Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Association between infection of different strains of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in subgingival plaque and clinical parameters in chronic periodontitis*

WU Yan-min[§1], YAN Jie[§2], CHEN Li-li[†‡1], GU Zhi-yuan[3]

([1]*Department of Stomatology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

([2]*Department of Medical Microbiology and Parasitology, School of Medicine, Zhejiang University, Hangzhou 310006, China*)

([3]*Department of Oral and Maxillofacial Surgery, the Affiliated Hospital of Stomatology, School of Medicine, Zhejiang University, Hangzhou 310003, China*)

[†]E-mail: yanchen_5657@163.com

Received Aug. 9, 2006; revision accepted Oct. 9, 2006

Abstract: Objective: The aim of this study was to investigate subgingival infection frequencies of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* strains with genetic variation in Chinese chronic periodontitis (CP) patients and to evaluate its correlation with clinical parameters. Methods: Two multiplex polymerase chain reaction (PCR) assays were developed to detect the *16SrDNA*, collagenase (*prtC*) and fimbria (*fimA*) genes of *P. gingivalis* and the *16SrDNA*, leukotoxin (*lktA*) and fimbria-associated protein (*fap*) genes of *A. actinomycetemcomitans* in 60 sulcus samples from 30 periodontal healthy subjects and in 122 subgingival plaque samples from 61 patients with CP. The PCR products were further T-A cloned and sent for nucleotide sequence analysis. Results: The *16SrDNA*, *prtC* and *fimA* genes of *P. gingivalis* were detected in 92.6%, 85.2% and 80.3% of the subgingival plaque samples respectively, while the *16SrDNA*, *lktA* and *fap* genes of *A. actinomycetemcomitans* were in 84.4%, 75.4% and 50.0% respectively. Nucleotide sequence analysis showed 98.62%~100% homology of the PCR products in these genes with the reported sequences. *P. gingivalis* strains with *prtC*+/*fimA*+ and *A. actinomycetemcomitans* with *lktA*+ were predominant in deep pockets (>6 mm) or in sites with attachment loss ≥5 mm than in shallow pockets (3~4 mm) or in sites with attachment loss ≤2 mm ($P<0.05$). *P. gingivalis* strains with *prtC*+/*fimA*+ also showed higher frequency in gingival index (*GI*)=3 than in *GI*=1 group ($P<0.05$). Conclusion: Infection of *P. gingivalis* with *prtC*+/*fimA*+ and *A. actinomycetemcomitans* with *lktA*+ correlates with periodontal destruction of CP in Chinese. Nonetheless *P. gingivalis fimA*, *prtC* genes and *A. actinomycetemcomitans lktA* gene are closely associated with periodontal destruction, while *A. actinomycetemcomitans fap* gene is not.

Key words: *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, Strain, Periodontitis, PCR
doi:10.1631/jzus.2007.B0121 **Document code:** A **CLC number:** R781

INTRODUCTION

Porphyromonas gingivalis, a black-pigmented gram-negative anaerobic coccobacillus, has been etiologically associated with various types of periodontal disease including chronic periodontitis (CP) (Chen *et al*., 2005; Cortelli *et al*., 2005a; Liu *et al*., 2003). This bacterium is frequently detected in deep periodontal pockets of CP patients and is occasionally found in healthy periodontal tissues without inflammation (Liu *et al*., 2003; van der Ploeg *et al*., 2004). There may be diversities in virulence among the organisms harbored by individuals who are periodontally healthy and those with periodontitis (Amano *et al*., 2000). Previous studies showed that the virulence of different *P. gingivalis* strains is closely associated with their ability to produce fimbria encoded by the

‡ Corresponding author
§ The two authors equally contributed to this work
* Project (No. 30471888) supported by the National Natural Science Foundation of China

fimbria (*fimA*) gene, collagenase encoded by the collagenase (*prtC*) gene and trypsin-like proteases (Amano *et al*., 2004; Imamura *et al*., 2003; Wittstock *et al*., 2000). However, a few *P. gingivalis* isolates lacking the *prtC* and/or *fimA* genes would result in virulence variation (Fujiwara *et al*., 1994; Slots *et al*., 1995; Wittstock *et al*., 2000). But little is known about the correlation between the *P. gingivalis* strains with virulence variation and the severity of CP in patients.

Actinobacillus actinomycetemcomitan is a gram-negative, facultative anaerobic coccobacillus. The presence of this bacterium and elevated serum antibody titers against the microbe is strongly correlated with the pathogenesis of aggressive periodontitis (Cortelli *et al*., 2005a; Leung *et al*., 2005; Nonnenmacher *et al*., 2001). Although it also exists in CP patients (Cortelli *et al*., 2005a; 2005b) and periodontally healthy individuals (Gafan *et al*., 2004; Yang *et al*., 2005), the proportions and serotypes of infected strains in different periodontal conditions are different (Lakio *et al*., 2002; Yang *et al*., 2005). Neutrophils are successful in eliminating most bacterial pathogens, but *A. actinomycetemcomitans* can produce a unique leukotoxin encoded by the leukotoxin (*lktA*) gene that contributes to the killing of human neutrophils and monocytes (Guthmiller *et al*., 2001; Poulsen *et al*., 2003). *A. actinomycetemcomitans* can also produce fimbria, which is closely associated with its ability to colonize various types of host cells. The fimbria-associated protein gene (*fap*) is strongly expressed in fimbriated *A. actinomycetemcomitans* strains but not in non-fimbriated ones (Ishihara *et al*., 1997). However, the relationship between *A. actinomycetemcomitans* heterogeneous strains and periodontal tissue destruction in CP seems to be rather complex and depends on the virulence of the bacteria (Johansson *et al*., 2005).

In order to evaluate the possible pathogenic role of different *P. gingivalis* and *A. actinomycetemcomitans* strains infecting Chinese CP patients, and to find the relationship between strains with genetic variation of the two microbes and periodontal tissue destruction of CP, two multiplex polymerase chain reaction (PCR) assays were established in this study to detect the *fimA* and *prtC* genes of *P. gingivalis*, and the *fap* and *lktA* genes of *A. actinomycetemcomitans* in subgingival plaque samples and in clinically isolated strains. The *16SrDNAs* of *P. gingivalis* and *A. actinomycetemcomitans* were also included in the PCR assays as internal positive controls for both pathogens.

MATERIALS AND METHODS

Subjects

The subjects were 61 Chinese CP patients [23 males aged 31 to 66 years, mean age was (43.6±9.8) years; 38 females aged 29 to 65 years, mean age was (41.2±7.6) years] and 30 periodontally healthy individuals [13 males aged 27 to 49 years, mean age was (38.5±7.6) years; 17 females aged 32 to 51 years, mean age was (36.2±6.9) years] who were referred to the dental clinic in the Second Affiliated Hospital of School of Medicine of Zhejiang University for dental or periodontal treatment or health monitoring. All the subjects were non-smokers without any systemic disease, and with at least 14 teeth remaining. Those who had received professional cleaning or had history of antibiotic therapy during the preceding 3 months were excluded. All of the patients and the healthy individuals underwent full-mouth examination. The criteria of diagnosis for chronic periodontitis were based on the Classification of Periodontal Diseases issued by the American Academy of Periodontology in 1999 (Armitage, 1999). Briefly, the patients had >30% sites showing periodontal probing depth ≥3 mm, clinical attachment loss >1 mm and radiographic evidence of alveolar bone loss. These individuals were considered periodontally healthy with periodontal probing depth <3 mm, without clinical attachment loss, with no inflammation of gingiva and no alveolar bone absorption on X-ray examination. All the subjects received detailed information concerning the nature of the study and the procedures involved, and their informed consent was obtained.

Sample collection

For each patient, two subgingival plaque samples were taken from the bottom of periodontal pockets from the deepest sites of one front tooth and one molar with separate sterile Gracy curettes after supragingival plaque was gently removed. For each periodontally healthy individual, two samples from the bottom of gingival sulcular of one front tooth and one molar were collected with the same method. Each

plaque sample was placed in 2 ml freshly prepared, pre-reduced fastidious anaerobe broth (Bioconnections, England, UK) for strain isolation and 200 μl lysis buffer (10 mmol/L Tris-HCl, 1.0 mmol/L EDTA, 1.0% Triton X-100, pH 8.0) for PCR assay. The broth containing the subgingival plaque sample was immediately transported to the laboratory while the lysis buffer was stored at −70 °C. The gingival index (*GI*), probing depth (*PD*) and attachment loss (*AL*) of each pocket were recorded.

Isolation and identification of *P. gingivalis* and *A. actinomycetemcomitans* strains

Broth (0.05 ml) containing subgingival plaque sample was inoculated into trypticase soy agar supplemented with haemin (5 μg/ml), vitamin K1 (1 μg/ml), 5% (v/v) sheep blood and menadione (1 μg/ml), or into tryptic soy medium containing 10% (v/v) horse serum, bacitracin (75 μg/ml) and vancomycin (5 μg/ml) respectively for selective recovery of *P. gingivalis* and *A. actinomycetemcomitans*. The bacteria from the subgingival plaque samples were grown in an anaerobic chamber with 10% H_2, 10% CO_2 and 80% N_2 at 37 °C for 7 d. *P. gingivalis* and *A. actinomycetemcomitans* colonies were identified by Gram-staining and morphological examination, biochemical reaction and *P. gingivalis 16SrDNA* or *A. actinomycetemcomitans 16SrDNA* gene detection by PCR. *P. gingivalis* strain ATCC 33277 and *A. actinomycetemcomitans* strain Y4 were also cultured with the above media, respectively.

DNA extraction

Each of the subgingival plaque samples in the lysis buffer was boiled for 10 min, and 10 μl of the supernatant was directly used as template in PCR. *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4 removed from the plates were resuspended in 10 mmol/L PBS (pH 8.0). Then genomic DNA of the bacterial strain, used to optimize PCR conditions and as positive control for PCR, was obtained using the phenol-chloroform method. An OD (optical density) value at 260 nm of the *P. gingivalis* ATCC 33277 or *A. actinomycetemcomitans* Y4 DNA preparation was measured with an ultraviolet spectrophotometer, and was used to calculate the DNA concentration. DNA preparations at 10-fold dilutions (0.1~100 ng) were used as templates for determination of PCR sensitivity.

PCR primers and amplification

Two multiplex PCR assays were established to detect the *P. gingivalis 16SrDNA*, *fimA* and *prtC* genes and the *A. actinomycetemcomitans 16SrDNA*, *lktA* and *fap* genes in the subgingival plaque samples. To detect *P. gingivalis*, PCR amplification was performed in a volume of 100 μl containing 10 μl of the template, 10 μl PCR buffer (20 mmol/L Tris-HCl, 50 mmol/L KCl, pH 8.4) and 5 U Taq polymerase (Promega), 0.25 mmol/L of each dNTP, 2.5 mmol/L $MgCl_2$, 25 pmol primers for the *P. gingivalis 16SrDNA* gene, 50 pmol primers for the *fimA* gene, and 25 pmol primers for the *prtC* gene. The sequences of primers specific for the *P. gingivalis 16SrDNA* gene are: 5′-AGG CAG CTT GCC ATA CTG CG-3′ (sense), and 5′-ACT GTT AGC AAC TAC CGA TGT-3′ (antisense) (Slots *et al*., 1995), for the *fimA* gene: 5′-ATA ATG GAG AAC AGC AGG AA-3′ (sense), and 5′-TCT TGC CAA CCA GTT CCA TTG C-3′ (antisense) (Watanabe and Frommel, 1993), and for the *prtC* gene: 5′-ACA ATC CAC GAG ACC ATC-3′ (sense), and 5′-TTC AGC CAC ACC GAG ACG-3′ (antisense) (Bodinka *et al*., 1994). Expected sizes of the PCR products amplified from the *P. gingivalis 16SrDNA*, *fimA* and *prtC* genes were 404 bp, 131 bp and 584 bp.

To detect *A. actinomycetemcomitans*, PCR amplification was conducted in a volume of 100 μl containing the same contents as mentioned above except for 25 pmol primers for the *A. actinomycetemcomitans 16SrDNA* gene, 50 pmol primers for the *A. actinomycetemcomitans fap* gene and 25 pmol primers for the *A. actinomycetemcomitans lktA* gene. The sequences of primers specific for the *A. actinomycetemcomitans 16SrDNA* gene are: 5′-GCT AAT ACC GCG TAG AGT CGG-3′ (sense), and 5′-ATT TCA CAC CTC ACT TAA AGG T-3′ (antisense) (Slots *et al*., 1995), for the *A. actinomycetemcomitans lktA* gene: 5′-TCG CGA ATC AGC TCG CCG-3′ (sense), and 5′-GCT TTG CAA GCT CCT CAC C-3′ (antisense) (Watanabe and Frommel, 1996), and for the *A. actinomycetemcomitans fap* gene: 5′-ATT AAA TAC TTT AAC TAC TAA AGC-3′ (sense), and 5′-GCA CTG TTA ACT GTA CTA GC-3′ (antisense) (Ishihara *et al*., 1997). Expected sizes of the PCR products amplified from the *A. actinomycetemcomitans 16SrDNA*, *lktA* and *fap* genes were 443 bp, 285 bp and 210 bp.

The PCR programs for detection of *P. gingivalis* and *A. actinomycetemcomitans* were identical, including an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min and extension at 72 °C for 1.5 min, and then a final extension step at 72 °C for 7 min. In each of the PCR assays, 10 ng of *P. gingivalis* ATCC 33277 DNA preparation and 10 ng of *A. actinomycetemcomitans* Y4 DNA were co-amplified with the subgingival plaque samples as positive and negative control respectively for detection of *P. gingivalis*. Ten nanograms of *A. actinomycetemcomitans* Y4 DNA preparation and 10 ng of *P. gingivalis* ATCC 33277 DNA, used as positive and negative controls, were co-amplified with the subgingival plaque samples respectively for detection of *A. actinomycetemcomitans*. All PCR assays were repeated once more, if two results were not consistent then a third time assay was carried out.

Detection of PCR products

Ten microlitres of each reaction product were mixed with 10 μl of 2× loading buffer and fractionated in a 2% agarose gel containing ethidium bromide (1 μg/ml), using a 100 bp DNA ladder (Promega) as a size marker, and visualized under UV light.

Sequence analysis

After being ligated into the plasmid *pUCm-T* vector (Promega), each of the PCR amplicons were transformed into *E. coli* DH5α. Plasmid DNA of white colonies was extracted and then digested with restriction endonuclease *Pst I* (Promega) for examination of the size of the inserted fragment. The plasmid containing the inserted fragment with expected size was used for nucleotide sequencing. Nucleotide sequences of the inserted fragments were analyzed by the dideoxy-chain termination method using an ABI PRISM™ 377 sequencer. The homology of nucleotide and amino acid sequences from the PCR products was compared with the sequences registered in GenBank.

Statistical analysis

Chi-square test was employed to compare the positive rates of *P. gingivalis* and *A. actinomycetemcomitans* in samples from both healthy individuals and CP patients. Fisher's exact test was used when the expected value was smaller than 1. Association between clinical parameters and genotypes was examined using multinomial logistic regression analysis with Stata 8.0 software. *P*-value equal to or below 0.05 was considered statistically significant.

RESULTS

Sensitivity and reproducibility of the PCR assay

Using as low as 0.1 ng DNA template of *P. gingivalis* ATCC 33277 or 10 ng DNA template of *A. actinomycetemcomitans* Y4, the amplification fragments with the expected sizes from the *P. gingivalis 16SrDNA*, *prtC* or *fimA* genes or the *A. actinomycetemcomitans 16SrDNA*, *lktA* or *fap* genes could be revealed in a 2% agarose gel, respectively (Figs.1 and 2). Repeated PCR assays showed 91.9% and 88.7% consistency between each detection of *P. gingivalis 16SrDNA*, *prtC* and *fimA* and *A. actinomycetemcomitans 16SrDNA*, *lktA* and *fap* genes, respectively.

Detection of *P. gingivalis* in subgingival plaque and sulcular samples and clinical isolates

In the 60 sulcular samples from the periodontally healthy individuals, 10.0% of the samples (6/60) were *P. gingivalis 16SrDNA* positive. Among the six *16SrDNA* positive samples, one was *prtC*+/*fimA*+, three were *prtC*+/*fimA*− and two were *prtC*−/*fimA*− (Table 1). Two *P. gingivalis* strains (*16SrDNA*+/*prtC*+/*fimA*+ and *16SrDNA*+/*prtC*+/*fimA*−) were isolated from the 60 sulcular samples.

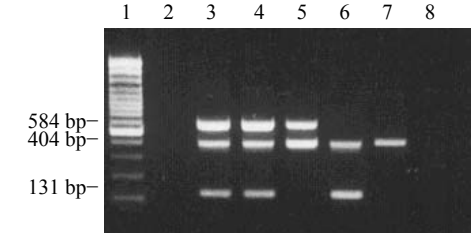

Fig.1 PCR detection of the *P. gingivalis 16SrDNA*, *prtC* and *fimA* genes in subgingival plaque samples
Lane 1: 100 bp marker; Lane 2: Negative control; Lane 3: Positive control; Lane 4: 3 amplification fragments from the *16SrDNA*, *prtC* and *fimA* genes from a plaque sample; Lane 5: 2 amplification fragments from *16SrDNA* and *prtC* from a plaque sample; Lane 6: 2 amplification fragments from *16SrDNA* and *fimA* from a plaque sample; Lane 7: 1 amplification fragment from *16SrDNA*; Lane 8: Blank control

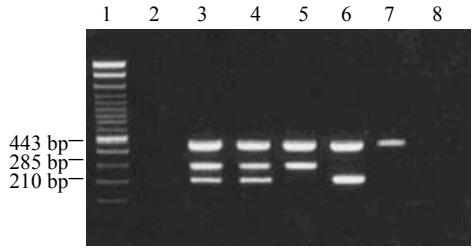

Fig.2 PCR detection of the *A. actinomycetemcomitans 16SrDNA*, *lktA* and *fap* genes in subgingival plaque samples

Lane 1: 100 bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control; Lane 4: 3 amplification fragments from the *16SrDNA*, *lktA* and *fap* genes from a plaque sample; Lane 5: 2 amplification fragments from *16SrDNA* and *lktA* from a plaque sample; Lane 6: 2 amplification fragments from *16SrDNA* and *fap* from a plaque sample; Lane 7: 1 amplification fragment from *16SrDNA*; Lane 8: Blank control

Table 1 Detection of different genotypes of *P. gingivalis* and *A. actinomycetemcomitans* in subgingival plaque samples and in clinical isolates

Genotypes	Clinical isolated strains		Subgingival plaque samples	
	Healthy	CP	Healthy	CP
P. gingivalis				
16SrDNA+/prtC+/fimA+	1	40	1	93
16SrDNA+/prtC+/fimA−	1	5	3	11
16SrDNA+/prtC−/fimA+	0	3	0	5
16SrDNA+/prtC−/fimA−	0	0	2	4
A. actinomycetemcomitans				
16SrDNA+/lktA+/fap+	1	17	1	58
16SrDNA+/lktA+/fap−	1	4	3	34
16SrDNA+/lktA−/fap+	0	2	0	3
16SrDNA+/lktA−/fap−	0	0	0	8

A hundred and thirteen out of the 122 subgingival plaque samples (92.6%) were *P. gingivalis 16SrDNA* positive while the rest of the 9 samples (7.4%) were not. As expected, none of the *P. gingivalis 16SrDNA* negative plaque samples were positive for *prtC* and/or *fimA*. Four genotypes (*16SrDNA+/prtC+/fimA+*, *16SrDNA+/prtC+/fimA−*, *16SrDNA+/prtC−/fimA+* and *16SrDNA+/prtC−/fimA−*) were found in the subgingival plaque samples (Table 1). In the 122 subgingival plaque samples, the positive rate of *P. gingivalis 16SrDNA* (92.6%) was significantly higher than that of *fimA* (98/122, 80.3%) (χ^2=7.890, P<0.01). However, there were no statistical differences between the positive rates of *P. gingivalis 16SrDNA* (92.6%) and *prtC* (104/122, 85.2%) (χ^2=3.370, P>0.05), nor between that of *prtC* and *fimA* (χ^2=1.04, P>0.05).

Fourty-eight strains of *P. gingivalis* were isolated from 38 out of the 61 patients (62.3%), all of which had detectable *16SrDNA*. But three of the strains (6.3%) were negative for *prtC*, and five (10.4%) were negative for *fimA*. Anyhow the genotypes of all isolated strains of *P. gingivalis* were consistent with those detected directly in the corresponding subgingival plaque samples.

The positive rate of *P. gingivalis 16SrDNA+/prtC+/fimA+* detected directly in subgingival plaque samples or in clinical isolates was significantly higher in CP than in periodontally healthy individuals (P=0.001; P=0.000), while the positive rates of the other three or two genotypes showed no statistical difference between CP and healthy controls (P= 0.132~0.645; P=0.355, 0.299).

Detection of *A. actinomycetemcomitans* in subgingival plaque and sulcular samples and clinical isolates

In the 60 sulcular samples from the periodontally healthy individuals, 6.7% of the samples (4/60) were *A. actinomycetemcomitans 16SrDNA* positive. One was positive for both *lktA* and *fap* and three were positive for *lktA* but negative for *fap* (Table 1). Two *A. actinomycetemcomitans* strains (*16SrDNA+/lktA+/fap+* and *16SrDNA+/lktA+/fap−*) were isolated from the 60 sulcular samples.

While 103 of the 122 subgingival plaque samples (84.4%) were *A. actinomycetemcomitans 16SrDNA* positive, only 19 samples (15.6%) showed no detectable *A. actinomycetemcomitans 16SrDNA*. As expected, none of the *A. actinomycetemcomitans 16SrDNA* negative samples were positive for either *lktA* or *fap*. There were four genotypes of *A. actinomycetemcomitans*: *16SrDNA+/lktA+/fap+*, *16SrDNA+/lktA+/fap−*, *16SrDNA+/lktA−/fap+*, and *16SrDNA+/lktA−/fap−* in the subgingival plaque samples (Table 1). In the 122 subgingival plaque samples, there was no statistical difference between the positive rates of *A. actinomycetemcomitans 16SrDNA* (84.4%) and *lktA* (92/122, 75.4%) (χ^2=3.090, P>0.05). However, the positive rate of *fap* was much lower than that of the other two genes (χ^2=16.820, 32.810; P<0.01).

Twenty-three strains of *A. actinomycetemcomitans* were isolated from 18 patients (29.5%). All of

the strains were positive for *A. actinomycetemcomitans 16SrDNA*, except that 4 of the strains were negative for the *fap* gene and 2 was negative for *lktA*. Furthermore, the genotype detected directly in the subgingival plaque samples was consistent with those of all isolated strains of *A. actinomycetemcomitans*.

In subgingival plaque samples, the positive rates of *A. actinomycetemcomitans 16SrDNA+/lktA+/fap+*, *16SrDNA+/lktA+/fap−* and *16SrDNA+/lktA−/fap−* were significantly higher in CP than that in healthy controls (P=0.000, 0.000, 0.038), while that of *16SrDNA+/lktA−/fap+* was not statistically different between the two groups (P=0.299). However, in clinical isolates, only *A. actinomycetemcomitans 16SrDNA+/lktA+/fap+* showed higher frequency in CP than in healthy controls (P=0.005), while the positive rates of the other two genotypes showed no significant difference between the two groups (P=0.466, 0.448).

Nucleotide sequence analysis

The PCR products of *P. gingivalis 16SrDNA* amplified from 10 representative subgingival plaque samples showed 98.62%~99.72% homology with the nucleotide sequence registered in GenBank (accession No. L16492), and the amplification fragments for the *prtC* and *fimA* genes of the same 10 samples showed between 99.09%~99.45% and between 98.89%~100% homology, respectively, with the nucleotide sequences registered in GenBank (accession Nos. AB006973 and AB004560).

In this study, the PCR products of *A. actinomycetemcomitans 16SrDNA* amplified from 10 subgingival plaque samples showed 98.75%~99.85% homology compared with the GenBank sequences (accession No. M75036) respectively, while that of the *lktA* and *fap* genes was between 99.46%~99.76% and between 99.45%~99.55%, respectively (accession Nos. X16829 and D83053).

Association between clinical parameters and *P. gingivalis* genotypes in subgingival plaque samples and clinically isolated strains

P. gingivalis with the *16SrDNA+/prtC+/fimA+* genotype was the predominant genotype detected in subgingival plaque samples and clinical isolates (93/113, 82.3%; 40/48, 83.8%). Multinomial logistic regression analysis suggested that both in the 122 subgingival plaque samples and in the 48 clinically isolated *P. gingivalis* strains, the *16SrDNA+/prtC+/fimA+* genotype might be closely associated with *PD* (P=0.023, OR=3.905; P=0.000, OR=17.953), *AL* (P=0.018, OR=5.408; P=0.000, OR=15.779), *GI* (P=0.004, OR=4.588; P=0.001, OR=3.202), and tooth site (P=0.026, OR=11.077; P=0.000, OR=5.996) while the other three genotypes did not show any association with these clinical parameters (P=0.395~0.925) (Table 2). The positive rate of *P. gingivalis* with the *16SrDNA+/prtC+/fimA+* genotype was higher in deep pockets (>6 mm) than that in shallow (3~4 mm) (P=0.000; P=0.001) or moderate depth ones (>4 mm and ≤6 mm) (P=0.002; P=0.003), yet without significant difference between shallow and moderate pockets (P=0.062; P=0.177). It was more frequently detected in AL≥5 mm sites than in AL≤2 mm sites (P=0.000; P=0.000) or in the 2 mm<AL<5 mm sites (P=0.012; P=0.000). But no statistical difference could be found between the AL≤2 mm and the 2 mm<AL<5 mm ones (P=0.299; P=0.035, α'=0.017). In the GI=3 groups, *P. gingivalis* with the *16SrDNA+/prtC+/fimA+* displayed much higher frequency than in GI=1 groups (P=0.000; P=0.013), but there was not discrepancy between GI=1 and GI=2 (P=0.024, α'=0.017; P=0.205) or between GI=2 and GI=3 groups (P=0.037, α'=0.017; P=0.123). In molars, its presence was also higher than that in front teeth (P=0.023; P=0.037).

Further analysis indicated that in subgingival samples and clinical isolates, *P. gingivalis* with *fimA+* genotype (*16SrDNA+/prtC+/fimA+* and *16SrDNA+/prtC−/fimA+*) might correlate with *PD* (P=0.007, OR=3.580; P=0.017, OR=5.215), *AL* (P=0.017, OR=2.813; P=0.008, OR=7.014), and *GI* (P=0.027, OR=2.339; P=0.013, OR=5.526) compared to *fimA−* genotype (*16SrDNA+/prtC+/fimA−* and *16SrDNA+/prtC−/fimA−*). No statistical difference could be found in the distribution of *P. gingivalis* with *fimA+* genotype in different tooth sites (P=0.108, OR=2.558; P=0.132, OR=4.364). In subgingival samples, *P. gingivalis* with *fimA+* was more frequently detected in deep pockets than in shallow ones (P=0.002), yet without significant difference between moderate and deep ones (P=0.031, α'=0.017) or between shallow and moderate ones (P=0.222). It also showed a higher frequency in AL≥5 mm sites than that in AL≤2 mm (P=0.004) or the 2 mm<AL<5 mm sites (P=0.016).

Table 2 Distribution of *P. gingivalis* and *A. actinomycetemcomitans* genotypes in subgingival plaque samples and in clinical isolates from CP patients and its correlation with clinical parameters

Groups	Cases (*n*)	*P. gingivalis 16SrDNA+*				*A. actinomycetemcomitans 16SrDNA+*			
		prtC+ fimA+	*prtC+ fimA−*	*prtC− fimA+*	*prtC− fimA−*	*lktA+ fap+*	*lktA+ fap−*	*lktA− fap+*	*lktA− fap−*
PD									
≤4 mm	49	29 (1)	7 (1)	4 (2)	3 (0)	18 (5)	12 (0)	2 (1)	5 (0)
>4 mm and ≤6 mm	39	30 (10)	4 (4)	1 (1)	1 (0)	18 (5)	10 (2)	1 (1)	3 (0)
>6 mm	34	34* (29*)	0 (0)	0 (0)	0 (0)	22* (7)	12* (2)	0 (0)	0 (0)
AL									
≤2 mm	51	31 (1)	6 (2)	4 (2)	3 (0)	19 (5)	13 (0)	2 (1)	6 (0)
>2 mm and <5 mm	38	29 (11)	5 (3)	1 (1)	1 (0)	19 (6)	9 (1)	1 (1)	2 (0)
≥5 mm	33	33* (28*)	0 (0)	0 (0)	0 (0)	20* (6)	12* (3)	0 (0)	0 (0)
GI									
1	18	9 (2)	3 (2)	1 (1)	2 (0)	6 (2)	4 (1)	1 (0)	3 (0)
2	46	33 (9)	3 (2)	2 (1)	2 (0)	22 (5)	14 (2)	1 (1)	2 (0)
3	58	51* (29*)	5 (1)	2 (1)	0 (0)	30 (10)	16 (1)	1 (1)	3 (0)
Tooth site									
Front tooth	61	39 (9)	7 (3)	4 (2)	3 (0)	33 (11)	18 (3)	1 (1)	3 (0)
Molar	61	54* (31*)	4 (2)	1 (1)	1 (0)	25 (6)	16 (1)	2 (1)	5 (0)

Data in the parenthesis are from *P. ginglvalis* or *A. actinomycetemcomtitans* clinical isolates; * $P<0.05$

But its positive rates between the *AL*≤2 mm and the 2 mm<*AL*<5 mm ones were not statistically different (P=0.666). In *GI*=3 group, the frequency of *P. gingivalis* with *fimA*+ was higher than that in *GI*=1 group (P=0.013), with no statistical difference between *GI*=3 and *GI*=2 (P=0.533) or between *GI*=1 and *GI*=2 groups (P=0.674). However, in clinical isolates, post hoc tests did not show any significant difference in the positive rates of *P. gingivalis* with *fimA*+ in different *PD* (P=0.121, 0.946), *AL* (P=0.019~0.366, α'=0.017) or *GI* groups (P=0.045~0.330, α'=0.017), except that between deep pockets and moderate depth pockets (P=0.010).

Both in subgingival samples and clinical isolates, *P. gingivalis* with *prtC*+ genotype (*16SrDNA+*/*prtC+*/*fimA+* and *16SrDNA+*/*prtC+*/*fimA−*) might associate with *PD* (P=0.023, *OR*=5.038; P=0.014, *OR*=16.420) and *AL* (P=0.026, *OR*=4.842; P=0.021, *OR*=13.072), compared to *prtC−* genotype (*16SrDNA+*/*prtC−*/*fimA+* and *16SrDNA+*/*prtC−*/*fimA−*). *P. gingivalis* with *prtC*+ genotype was more frequently detected in deep pockets than in shallow pockets (P=0.013; P=0.011), but was no discrepancy between deep and moderate ones (P=0.261; P=0.341) or between shallow and moderate ones (P=0.127; P=0.097). In subgingival plaque samples, the positive rate of the *prtC*+ genotype in *AL*≥5 mm sites was higher than that in *AL*≤2 mm sites (P=0.016), with no difference between *AL*≥5 mm and the 2 mm<*AL*<5 mm ones (P=0.269) or between *AL*≤2 mm and the 2 mm<*AL*<5 mm ones (P=0.135). However, in clinical isolates post hoc tests indicated that the frequency of the *prtC*+ genotype in the different *AL* groups showed only slight difference (P=0.019~0.349, α'=0.017). Although multinomial logistic regression analysis of subgingival plaque samples suggested that *P. gingivalis* with *prtC*+ genotype might also correlate with *GI* (P=0.035, *OR*=2.756), but further analysis showed that its positive rate was not statistically different among the three *GI* groups (P=0.056~0.284). Compared to the *prtC−* genotype, no significant difference was found in the distribution of *P. gingivalis* with *prtC*+ genotype in different tooth sites in subgingival plaque samples and in clinical isolates (P=0.072, *OR*=4.413; P=0.180, *OR*=5.500) or that in the three *GI* groups in clinical isolates (P=0.147, *OR*=3.052).

Association between clinical parameters and *A. actinomycetemcomitans* genotypes in subgingival plaque samples and clinically isolated strains

Multinomial logistic regression analysis suggested that in the 122 subgingival plaque samples *A. actinomycetemcomitans* with *16SrDNA+*/*lktA+*/*fap+* or *16SrDNA+*/*lktA+*/*fap−* genotypes might associate

with *PD* (*P*=0.002, *OR*=3.575; *P*=0.008, *OR*=3.215) and *AL* (*P*=0.015, *OR*=2.510; *P*=0.035, *OR*=2.340) rather than the other two genotypes (*P*=0.415~0.980). No correlation was found between any one of the four genotypes or the *lktA*+ genotypes and *GI* or tooth site (*P*=0.061~0.952). *A. actinomycetemcomitans* with *lktA*+ genotype (*16SrDNA*+/*lktA*+/*fap*+ and *16SrDNA*+/*lktA*+/*fap*−) was more frequently detected in deep pockets than that in shallow pockets (*P*=0.008), with no significant difference between deep and moderate ones (*P*=0.050) or between moderate and shallow ones (*P*=0.348). In sites with *AL*≥5 mm, the positive rate of *A. actinomycetemcomitans* with *lktA*+ was higher than that in *AL*≤2 mm sites (*P*=0.006), while no statistical difference was shown between *AL*≥5 mm and the 2 mm<*AL*<5 mm sites (*P*=0.113) or between the 2 mm<*AL*<5 mm and *AL*≤2 mm ones (*P*=0.196). However, in the 23 clinically isolated *A. actinomycetemcomitans* strains, no association could be established between any one of the four genotypes with *PD*, *AL*, *GI* or tooth site (*P*=0.065~0.921). Moreover, in subgingival plaque samples and clinical isolates, statistical difference did not appear in the positive rate of *A. actinomycetemcomitans* with *fap*+ genotype (*16SrDNA*+/*lktA*+/*fap*+ and *16SrDNA*+/*lktA*−/*fap*+) in different *PD*, *AL*, *GI* or tooth site groups (*P*=0.139~0.654) compared with that of the *fap*− genotype (*16SrDNA*+/*lktA*+/*fap*− and *16SrDNA*+/*lktA*−/*fap*−).

DISCUSSION

There is a growing body of evidence linking *P. gingivalis* and *A. actinomycetemcomitans* with pathogenesis of periodontitis (Cortelli *et al*., 2005a; Liu *et al*., 2003; Yuan *et al*., 2001). In previous studies, *P. gingivalis* isolates lacking *prtC* and/or *fimA* and *A. actinomycetemcomitans* isolates lacking *fap* were found (Fujiwara *et al*., 1994; Slots *et al*., 1995; Ishihara *et al*., 1997). However, the clinical significance of this virulence variation for both microbes was not understood. Routine diagnosis assays such as culture methods and biochemical reactions, enzyme-linked immunosorbent assays (ELISA) and immunofluorescence had been used to identify *P. gingivalis* and *A. actinomycetemcomitans* in clinical samples; but none of these diagnostic methods had focused on potential virulence factors. In the present study, multiplex PCR assays were adopted to detect the toxic genes of *P. gingivalis* and *A. actinomycetemcomitans*, using *16SrDNA* as internal positive control. Multiplex PCR assays revealed clear PCR products with expected sizes (Figs.1 and 2). Repeated PCR amplification also demonstrated high reproducibility of the assays (91.9% and 88.7%). The genotypes of all cultured strains of *P. gingivalis* and *A. actinomycetemcomitans* were consistent with those detected directly in the corresponding subgingival plaque samples. Moreover, nucleotide sequence analysis showed high homology (98.62%~100%) of the PCR products for these toxic genes from partial subgingival plaque samples with the reported sequences. All the results from this study indicated that multiplex PCR could be used as a reliable method for diagnosis of the toxic genes of these two microbes in clinical samples.

The correlation between the infection of *P. gingivalis* and *A. actinomycetemcomitans* with different genotypes and periodontal destruction of CP is an interesting and important subject for investigation. Previous reports indicated that *P. gingivalis* was frequently detected in serious periodontal destruction sites with deep pockets (Noiri *et al*., 2001; Darout *et al*., 2003; Takeuchi *et al*., 2001), whereas the presence of *A. actinomycetemcomitans* in deep pockets was only occasionally (Noiri *et al*., 2001; Hamlet *et al*., 2001). But in this study, it was shown that *P. gingivalis* with *prtC*+/*fimA*+ and *A. actinomycetemcomitans* with *lktA*+ were predominant in teeth with deep pockets or serious attachment loss, suggesting that these genotypes might exhibit greater periodontal destruction potential than strains lacking either one or two of the toxic genes. *P. gingivalis* strains with *prtC*+/*fimA*+ were also found to be correlated with *GI* and tooth site. It was more frequently detected in sites with *GI*=3 and in molars, which implied its possible role in inflammation of periodontal tissue and potential inclination for colonization in molars rather than front teeth. *A. actinomycetemcomitans* with *lktA*+ was not associated with *GI* or tooth site, which correlated with previous reports (Tan *et al*., 2001). These studies indicated *P. gingivalis*'s presence in periodontal pockets was related with higher gingival index or deeper pocket depth (Yuan *et al*., 2001; Chen *et al*., 2005), while *A. actinomycetemcomitans*'s association

with *GI* was not found (Tan *et al*., 2001; Nogueira *et al*., 2004). However, unlike the same results obtained from clinically isolated *P. gingivalis* strains and from subgingival plaque samples, in *A. actinomycetemcomitans* clinical isolates, no correlation could be established between *lktA*+ strains and *PD* or *AL*. This might be explained by the relatively low culture frequency of this microbe in this study.

Fimbriae were crucial pathogenic factors for *P. gingivalis* during adherence and colonization of periodontal epithelial cells (Amano *et al*., 1999; Amano *et al*., 2004). *P. gingivalis* expressed two distinct fimbria molecules, major and minor fimbriae, on its cell surfaces (Amano *et al*., 2004; Amano, 2003). The *fimA* gene, which encoded the major fimbriae—fimbrillin, has been classified into six variants (type I through V and Ib) on the basis of their nucleotide sequences. It was demonstrated that the fimbria variations might have an influence on the development of periodontal disease (Amano *et al*., 2004; Beikler *et al*., 2003a; Tamura *et al*., 2005), and that type II *fimA* was predominant in deep pockets (Amano *et al*., 1999; 2004). In the present study, PCR primers for *fimA* were designed based on DNA alignment of type I *fimA* gene by Watanabe and Frommel (1993), which could not detect types IV and V *fimA*. It was found that *P. gingivalis* with *fimA*+ was more frequently detected in deep pockets or in serious attachment loss sites than *P. gingivalis* strains with *fimA*−, indicating that the *fimA* gene of *P. gingivalis* was associated with periodontal destruction. It showed higher frequency in *GI*=3 sites which also suggested its correlation with inflammation of gingiva. As collagen is an important component of the periodontium, collagenase activity might play a role in tissue destruction and progression of periodontitis (Wittstock *et al*., 2000; Potempa *et al*., 2000; Beikler *et al*., 2003b). The *prtC* gene described by Kato *et al*.(1992) was 1002 bp in length and encoded a protein with 333 amino acids and a calculated mass of 37.8 kDa. Specific cleavage of type I collagen had been attributed to the function of the *prtC* gene product, which was referred to as collagenase (Kato *et al*., 1992). But the relationship between *prtC* and severity of the disease remained controversial (Bodinka *et al*., 1994; Wittstock *et al*., 2000; Potempa *et al*., 2000). This study showed that *P. gingivalis* with *prtC*+ was more frequently found in deep pockets or in serious attachment loss sites than *P. gingivalis* strains with *prtC*−, suggesting that the *prtC* gene of *P. gingivalis* was also associated with periodontal destruction.

Leukotoxin, a unique toxic factor produced by *A. actinomycetemcomitans*, could kill polymorphonuclear leukocytes in periodontal tissue, thus enabling the bacterium to escape an important part of the innate host immune response and was thus considered a significant virulence factor in periodontitis (Guthmiller *et al*., 2001; Poulsen *et al*., 2003). Greater mean attachment loss was found in subjects with highly leucotoxic *A. actinomycetemcomitans* than in subjects with minimally leucotoxic or subjects not infected (Cortelli *et al*., 2005a). In this study, it was found that *A. actinomycetemcomitans* with *lktA*+ genotype associated with periodontal destruction and that the frequency of *lktA*+ genotypes was significantly higher in deep pockets or in serious periodontal destruction sites, which demonstrated the clinical importance of the *lktA* of *A. actinomycetemcomitans* in the pathogenesis of CP. The fimbriae were generally critical for the bacteria to adhere to cells, which was an important step in pathogenesis. However, no correlation could be established between *A. actinomycetemcomitans* strains with *fap*+ (encoding fimbriae) and periodontal destruction in the present study. Hence it suggested that rather than directly adhere to cells and exert destructive effect on periodontal tissue, *A. actinomycetemcomitans* might inhibit local immune response by producing leukotoxin and thus enable the host prone to infection by other periodontal pathogens to develop chronic peridontitis.

A. actinomycetemcomitans was thought to be a major pathogen of aggressive periodontitis (Leung *et al*., 2005; Nonnenmacher *et al*., 2001). Although the primer set for *16SrDNA* of *A. actinomycetemcomitans* designed by Slots *et al*.(1995) is known to cross-react with *16SrDNA* of *Haemophilus* species, with the *lktA* specific primers, a high infection rate of *A. actinomycetemcomitans* was shown in CP patients by this study, which suggested that this microbe might also be an important factor in the pathogenesis of Chinese CP patients. The positive culture rate of *A. actinomycetemcomitans* from subgingival plaque samples was much lower when compared with that of PCR. This discordance between the positive rates of the PCR assay and the culture method might be due to the fact that a positive culture required a larger number of

bacteria in clinical samples than in PCR. Further study would be needed to define its role in periodontal tissue destruction in CP. Although a long time study is necessary to establish whether *P. gingivalis* with *prtC*+/*fimA*+ or *A. actinomycetemcomitans* with *lktA*+ is a definitive factor for establishing periodontitis, this genotype is most likely to be an infectious agent contributing to the etiology. Future study should focus on the genotypic diversity of the *fimA* and *prtC* genes of *P. gingivalis* and the *lktA* gene of *A. actinomycetemcomitans* to elucidate its association with the pathogenesis of periodontitis.

References

Amano, A., 2003. Molecular interaction of *Porphyromonas gingivalis* with host cells: implication for the microbial pathogenesis of periodontal disease. *J. Periodontol.*, **74**(1):90-96. [doi:10.1902/jop.2003.74.1.90]

Amano, A., Nakagawa, I., Kataoka, K., Morisaki, I., Hamada, S., 1999. Distribution of *Porphyromonas gingivalis* strains with *fimA* genotypes in periodontitis patients. *J. Clin. Microbiol.*, **37**(5):1426-1430.

Amano, A., Kuboniwa, M., Nakagawa, I., Akiyama, S., Morisaki, I., Hamada, S., 2000. Prevalence of specific genotypes of *Porphyromonas gingivalis* fimA and periodontal health status. *J. Dent. Res.*, **79**(9):1664-1668.

Amano, A., Nakagawa, I., Okahashi, N., Hamada, N., 2004. Variations of *Porphyromonas gingivalis* fimbriae in relation to microbial pathogenesis. *J. Periodontal Res.*, **39**(2):136-142. [doi:10.1111/j.1600-0765.2004.00719.x]

Armitage, G.C., 1999. Development of a classification system for periodontal disease and conditions. *Ann. Periodontol.*, **4**(1):1-6. [doi:10.1902/annals.1999.4.1.1]

Beikler, T., Peters, U., Prajaneh, S., Prior, K., Ehmke, B., Flemmig, T.F., 2003a. Prevalence of *Porphyromonas gingivalis* fimA genotypes in Caucasians. *Eur. J. Oral Sci.*, **111**(5):390-394. [doi:10.1034/j.1600-0722.2003.00065.x]

Beikler, T., Ehmke, B., Wittstock, M., Schmidt, H., Karch, H., Flemmig, T.F., 2003b. Serum antibody reactivity against recombinant PrtC of Porphyromonas gingivalis following periodontal therapy. *J. Periodontal Res.*, **38**(3):276-281.

Bodinka, A., Schmidt, H., Henkel, B., Flemmig, T.F., Klaiber, B., Karch, H., 1994. Polymerase chain reaction for the identification of *Porphyromonas gingivalis* collagenase genes. *Oral Microbiol. Immunol.*, **9**(3):161-165.

Chen, L.L., Wu, Y.M., Yan, J., Sun, W.L., Sun, Y.Z., Ojcius, D., 2005. Association between coinfection of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Treponema denticola* and periodontal tissue destruction in chronic periodontitis. *Chin. Med. J. (Engl.)*, **118**(11):915-921.

Cortelli, J.R., Cortelli, S.C., Jordan, S., Haraszthy, V.I., Zambon, J.J., 2005a. Prevalence of periodontal pathogens in Brazilians with aggressive or chronic periodontitis. *J. Clin. Periodontol.*, **32**(8):860-866. [doi:10.1111/j.1600-051X.2005.00777.x]

Cortelli, S.C., Feres, M., Rodrigues, A.A., Aquino, D.R., Shibli, J.A., Cortelli, J.R., 2005b. Detection of *Actinobacillus actinomycetemcomitans* in unstimulated saliva of patients with chronic periodontitis. *J. Periodontol.*, **76**(2):204-209. [doi:10.1902/jop.2005.76.2.204]

Darout, I.A., Skaug, N., Albandar, J.M., 2003. Subgingival microbiota levels and their associations with periodontal status at the sampled sites in an adult Sudanese population using miswak or toothbrush regularly. *Acta Odontol. Scand.*, **61**(2):115-122. [doi:10.1080/00016350310002784]

Fujiwara, T., Nakagawa, I., Morishima, S., Takahashi, I., Hamada, S., 1994. Inconsistency between the fimbrillin gene and the antigenicity of lipopolysaccharides in selected strains of *Porphyromonas gingivalis*. *FEMS Microbiol. Lett.*, **124**(3):333-341. [doi:10.1111/j.1574-6968.1994.tb07305.x]

Gafan, G.P., Lucas, V.S., Roberts, G.J., Petrie, A., Wilson, M., Spratt, D.A., 2004. Prevalence of periodontal pathogens in dental plaque of children. *J. Clin. Microbiol.*, **42**(9):4141-4146. [doi:10.1128/JCM.42.9.4141-4146.2004]

Guthmiller, J.M., Lally, E.T., Korostoff, J., 2001. Beyond the specific plaque hypothesis: are highly leukotoxic strains of *Actinobacillus actinomycetemcomitans* a paradigm for periodontal pathogenesis? *Crit. Rev. Oral Biol. Med.*, **12**(2):116-124.

Hamlet, S.M., Cullinan, M.P., Westerman, B., Lindeman, M., Bird, P.S., Palmer, J., Seymour, G.J., 2001. Distribution of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* in an Australian population. *J. Clin. Periodontol.*, **28**(12):1163-1171. [doi:10.1034/j.1600-051X.2001.281212.x]

Imamura, T., Travis, J., Potempa, J., 2003. The biphasic virulence activities of gingipains: activation and inactivation of host proteins. *Curr. Protein Pept. Sci.*, **4**(6):443-450. [doi:10.2174/1389203033487027]

Ishihara, K., Honma, K., Miura, T., 1997. Cloning and sequence analysis of the fimbriae associated protein (fap) gene from *Actinobacillus actinomycetemcomitans*. *Microb. Pathog.*, **23**(2):63-69. [doi:10.1006/mpat.1997.0137]

Johansson, A., Buhlin, K., Koski, R., Gustafsson, A., 2005. The immunoreactivity of systemic antibodies to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in adult periodontitis. *Eur. J. Oral Sci.*, **113**(3):197-202. [doi:10.1111/j.1600-0722.2005.00218.x]

Kato, T., Takahashi, N., Kuramitsu, H.K., 1992. Sequence analysis and characterization of the *Porphyromonas gingivalis prtC* gene, which expresses a novel collagenase activity. *J. Bacteriol.*, **174**(12):3889-3895.

Lakio, L., Kuula, H., Dogan, B., Asikainen, S., 2002. *Actinobacillus actinomycetemcomitans* proportion of subgingival bacterial flora in relation to its clonal type. *Eur. J. Oral Sci.*, **110**(3):212-217. [doi:10.1034/j.1600-0447.

2002.201238.x]

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. *J. Periodontal Res.*, **40**(3):258-268. [doi:10.1111/j.1600-0765.2005.00805.x]

Liu, L., Wen, X., He, H., Shi, J., Ji, C., 2003. Species-specific DNA probe for the detection of *Porphyromonas gingivalis* from adult Chinese periodontal patients and healthy subjects. *J. Periodontol.*, **74**(7):1000-1006. [doi:10.1902/jop.2003.74.7.1000]

Nogueira Moreira, A., Chiappe, V., Fernandez Caniggia, L., Alonso, C., Piovano, S., 2004. Clinical and microbiological associations in chronic periodontitis. *Acta Odontol. Latinoam.*, **17**(1-2):15-21.

Noiri, Y., Li, L., Ebisu, S., 2001. The localization of periodontal-disease-associated bacteria in human periodontal pockets. *J. Dent. Res.*, **80**(10):1930-1934.

Nonnenmacher, C., Mutters, R., de Jacoby, L.F., 2001. Microbiological characteristics of subgingival microbiota in adult periodontitis, localized juvenile periodontitis and rapidly progressive periodontitis subjects. *Clin. Microbiol. Infect.*, **7**(4):213-217. [doi:10.1046/j.1469-0691.2001.00210.x]

Potempa, J., Banbula, A., Travis, J., 2000. Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontology 2000*, **24**(1):153-192. [doi:10.1034/j.1600-0757.2000.2240108.x]

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. *J. Clin. Microbiol.*, **41**(10):4829-4832. [doi:10.1128/JCM.41.10.4829-4832.2003]

Slots, J., Ashimoto, A., Flynn, M.J., Li, G.L., Chen, C., 1995. Detection of putative pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin. Infect. Dis.*, **20**(Suppl. 2):304-307.

Takeuchi, Y., Umeda, M., Sakamoto, M., Benno, Y., Huang, Y., Ishikawa, I., 2001. *Treponema socranskii*, *Treponema denticola*, and *Porphyromonas gingivalis* are associated with severity of periodontal tissue destruction. *J. Periodontol.*, **72**(10):1354-1363. [doi:10.1902/jop.2001.72.10.1354]

Tamura, K., Nakano, K., Nomura, R., Miyake, S., Nakagawa, I., Amano, A., Ooshima, T., 2005. Distribution of *Porphyromonas gingivalis* fimA genotypes in Japanese children and adolescents. *J. Periodontol.*, **76**(5):674-679. [doi:10.1902/jop.2005.76.5.674]

Tan, K.S., Woo, C.H., Ong, G., Song, K.P., 2001. Prevalence of *Actinobacillus actinomycetemcomitans* in an ethnic adult Chinese population. *J. Clin. Periodontol.*, **28**(9):886-890. [doi:10.1034/j.1600-051x.2001.028009886.x]

van der Ploeg, J.R., Giertsen, E., Ludin, B., Morgeli, C., Zinkernagel, A.S., Gmur, R., 2004. Quantitative detection of *Porphyromonas gingivalis* fimA genotypes in dental plaque. *FEMS Microbiol. Lett.*, **232**(1):31-37. [doi:10.1016/S0378-1097(04)00064-3]

Watanabe, K., Frommel, T.O., 1993. Detection of *Porphyromonas gingivalis* in oral plaque samples by use of the polymerase chain reaction. *J. Dent. Res.*, **72**(6):1040-1044.

Watanabe, K., Frommel, T.O., 1996. *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Treponema denticola* detection in oral plasque samples using the polymerase chain reaction. *J. Clin. Periodontol.*, **23**(3):212-219. [doi:10.1111/j.1600-051X.1996.tb02078.x]

Wittstock, M., Schmidt, H., Flemmig, T.F., Karch, H., 2000. Heterogeneity of the prtC gene of *Porphyromonas gingivalis*. *Oral Microbiol. Immunol.*, **15**(1):33-39. [doi:10.1034/j.1399-302x.2000.150106.x]

Yang, H.W., Huang, Y.F., Chan, Y., Chou, M.Y., 2005. Relationship of *Actinobacillus actinomycetemcomitans* serotypes to periodontal condition: prevalence and proportions in subgingival plaque. *Eur. J. Oral Sci.*, **113**(1):28-33. [doi:10.1111/j.1600-0722.2004.00192.x]

Yuan, K., Chang, C.J., Hsu, P.C., Sun, H.S., Tseng, C.C., Wang, J.R., 2001. Detection of putative periodontal pathogens in non-insulin-dependent diabetes mellitus and non-diabetes mellitus by polymerase chain reaction. *J. Periodontal Res.*, **36**(1):18-24. [doi:10.1034/j.1600-0765.2001.90613.x]