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Original article

Association between coinfection of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Treponema denticola* and periodontal tissue destruction in chronic periodontitis

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Background   The association between the infection of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Treponema denticola* in chronic periodontitis (CP) and the severity of periodontal disease remains to be elucidated. The aim of this study was to investigate the subgingival infection frequencies of three periodontopathic bacteria in Chinese CP patients and to evaluate the correlations between infection by these bacteria and periodontal destruction.

Methods   A multiple PCR assay using primers derived from *16SrDNA* genes of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* was established to measure simultaneously the presence of the three microbes in 162 subgingival samples from 81 Chinese CP patients.

Results   The positive rates of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* in the subgingival samples were 84.6%, 83.3% and 88.3%, respectively. Of the subgingival samples, 68% revealed the coinfection of all the three microbes. The infection rates with *P. gingivalis*, *A. actinomycetemitans* or *T. denticola* alone was 5.9% (1/17), 17.6% (3/17) and 76.5% (13/17), respectively. A close association was present between the *A. actinomycetemitans* infection and gingival index (GI) (P < 0.01), but not between *P. gingivalis* or *T. denticola* infection and GI (P > 0.05). *P. gingivalis* and *A. actinomycetemitans* were more frequently detectable in middle and deep pockets than in shallow ones (P < 0.01), while *T. denticola* was found remarkably often in deep pockets (P < 0.05). The coinfection rate of the three microbes was significantly higher in sites with severe periodontitis than in those with mild periodontitis (P < 0.01).

Conclusions   The multiple PCR established in this study can be used as a sensitive and specific method to simultaneously detect all three microbes in subgingival samples. *A. actinomycetemitans* infection may be associated with CP and play an important role in the periodontal tissue destruction. The coinfection of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* can cause more serious periodontal destruction than infection of any one or two of the three microbes.

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Periodontitis is a common oral disease characterized by alveolar bone destruction and pocket formation. This disease is generally divided into two clinical types: chronic periodontitis (CP) and aggressive periodontitis (AgP). The former exhibits obvious local inflammation and the latter is characterized by destructive immunoreactions, which result in adoption of different therapeutic

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strategies.[1] It is widely accepted that periodontitis occurs as a result of infection by subgingival bacteria, particularly gram-negative anaerobes.[1,2] In the earlier epidemiological data, Porphyromonas gingivalis was considered to be responsible for CP,[3,4] whereas Actinobacillus actinomycetem-comitans was confirmed to be a specific causative agent of AgP.[2,3] Treponema denticola infection was proved to be closely associated with periodontal diseases such as early onset periodontitis, necrotizing ulcerative gingivitis and acute pericoronitis.[5,6] Recent basic research as well as clinical evidence suggested that T. denticola may play an important role in periodontal tissue destruction,[5,6] but it has been found occasionally in subgingival samples of CP.[7] However, correlation between coinfection of the three microbes and periodontal tissue destruction is not well characterized.[1,4] Coinfection of multiple subgingival anaerobes could result in more serious destruction of periodontal tissue in Caucasian CP patients,[5,8] but conflicting conclusions have been reported also.[9]

The purpose of this study was to investigate the subgingival infection frequencies of P. gingivalis, A. actinomycetemitans and T. denticola in Chinese CP patients as measured by a multiple PCR using specific primers derived from the 16SrDNA genes of these three microbes, and to evaluate the correlations among the three microbes and periodontal destruction.

METHODS

Subjects
Eighty-one untreated CP patients (38 men and 43 women, 27 – 65 years, mean 43 years) with at least 14 teeth remaining were recruitedin the dental clinic of the Second Affiliated Hospital, Medical School of Zhejiang University. The patients were diagnosed according to their clinical examination results: the average periodontal probing depth (PD) $\geqslant 3$ mm, clinical attachment loss > 0.5 mm and with alveolar bone loss on X-ray examination. Samples from 30 periodontally healthy individuals (12 men and 18 women, 19 – 39 years, mean 32 years) were also studied. All of the patients and the healthy individuals were nonsmokers without any systemic disease. Individuals who were under orthodontic treatment or had antibiotic therapy during the preceding 3 months were excluded. All of the individuals received detailed information concerning

the nature of the study and the procedures involved, and their consent was obtained.

Sample collection
For all the patients, two subgingival plaque samples from periodontal pockets with a minimum depth of 3 mm from two different tooth sites were collected with a separate curette for each sample to avoid cross contamination. For the healthy population, one sample of gingival sulcus was collected from each of the individuals by the same method. The plaque samples were placed in 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[10] and stored at −20℃ until used. The gingival index (GI)[11] and attachment loss of each pocket was recorded. AL data were classified into 3 grades: $\leqslant 2$ mm, 2 mm to 5 mm, and > 5 mm.[12] The clinical severity of periodontitis of the sampled tooth sites was classified into three grades according to Hugoson's categories.[13] Mild periodontitis: tooth with attachment loss $\leqslant 2$ mm, alveolar bone loss $\leqslant 1/3$ of root length, and no tooth mobility. Moderate periodontitis: tooth with attachment loss between 3 mm to 5 mm, alveolar bone loss $\leqslant 1/2$, slight furcation involvement and slight tooth mobility. Severe periodontitis: tooth with attachment loss > 5 mm, alveolar bone loss $> 1/2$, obvious furcation involvement and obvious tooth mobility.[13]

Bacteria and growth condition
P. gingivalis strain ATCC 33277, A. actinomycetemitans strain Y4 and T. denticola strain FM were used as positive controls. P. gingivalis strain ATCC 33277 was grown in trypticase soy agar supplemented with haemin (5 μg/ml) and vitamin K1 (1 μg/ml), 5% (5 ml/100 ml) sheep blood and menadione (1 μg/ml).[14] A. actinomycetemitans strain Y4 was cultured on TSBV selective agar medium. The TSBV medium contains tryptic soy medium (Oxoid), 10% (10 ml/100 ml) horse serum, bacitracin (75 μg/ml) and vancomycin (5 μg/ml).[15] T. denticola strain FM was grown in new oral spirochete medium with 10% heat inactivated rabbit serum and 10 μg/ml cocarboxylase.[16] The above anaerobes were cultured in an anaerobic chamber at 36℃ with an atmosphere of 85% N_2, 5% CO_2 10% H_2. E. coli strain DH 5α was used as negative control and cultured by using MH medium (Oxoid, England).

DNA extraction
Each of the subgingival plaque samples in the lysis

buffer was boiled for 10 minutes, and 10 μl of the supernatant was directly used as template in PCR. [10] Cultured *P. gingivalis* strain ATCC 33277, *A. actinomycetemitans* strain Y4, *T. denticola* strain FM, and *E. coli* strain DH 5α were suspended in 0.01 mol/L PBS (pH 8.0). Genomic DNAs of the bacterial strains, which would be used as controls in PCR, were obtained by the phenol chloroform method.

PCR primers and amplification

A multiple PCR assay was developed to detect the *16SrDNA* genes of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* in the subgingival plaque samples. PCR amplification was carried out in a volume of 100 μl containing 10 μl of the template, 10 μl PCR buffer (20 mmol/L Tris-Cl, 50 mmol/L KCl, pH 8.4) and 5 U Taq polymerase (Sangon, East Markham Ontario L3R 2R5, Canada), 0.25 mmol/L of each dNTP, 2.5 mmol/L MgCl$_2$, and 25 pmol/L primers specific for the *16SrDNA* genes of *P. gingivalis*, *A. actinomycetemitans 16SrDNA* and *T. denticola*. Primers specific for *P. gingivalis 16SrDNA* gene were: 5'-AGG CAG CTT GCC ATA CTG CG-3' (sense), 5'-ACT GTT AGC AAC TAC CGA TGT-3' (antisense). [7] Primers specific for *A. actinomycetemitans 16SrDNA* gene were: 5'-ATG CCA AAT TGA CGT TAA AT-3' (sense), 5'-AAA CCC ATC TCT GAG TTC TTC TTC-3' (antisense). [17] Primers specific for *T. denticola 16SrDNA* gene were: 5'-TAA TAC CGA ATG TGC TCA TTT ACA T-3' (sense), 5'-TCA AAG AAG CAT TCC CTC TTC TTC TTA-3' (antisense). [7] Expected sizes of the target fragments amplified from the *16SrDNA* genes of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* were 404 bp, 557 bp and 316 bp, respectively. [7,17] The PCR program includes an initial denaturation step at 94℃ for five minutes followed by 35 cycles of denaturation at 94℃ for one minute, primer annealing at 54℃ for 1 minute and extension at 72℃ for 1.5 minutes, and then a final step at 72℃ for seven minutes. Ten μl of distilled water was added instead of 10 μl template in the PCR as blank control, and 10 μl DNA template of *E. coli* DH 5α was used as negative control. Ten μl of mixed DNA templates of *P. gingivalis* strain ATCC33277, *A. actinomycetemitans* strain Y4 and *T. denticola* strain FM was used as positive controls. To guarantee the reproducibility of PCR reaction, we repeated each reaction three times in our trial test to set up PCR conditions using the same DNA templates. When used in clinical samples, each reaction was repeated twice using the same sample. If the two PCR results were not consistent, a third time reaction was carried out.

Detection of PCR Products

Ten μl of each reaction product mixed with 10 μl of 2 × loading buffer was fractionated on 2% agarose gel stained with 1 μg/ml ethidium bromide, using a 100 bp DNA ladder (Sangon, Canada) as a size marker.

Statistical analysis

Chi-square test by using SPSS9.0 software was performed for statistical analysis.

RESULTS

Detection of *P. gingivalis 16SrDNA*, *A. actinomycetemitans 16SrDNA* and *T. denticola 16SrDNA* in healthy individuals by multiple PCR
By using multiple PCR, the detection results of all the three microbes in the plaque or sulcus samples could be simultaneously obtained. In addition, clear and exact amplification fragments representative for one, two or all of the three microbes could be shown (Fig.). In the 30 samples from 30 periodontally healthy individuals, 3 (10.0%) of the samples were *P. gingivalis 16SrDNA* positive, 2 (6.7%) samples were *A. actinomycetemitans 16SrDNA* positive, and 1 (3.3%) sample was *T. denticola 16SrDNA* positive. Furthermore, only one sulcus sample was found to be positive for both *16SrDNA*s of *P. gingivalis* and *A. actinomycetemitans*.

Detection of *P. gingivalis 16SrDNA*, *A. actinomycetemitans 16SrDNA* and *T. denticola 16SrDNA* in the subgingival plaque samples by multiple PCR
The positive rates of *P. gingivalis 16SrDNA*, *A. actinomycetemitans 16SrDNA* and *T. denticola 16SrDNA* in 162 subgingival plaque samples from the patients were 84.6%, 83.3% and 88.3%, respectively (Tables 1 & 2). Compared with samples from periodontal healthy individuals, the positive rates of *16SrDNA*s from the three anaerobes in subgingival plaque samples from the patients were significantly higher ($\chi^2 \leqslant 72.789$, $P < 0.01$). Using *16SrDNA* as a genetic marker for the bacteria in the 162 subgingival plaque samples, 110 samples were positive for the three anaerobes, one sample was negative for all three anaerobes, and

Table 1. Detection rates of the *16SrDNA* genes of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* in subgingival plaque samples with different gingival index (GI)

GI	samples (n)	P. gingivalis 16SrDNA		A. actinomycetemitans16SrDNA		T. denticola 16SrDNA	
		negative	positive	negative	positive	negative	positive
1	31	6	25 (80.6%)	7	24 (77.4%)	7	24 (77.4%)
2	77	15	62 (80.5%)	19	58 (75.3%)	6	71 (92.2%)
3	54	4	50 (92.6%)	1	53 (98.1%)	6	48 (88.9%)
Total	162	25	137 (84.6%)	27	135 (83.8%)	19	143 (88.3%)
P value			0.136		0.002		0.095

Table 2. Detection rates of the *16SrDNA* genes of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* in subgingival plaque samples with attachment loss (AL)

AL (mm)	samples (n)	P. gingivalis16SrDNA		A. actinomycetemitans16SrDNA		T. denticola 16SrDNA	
		negative	positive	negative	positive	negative	positive
≤2	74	20	54 (73.0%)	20	54 (73.0%)	13	61 (82.4%)
2-5	54	3	51 (94.4%)	5	49 (90.7%)	6	48 (88.9%)
>5	34	2	32 (94.1%)	2	32 (94.1%)	0	34 (100%)
Total	162	25	137 (84.6%)	27	135 (83.3%)	19	143 (88.3%)
P value			0.001		0.005		0.031

the remaining 51 samples were positive for either one or two anaerobes (Table 3).

Table 3. Distribution of the multiple PCR detection results for the *16SrDNA* genes of *P. gingivalis* (Pg), *A. actinomycetemitans* (Aa) and *T. denticola* (Td) and its relation with severity of periodontitis

Detection results	samples	severity of periodontitis		
		mild	moderate	severe
Pg + Aa + Td +	110	32	36	42
Pg + Aa + Td − *	14	8	6	0
Pg + Aa − Td + *	12	4	5	3
Pg − Aa + Td + *	8	2	1	5
Pg + Aa − Td − *	1	1	0	0
Pg − Aa − Td + *	13	13	0	0
Pg − Aa + Td − *	3	2	1	0
Pg − Aa − Td −	1	1	0	0
Total	162	63	49	50
P value	0.001			

+: positive; −: negative; *: data in these rows were combined.

Infection of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* in the patients

Two samples were taken from two different teeth of each patients. Only one patient had one sample positive for all three anaerobes, but another was negative for all three. In 80 out of the 81 patients, there was at least one of the two subgingival plaque samples positive for one of the three microbes. Coinfection of the three microbes was found in both samples from 39 patients and in one of the two samples from 31 out of the 80 patients. Six of the remaining 10 infected patients had one sample infected with two of the three anaerobes while the
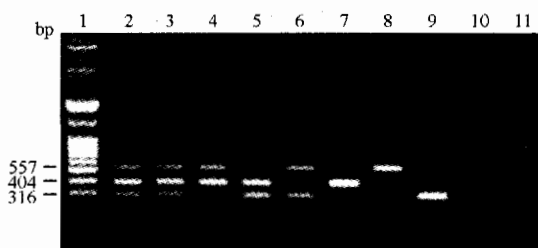

Fig. Detection by multiple PCR of the *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* 16SrDNAs in subgingival plaque samples. Lane 1: 100 bp marker; Lane 2: positive control (mixed DNA templates from *P. gingivalis* strain ATCC33277, *A. actinomycetemitans* strain Y4 and *T. denticola* strain FM); Lane 3: a positive sample for *P. gingivalis*, *A. actinomycetemitans* and *T. denticola*; Lane 4: a positive sample for *P. gingivalis* positive and *A. actinomycetemitans* but negative for *T. denticola*; Lane 5: a positive sample for *P. gingivalis* positive and *T. denticola* but negative for *A. actinomycetemitans*; Lane 6: a positive sample for *A. actinomycetemitans* and *T. denticola* but negative for *P. gingivalis*; Lane 7: a positive sample for *P. gingivalis* but negative for *A. actinomycetemitans* and *T. denticola*; Lane 8: a positive sample for *A. actinomycetemitans* but negative for *T. denticola* and *P. gingivalis*; Lane 9: a positive sample for *T. denticola* but negative for *P. gingivalis* and *A. actinomycetemitans*; Lane 10: negative control (DNA template from *E. coli* strain DH5α); and Lane 11: blank control.

other had one infected with only one anaerobe. One patient had two samples both positive for two anaerobes, while the other three patients had both samples positive for only one anaerobe. The consistency between two samples from the same

patient was 53. 1% （43/81）.

Association between clinical signs and positive rates of *P. gingivalis* 16SrDNA, *A. actinomycetemitans* 16SrDNA and *T. denticola* 16SrDNA

No correlation could be found between the positive rates of *P. gingivalis* and *T. denticola* and GI （χ^2 = 3. 997, P = 0. 136; χ^2 = 4. 699, P = 0. 095; α' = 0. 05） . However, the positive rates of *A. actinomycetemitans* seemed to be related with GI （χ^2 = 12. 870, P = 0. 002, α' = 0. 0167） . It was more significant when GI was 3, compared to the GI = 1 or 2 （χ^2 = 9. 925, P = 0. 002; χ^2 = 12. 781, P = 0. 000; α' = 0. 0167） （Table 1）.

The detection rates of *P. gingivalis*, *A. Actinomycetemcomitans* and *T. denticola* varied with different AL degrees （χ^2 = 14. 035, P = 0. 001; χ^2 = 10. 699, P = 0. 005; χ^2 = 6. 974, P = 0. 031; α' = 0. 05） . It showed that the positive rates of *P. gingivalis* and *A. actinomycetemitans* in middle or deep pockets with AL 2 mm – 5 mm or >5 mm were much higher than in shallow ones with AL ≤2 mm （χ^2 = 9. 764, P = 0. 002; χ^2 = 6. 421, P = 0. 011; and χ^2 = 6. 271, P = 0. 012; χ^2 = 6. 421 P = 0. 011; α' = 0. 0167） （Table 2）. However, *T. denticola* was found more frequently in deep pockets （AL >5 mm） than in shallow pockets （AL ≤2 mm） （χ^2 = 6. 790, P = 0. 009; α' = 0. 0167） . No differences could be found between detection rates of *T. denticola* in moderate （2 mm < AL ≤5 mm） and shallow pockets （AL ≤2 mm） （χ^2 = 4. 054, P = 0. 044; α' = 0. 05） （Table 2）.

Association between severity degree of periodontitis and positive rates of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola*

Compared to the frequencies between cases infected with one and/or two microbes, cases infected with all three microbes in sites with different degrees of severity of periodontitis showed a statistically significant difference （χ^2 = 13. 725, P = 0. 001; α' = 0. 05） . The coinfection rates of the three anaerobes in sites with severe periodontitis was significantly higher than in those with mild disease （χ^2 = 12. 951, P = 0. 000; α' = 0. 0167） . However, no statistically significant differences of the coinfection rates between severe and moderate periodontitis and between moderate and mild periodontitis could be found （χ^2 = 2. 263, P = 0. 133; χ^2 = 4. 492, P = 0. 034; α' = 0. 0167）

（Table 3） . Due to the small number of cases infected with one and/or two anaerobes, no statistical differences could be drawn among the infection rates and the different state of severity of the disease.

DISCUSSION

In the previous reports, PCR assay was used for rapid clinical diagnosis as a routine method to detect the *16SrDNA* of *P. gingivalis*, *A. actinomycetemitans* or *T. denticola* in subgingival plaque samples. In this study, we established a new multiple PCR assay to detect simultaneously the *16SrDNA* genes of the three microbes.[7,10] In repeated multiple PCR in the same clinical samples, the reproducibility was found to be reliable. All the results in this study indicate that multiple PCR can be used as a diagnostic method for the three microbes in clinical samples.

For each of the positive detection rates of the three microbes, significantly more patients were infected with *P. gingivalis* （84. 6%）, *A. actinomycetemitans* （83. 3%） and *T. denticola* （88. 3%）, respectively, than the periodontally healthy individuals, indicating the three microbes were the prevalent bacteria in CP patients. *A. actinomycetemitans* was generally believed to be a specific pathogen only associated with AgP.[2,3] However, in this study, a high infection rate of *A. actinomycetemitans* in the CP patients was found. In our previous study, serum antibody against *A. actinomycetemitans* in approximate 30% of Chinese CP patients had been demonstrated. Sirinian as well as Umeda pointed out that distribution of *A. actinomycetemitans* in different ethnic groups were distinct and Asians may have an increased risk for harbouring this microbe in periodontal pockets.[18-20] These data indicated that the real role of *A. actinomycetemitans* in CP in different ethnic populations remains to be determined.

Previous data reported that presence of *P. gingivalis* and *T. denticola* in periodontal pockets was related with high GI scores,[6,21] while *A. actinomycetemitans*' s association with GI was tenuous.[22] However, our data showed that no association between infection of *P. gingivalis* or *T. denticola* and GI could be found, but infection of *A. actinomycetemitans* was closely associated with GI （Table 1）. Besides, some reports indicated that *P. gingivalis* and *T. denticola* were frequently detected

in middle (>4 mm) or deep (>6 mm) periodontal pockets,[23,24] whereas the presence of *A. actinomycetemitans* in deep pocket was only occasional.[23,25] We found that *A. actinomycetemitans*, like *P. gingivalis* and *T. denticola*, was more frequently detectable in deep pockets than in shallow ones (Table 2). Since GI and AL are important clinical indicators showing gingival inflammation and periodontal tissue destruction, the close associations between *A. actinomycetemitans* and the two indices found in our study implied a possible pathogenic role for this microbe in CP in the Chinese population.

Particular attention has been paid recently to the clinical significance of coinfection of periodontal bacterial pathogens.[6,8] *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* as well as other subgingival bacteria form a mixed infection, which causes more serious periodontal destruction than a single infection.[8,9] In this study, most of the samples (68.0%) showed the presence of *P. gingivalis*, *A. actinomycetemitans* and *T. denticola*. In addition, this coinfection was more frequent in the samples from severe periodontitis sites than in those from mild periodontitis, whereas single infection with any of the three microbes was only found in mild or moderate periodontitis sites (Table 3). These data suggested that coinfection with *P. gingivalis*, *A. actinomycetemitans* and *T. denticola* might have a higher pathogenic capacity in periodontal destruction than infection with only one or two of the microbes. It was interesting to note that a relatively high frequency of samples infected with only one microbe contained *T. denticola*, rather than *P. gingivalis* or *A. actinomycetemcomitan*, which implied a possibility of stronger etiologic role of *T. denticola* than the other two in Chinese CP patients.

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Selected Practice Recommendations for Contraceptive Use (Second edition)

This publication is the companion guideline to WHO's *Medical Eligibility Criteria for Contraceptive Use.*

It aims to improve access to quality care in family planning by providing guidance on the safe and effective use of contraceptive methods once they are deemed to be medically appropriate.

The book is intended to be used by policy-makers, programme managers and the scientific community, and aims to provide guidance to national family planning and reproductive health programmes in the preparation of guidelines for service delivery of contraceptives.

It contains 33 specific questions with recommendations, including 10 new questions for this second edition. Recommendations are given on initiation/continuation of methods; incorrect method use; problems during use, such as vomiting and/or diarrhoea, menstrual abnormalities, pelvic inflammatory disease, and pregnancy; and programmatic issues, such as exams and tests required for method use. Recommendations are based on the latest clinical and epidemiological data, and developed through consensus at an International Expert Working Group meeting.

The book covers the following family planning methods: combined oral contraceptives, combined injectable contraceptives, progestogen-only pills, DMPA, NET-EN, levonorgestrel implants, emergency contraceptive pills, copper-bearing IUDs, levonorgestrel-releasing IUDs, fertility awareness-based methods, and male & female sterilization.

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