[4] J. Kaplan, M. Perry, L. Maclean, D. Furgang, M. Wilson, D. Fine, Structural and genetic analyses of O polysaccharide from *Actinobacillus actinomycetemcomitans* serotype f, Infect. Immun. 69 (2001) 5375–5384.
[5] N. Suzuki, Y. Nakano, Y. Yoshida, D. Ikeda, T. Koga, Identification of *Actinobacillus actinomycetemcomitans* serotypes by multiplex PCR, J. Clin. Microbiol. 39 (2001) 2002–2005.
[6] O. Tsuzukibashi, M. Saito, T. Kobayashi, U. Koji, N. Fumio, F. Nagahama, T. Hiroi, M. Hirasawa, K. Takada, A gene cluster for the synthesis of serotype g-specific polysaccharide antigen in *Aggregatibacter actinomycetemcomitans*, Arch. Microbiol. 196 (2014) 261–265.
[7] D. Haubek, A. Johansson, Pathogenicity of the highly leukotoxic JP2 clone of Aggregatibac- ter actinomycetemcomitans and its geographic dissemination and role in aggressive periodontitis, J. Oral Microbiol. 6 (2014) 1–22.
[8] C.H. Åberg, D. Haubek, F. Kwamin, A. Johansson, R. Claesson, Leukotoxic activity of *Aggregatibacter actinomycetemcomitans* and periodontal attachment loss, Plos One 9 (2014) 1–11.
[9] J. Brogan, E. Lally, K. Poulsen, M. Kilian, D. Demuth, Regulation of Actinobacillus actinomycetemcomitans leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains, Infect. Dis. 62 (1994) 672–683.
[10] B. Henderson, J. Ward, D. Ready, Aggregatibacter (Actinobacillus) actinomycetemcomitans: a triple A* periodontopathogen? Periodontol 2000 54 (2010) 78–105.
[11] P.M. Fives-Taylor, D.H. Meyer, K.P. Mintz, C. Brissette, Virulence factors of *Actinobacillus actinomycetemcomitans*, Periodontol 2000 20 (1999) 136–167.
[12] D.H. Meyer, P.K. Sreenivasan, P.M. Fives-Taylor, Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*, Infect. Immun. 59 (1991) 2719–2726.
[13] B.J. Shenker, M.E. Kushner, C.C. Tsai, Inhibition of fibroblast proliferation by Actinobacil- lus actinomycetemcomitans, Infect. Immun. 38 (1982) 986–992.
[14] E.T. Lally, E.E. Golub, I.R. Kieba, N.S. Taichman, J. Rosenbloom, J.C. Rosenbloom, C.W. Gibson, D.R. Demuth, Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Delineation of unique features and comparison to homologous toxins, J. Biol. Chem. 264 (1989) 15451–15456.
[15] D. Haubek, J.M. Dirienzo, E.M. Tinoco, J. Westergaard, N.J. Lopez, C.P. Chung, K. Poulsen, M. Kilian, Racial tropism of a highly toxic clone of *Actinobacillus actinomycetemcomitans* associated with juvenile periodontitis, J. Clin. Microbiol. 35 (1997) 3037–3042.
[16] R. Claesson, J. Gudmundson, C.H. Aberg, D. Haubek, A. Johansson, Detection of a 640-bp deletion in the *Aggregatibacter actinomycetemcomitans* leukotoxin promoter region in isolates from an adolescent of Ethiopian origin, J. Oral Microbiol. 7 (2015) 1–4.
[17] K. Poulsen, O.K. Ennibi, D. Haubek, Improved PCR for detection of the highly leukotoxic JP2 clone of *Actinobacillus actinomycetemcomitans* in subgingival plaque samples, J. Clin. Microbiol. 41 (2003) 4829–4832.
[18] K.S. Tan, K.P. Song, G. Ong, Cytolethal distending toxin of Actinobacillus actinomycetem- comitans. occurrence and association with periodontal disease, J. Periodontal Res. 37 (2002) 268–272.
[19] J. Umeda, P. Longo, M. Simionato, M. Mayer, Differential transcription of virulence genes in *Aggregatibacter actinomycetemcomitans* serotypes, J. Oral Microbiol. 5 (2013) 1–8.
[20] T. He, T. Nishihara, D.R. Demuth, I. Ishikawa, A novel insertion sequence increases the expression of leukotoxicity in *Actinobacillus actinomycetemcomitans* clinical isolates, J. Periodontol. 70 (1999) 1261–1268.
[21] G. Dahlen, F. Widar, R. Teanpaisan, P.N. Papapanou, V. Baelum, O. Fejerskov, *Actinobacillus actinomycetemcomitans* in a rural adult population in southern Thailand, Oral Microbiol. Immun. 17 (2002) 137–142.
[22] P. Bandhaya, P. Saraithong, K. Likittanasombat, B. Hengprasith, K. Torrungruang, *Aggregatibacter actinomycetemcomitans* serotypes, the JP2 clone and cytolethal distending toxin genes in a Thai population, J. Clin. Periodontol. 39 (2012) 519–525.
[23] A. Mombelli, R. Gmur, N.P. Lang, E. Corbert, J. Frey, *Actinobacillus actinomycetemcomitans* in Chinese adults: serotype distribution and analysis of the leukotoxin gene promoter locus, J. Clin. Periodontol. 26 (1999) 505–510.
[24] Y. Yoshida, N. Suzuki, Y. Nakano, K. Shibuyu, Y. Ogawa, T. Koga, Distribution of *Actinobacillus actinomycetemcomitans* serotypes and Porphyromonas gingivalis in Japanese adults, Oral Microbiol. Immunol. 18 (2003) 135–139.
[25] J.B. Kaplan, H.C. Schreine, D. Furgang, D.H. Fine, Population structure and genetic diversity of *Actinobacillus actinomycetemcomitans* strains isolated from localized juvenile periodontitis patients, J. Clin. Microbiol. 40 (2002) 1181–1187.
[26] D. Kawamoto, E.S. Ando, P.L. Longo, A.C. Nunes, M. Wikstrom, M.P. Mayer, Genetic diversity and toxic activity of *Aggregatibacter actinomycetemcomitans* isolates, Oral Microbiol. Immunol. 24 (2009) 493–501.
[27] P. Arirachakaran, W. Apinhasmit, P. Paungmalit, P. Jeramethakul, P. Rerkyen, R. Mahanonda, Infection of human gingival fibroblasts with *Aggregatibacter actinomycetemcomitans*: an in vitro study, Arch. Oral Biol. 57 (2012) 964–972.
[28] R.S. Percival, S.J. Challacombe, P.D. Marsh, Age-related microbiological changes in the salivary and plaque microflora of healthy adults, J. Med. Microbiol. 35 (1991) 5–11.
[29] L.S. Percival, Ageing Theories, Diseases and Microorganisms. Microbiology and Aging, Humana Press, 2009, pp. 1–13.

APPENDIX C

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Search for protein from lactobacilli inhibit growth of Aggregatibacter actinomycetemcomitans

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Abstract- Lactobacilli can provide health benefits to the host due to their antimicrobial components which inhibit the growth of oral pathogens including periodontal pathogens. The aim of this study was to evaluate whether protein produced from lactobacilli could inhibit the growth of *Aggregatibacter actinomycetemcomitans*, one of etiologic bacteria for aggressive periodontitis. Materials and methods: Seven strains of *Lactobacillus*, *L. paracasei* SD1, *L. casei* SD2, *L. salivarius* SD3, *L. plantarum* SD4, *L. rhamnosus* SD5, *L. fermentum* SD6 and *L. fermentum* SD11 were included in the study. The strains were cultured in MRS broth at 37°C, 24-48 h in anaerobic condition and were centrifuged at 8,000 rpm 5 min. The whole proteins in the supernatant were prepared by dialysis bag and then were concentrated using carboxymethlycellulose sodium salt. The antimicrobial activities of proteins against *A. actinomycetemcomitans* ATCC 33384 were determined using micro-dilution method. The results showed that crude proteins of *L. fermentum* SD11 and *L. paracasei* SD1 gave the strongest activity with Minimum inhibitory concentration (MIC) of 4.46 and 5.52 µg/ml. *L. casei* SD2, *L. plantarum* SD4 and *fermentum* SD6 showed the moderate activity with MIC of 9.12, 12.11 and 14.71 µg/ml, respectively. *L. salivarius* SD3 and *L. rhamnosus* SD5 had the lowest activity with MIC of 41.10 and 28.30 µg/ml. Conclusion: It is promising that the proteins extracted of certain *Lactobacillus* e.g. *L. fermentum* SD11 and *L. paracasei* SD1 may be useful for treatment of periodontal diseases.

Introduction

The application of using of probiotics for prevention of periodontal diseases has been an interest recently. Mayanagi et al. (1) found that probiotic bacteria may affect on the oral microbial ecology, nevertheless, the effects of various probiotic in the oral cavity are not easy to predict. Most probiotics belong to the genera of *Lactobacillus* which are gram positive bacteria, negative catalase and rod shapes. Lactobacilli can provide health benefits to the host due to their antimicrobial components, for example organic acid (lactic acid and acetic acid) and antimicrobial substance. There were reports showed that *Lactobacillus* strains could inhibit the growth of oral pathogens such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia*, and *Streptococcus mutans* (2, 3). Therefore, in this study investigated potential of protein from *Lactobacillus* strains to inhibit growth of *A. actinomycetemcomitans,* a periodontal pathogen.

Materials and methods

Seven strains of *Lactobacillus*, *L. paracasei* SD1, *L. casei* SD2, *L. salivarius* SD3, *L. plantarum* SD4, *L. rhamnosus* SD5, *L. fermentum* SD6 and *L. fermentum* SD11 (a collection strains of the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Thailand) were included in the study. The strains were cultured in MRS (de Man Rogosa and Shape) broth at 37°C, 24-48 h in anaerobic condition and were centrifuged at 8,000 rpm 5 min. The whole proteins in the supernatant were prepared by dialysis bag and then were concentrated using carboxymethlycellulose sodium salt. *A. actinomycetemcomitans* ATCC 33384 was cultured in BHI (brain heart infusion) broth and was incubated at 37°C, 24-48 h in anaerobic condition. The antimicrobial activities of MIC of proteins against *A. actinomycetemcomitans* ATCC 33384 were determined using micro-dilution method.

Results and Discussions

The crude proteins of *L. fermentum* SD11 and *L. paracasei* SD1 gave the strongest activity with MIC (Minimum Inhibitory Concentration) of 4.46 and 5.52 µg/ml. *L. casei* SD2, *L. plantarum* SD4 and *L. fermentum* SD6 showed the moderate activity with MIC of 9.12, 12.11 and 14.71 µg/ml, respectively. *L. salivarius* SD3 and *L. rhamnosus* SD5 had the lowest activity with MIC of 41.10 and 28.30 µg/ml (Table 1).

Table 1 Antimicrobial activities and MIC of proteins of various *Lactobacillus* strains against *A. actinomycetemcomitans* ATCC 33384

Proteins	Antimicrobial activities	MIC (µg/mL)	Proteins	Antimicrobial activities	MIC (µg/mL)
L. paracasei SD1	+	5.52	*L. rhamnosus* SD5	+	28.30
L. casei SD2	+	9.12	*L. fermentum* SD6	+	14.71
L. salivarius SD3	+	41.10	*L. fermentum* SD11	+	4.46
L. plantarum SD4	+	12.11			

Conclusion

The results presented that proteins derived from Lactobacillus strains tested could inhibit the growth of *A. actinomycetemcomitans* ATCC 33384. Thus, it is promising that the proteins extracted of certain *Lactobacillus* e.g. *L. fermentum* SD11 and *L. paracasei* SD1 may be useful for treatment of periodontal diseases.

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References

1)Mayanagi G., Kimura M., Nakaya S., Hirata H., Sakamoto M., Benno Y. and Shimauchi H. Probiotic effects of orally administered *Lactobacillus salivarius* WB21-containing tablets on periodontopathic bacteria: a double-blinded, placebo-controlled, randomized clinical trial. J Clin Periodontol. 2009 36: 506-513.

2) Koll P., Mandar R., Marcotte H., Leibur E., Mikelsaar M. and Hammarstrom L. Characterization of oral lactobacilli as potential probiotics for oral health. Oral Microbiol Immunol. 2008 23: 139–147.

3)Teanpisan R., Piwat S. and Dahlen G. Inhibitory effect of oral Lactobacillus against oral pathogens. Lett in Appl Microb. 2011 53: 452–459.

APPENDIX D

Documentary Proof of Ethical Clearance Research Ethics Committee (REC) Faculty of Dentistry, Prince of Songkla University (EC5509-36-P)

ที่ ศธ 0521.1.03/ 0093

คณะทันตแพทยศาสตร์
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ตู้ไปรษณีย์เลขที่ 17
ที่ทำการไปรษณีย์โทรเลขคอหงส์
อ.หาดใหญ่ จ.สงขลา 90112

หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัยเรื่อง "ความสัมพันธ์ของซีโรไทป์ของ *Aggregatibacter actinomycetemcomitans* และปัจจัยก่อความรุนแรงของ *A. actinomycetemcomitans*"

รหัสโครงการ EC5509-36-P

หัวหน้าโครงการ นางสาวนันทิยา พาหุมันโต

สังกัดหน่วยงาน นักศึกษาหลังปริญญา ภาควิชาโอษฐวิทยา คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Research Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคนของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นสากล ได้แก่ Declaration of Helsinki, the Belmont Report, CIOMS Guidelines และ the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

ในคราวประชุมครั้งที่ 9/2555 **เมื่อวันที่** 21 ธันวาคม 2555

ให้ไว้ ณ วันที่ 30 มี.ค. 2556

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Documentary Proof of Ethical Clearance

Research Ethics Committee (REC)

Faculty of Dentistry, Prince of Songkla University

The Project Entitled Relationship of serotypes of *Aggregatibacter actinomycetemcomitans* and virulent factors

REC Project No. : EC5509-36-P

Principal Investigator : Miss Nuntiya Pahumunto

Approved by Research Ethics Committee (REC), Faculty of Dentistry, Prince of Songkla University.

This is to certify that REC is in full Compliance with International Guidelines for Human Research Protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Date of Approval : 30 JANUARY 2013

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(Asst. Prof.Surapong Vongvatchranon)

(Asst. Prof. Dr. Angkana Thearmontree)

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Nuntiya Pahumunto, Praphansri Ruangsri, Mutita Wongsuwanlert, Supatcharin Piwat, Gunnar Dahlen and Rawee Teanpaisan. *Aggregatibacter actinomycetemcomitans* Serotypes and DGGE Subtypes in Thai Adults with Chronic Periodontitis. ***Arch Oral Biol.*** 2015; 60:1789-96.

Nuntiya Pahumunto, Praphansri Ruangsri, Mutita Wongsuwanlert, Supatcharin Piwat, Gunnar Dahlen and Rawee Teanpaisan. Virulence of *Aggregatibacter actinomycete-mcomitans* serotypes and DGGE subtypes isolated from chronic adult periodontitis in Thailand. ***Anaerobe*** 2015; 36: 60-4.

Pahumunto N. and Teanpaisan R. 2012. Search for protein from lactobacilli inhibit growth of *Aggregatibacter actinomycetemcomitans*. The 2nd Current Drug Development International Conference. 2nd-4th May 2012.