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Prevalence and distribution of *Aggregatibacter actinomycetemcomitans* and its *cdtB* gene in subgingival plaque of Chinese periodontitis patients

Xiaoqian Wang[1,2†], Lu Li[1,2†], Mifang Yang[1], Ying Geng[2], Huiping Chen[2], Yan Xu[1,2*†] and Ying Sun[1,2*†]

Abstract

Background: *Aggregatibacter actinomycetemcomitans* (*A.actinomycetemcomitans*) is an important periodontal pathogen that can participate in periodontitis and other non-oral infections. The cytolethal distending toxin (Cdt) is among the virulence factors produced by this bacterium. This study was to elucidate the distribution of *A.actinomycetemcomitans* and the prevalence of its *cdtB* gene in Chinese subjects.

Methods: A total of 255 subgingival samples were obtained from 30 subjects. Samples were collected from periodontal healthy sites as well as shallow, moderate and deep pockets. The absolute quantity of *A.actinomycetemcomitans* and *cdtB* gene were determined by real-time polymerase chain reaction.

Results: *A.actinomycetemcomitans* was detected in 92 of 105 (87.6%) samples of aggressive periodontitis (AgP) patients, in 73 of 79 (92.4%) samples of chronic periodontitis (CP) patients and in 5 of 71 (7.0%) samples of periodontal healthy subjects. The *cdt*B gene was detected in 72 sites (78.3%) with AgP infected with *A.actinomycetemcomitans*, 54 sites (74.0%) with CP infected with *A.actinomycetemcomitans* and none in healthy sites infected with *A.actinomycetemcomitans*. In addition, quantity of *A.actinomycetemcomitans* and *cdt* gene in samples from deep pockets were significant larger than moderate, shallow and healthy sites ($P < 0.05$). In comparison to CP, AgP patients were infected with increased numbers of *cdt* genotype in deep pockets ($P < 0.05$).

Conclusion: This study suggests that the *cdtB* gene are prevalent in *A.actinomycetemcomitans*, and the distribution of *cdt* genotype strain may be correlated with AgP and serious periodontal inflammation.

Keywords: *Aggregatibacter actinomycetemcomitans*, Cytolethal distending toxin, Subgingival plaque, Real-time PCR

Background

Periodontitis is a chronic inflammatory disease leading to the loss of periodontal tissues, which is highly prevalent and is the major cause of tooth loss in adults. Aggressive periodontitis (AgP) is characterized with rapid development and serious bone resorption, generally affects younger patients than does the chronic form.

A.actinomycetemcomitans is a Gram-negative, non motile rod, facultative anaerobic and commensal bacterium, which has long been strongly associated with AgP and may also contribute to chronic periodontitis (CP). Apart from oral infection, this bacterium has also been responsible for some systemic infectious diseases, such as endocarditic, meningitis, osteomyelitis, glomerulonephritis and arthritis [1].

This microorganism expresses several potential virulence factors that are involved in the colonization in oral cavity, inhibition of regeneration of periodontal tissues and interference with host defense mechanisms. In 1998, Ohguchi [1] revealed that *A.actinomycetemcomitans* could produce cytolethal distending toxin (CDT), which was secreted into

* Correspondence: yanxu@njmu.edu.cn; ebolasun@njmu.edu.cn
†Equal contributors
[1]Laboratory of Oral Infection and Immunology, Institute of Stomatology, Nanjing Medical University, Nanjing, Jiang Su, China
[2]Department of Periodontology, College of Stomatology, Nanjing Medical University, Nanjing, Jiang Su, China

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the bacterial culture supernatant. It is clear that CDT is encoded by three genes designated *cdtA*, *cdtB*, and *cdtC*, which are arranged as an apparent operon. These three genes specify three polypeptides designated CdtA, CdtB and CdtC with apparent molecular masses of 28, 32 and 20 kDa respectively, which form a heterotrimeric holotoxin [2-5]. CdtA and CdtC are necessary for the secretion of the toxin, while CdtB is responsible for the biologic activity [6]. CdtB has a sequence homology with mammalian DNase I, indicating a critical role for nuclease activity in host parasite interactions [7].

Among periodontal pathogenic bacteria, *A.actinomycetemcomitans* is the unique bacterium that can produce CDT. Accumulating evidences show that CDT is associated with the persistence of infection in animal models [7], increased expression of RANKL (receptor activation of nuclear factor-κB ligand) and the consequent osteoclastogenesis [3]. There was report showing that *cdtABC* was absent [8] in *A.actinomycetemcomitans* isolated in Japan [9], but Kawamoto confirmed that *cdtABC* was frequently found in the genome of *A.actinomycetemcomitans* [10].

Till now, there have been few reports on the prevalence of *cdt* genotype strain of *A.actinomycetemcomitans* in Chinese periodontitis patients. Accumulating evidences show that CDT is associated with the persistence of infection in animal models [7], increased expression of RANKL (receptor activation of nuclear factor-κB ligand) and the consequent osteoclastogenesis [3]. The association of CDT genetic diversity within *A.actinomycetemcomitans* should be better evaluated. In this study, we detected the distribution of *A.actinomycetemcomitans* and the prevalence of its putative virulence factor CDT encoding gene *cdt*B in subgingival plaque obtained from Chinese patients suffering from CP and AgP by real-time PCR, to evaluate the association of *A.actinomycetemcomitans* CDT genetic diversity and clinical features.

Methods

Subjects

This study was approved by the Ethical Committee of Stomatological Hospital affiliated to Nanjing Medical University, Nanjing, China. The purposes and procedures of the study were explained and informed consents were obtained from all recruits.

Participants included in the present study were recruited at the Stomatological Hospital affiliated to Nanjing Medical University, from December 2008 to March 2009. 10 CP patients, 10 AgP patients and 10 healthy subjects were recruited in this study.

The criteria for patient inclusion were as follows: (i) ethnic Han and<40 years old; (ii) no history of periodontal therapy; (iii) no systemic antibiotics or anti-inflammatory drugs taken within 3 months; (iv) healthy systemic conditions; (v) no pregnancy , and (vi) were not current users of tobacco products or nicotine replacement medication [10]. The periodontal healthy subjects had no sites with probing depth (PD) >3 mm or clinical attachment loss (CAL) >1 mm, and no more than 10% of sites with bleeding on probing (BOP).The diagnoses of CP and AgP were made based on criteria defined at the workshop sponsored by the American Academy of Periodontology (AAP) in 1999 [11-13].

Sample sites were classified as shallow, moderate or deep according to the levels of PD and CAL. The level of PD was about 3 mm or the level of CAL was 1–2 mm in shallow group; and 4–6 mm of PD or 3–4 mm of CAL in moderate group. In deep group, the level of PD was over 6 mm or CAL ≥ 5 mm [12].

Clinical measurements

Before sampling, a complete periodontal examination was conducted to record clinical periodontal parameters by using FP32 probe (Florida probe, USA), including PD, CAL and BOP. All measurements were performed by a calibrated examiner. To avoid any contaminations that might result from bleeding on probing, clinical probing and measurement were carried out at least 7 days in advance of bacteria sampling [14].

Sampling of subginginval bacteria plaque

8–10 samples were taken from shallow, moderate and deep sites of each enrolled patients suffering from CP and AgP, as well as from gingival sulci of healthy controls. After careful removal of supragingival plaque deposits, the sampling site was isolated with cotton rolls and gently air-dried. Then, a 30# paper point [15,16] was inserted into the pockets/gingival sulci and left in place for 30s. The paper point from each sampling site was immediately placed into an empty 1.5 ml microfuge tube. Samples for PCR analysis were stored at −80°C.

Positive control bacterial strains

A.actinomycetemcomitans ATCC 29522, ATCC 29523, ATCC 24523 were grown anaerobically (75% N_2, 10% CO_2, 15% H_2) at 37°C on 5% sheep blood agar plates (Oxoid, UK) enriched with haemin (5 mg/l) and menadione (1 mg/l) for 3–5 days, and then inoculated into brain heart infusion broth until grown to the late logarithmic phase of growth. The bacteria were harvested by centrifugation, washed in PBS, and re-suspended at a concentration with optical density at 690 nm of 1, corresponding to approximately 1×10^8 colony-forming units (CFU)/ml [17].

Oligonucleotide primers and TaqMan probes

Identification of conserved regions was done by multiple sequence alignment with ClustalW software based on

the published 16S rDNA and *cdtB* sequences. The fluorescent dyes at the 5′ and 3′ ends of the probe were FAM (6-carboxyfluorescein; reporter) and TAMRA (6-carboxytetramethylrhodamine; quencher), respectively. All primers and probes were checked for possible cross-hybridization with bacterial genes using the database similarity search program BLAST. Primers and probes used for quantification of *A actinomycetemcomitans* 16S rDNA and *cdt* gene were shown in Table 1.

Standard curves construction

In order to establish the quantitative assay, plasmids containing the target sequences of *A.actinomycetemcomitans* 16S rDNA and *cdt* gene were cloned using the pMD 19 T-Vector (Takara, Japan). PCR products for *A. actinomycetemcomitans* 16S rDNA and *cdt* gene were inserted into plasmid vectors respectively, and the recombinant vectors were transformed into *E. coli*. Then, the plasmids were purified with Mini BEST Plasmid Purification Kit (Takara, Japan). The purified plasmids were quantified by spectrophotometry. Standard curves were constructed by using serial diluted purified plasmids with predetermined concentrations on the basis of the linear relationship between the Ct and the logarithm of the starting gene amount. Sensitivity of the developed real-time PCR assay was evaluated by using 10^7–10° plasmid copies of *A. actinomycetemcomitans* and *cdtB* gene (data not shown), limit of approximately 10 cells was established in the PCR reaction mixture.

DNA extraction and Real-time PCR

Each sample was diluted in 500 μl distilled water, dispersed in vortex for 1 min, took out the paper points before DNA extraction. Then, DNA was purified by the Mini BEST Bacteria Purification Kit (Takara, Japan), and eluted in 60 μl elution buffer.

TaqMan Universal PCR Master Mix (Applied Biosystems, USA) was used for PCR analysis. The final concentration was used in a total volume of 10 μl contained 5 μl of 2 × Master Mix, 3 μl of DNA template, 0.9 mM of each primer and 0.25 mM of probe. Real-time PCR was carried out in duplicates in ABI 7900 HT system (Applied Biosystems, USA) with the following sequence: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Table 1 Primers and TaqMan probes sequences

Primer	Sequence	Products size
Aa-F	GCTGGTCTGAGAGGATGGC	
Aa-R	CGAAAGAACTTTACAACCCGA	153 bp
Aa-P	CCTACGGGAGGCAGCAGTGG	
cdtB-F	ATTCTTCTGTGCTTCAATCTCG	
cdtB-R	GGTGATGATGGTGATGAGGTAA	151 bp
cdtB-P	CACAGGTGGTTCTGATGCGGTAA	

Aa-F, *Aa*-R and *Aa*-P stand for primers and TaqMan probe for *A. actinomycetemcomitans* 16 s rDNA; *cdtB*-F, *cdtB*-R and *cdtB*-P stand for primers and TaqMan probe for *A.actinomycetemcomitans cdtB*.

Statistical analysis

Descriptive analysis was performed for all variables. Quantitative variables were described by mean values, standard deviations, as well as minimum and maximum values. Qualitative variables were described by absolute and relative frequencies. Comparisons were made between groups (AgP, CP and healthy subjects) for independent variables (PD) by applying analysis of variance (ANOVA). All tests were performed using SPSS for Windows Release 12.0 (USA) and P values less than 0.05 were considered significant.

Results

Clinical analysis

We analyzed plaque samples collected from 30 subjects following the protocols described above. This study consisted of 255 subgingival plaque samples, which were collected from 10 AgP patients, 10 CP patients and 10 periodontal healthy subjects. The study population had no previous history of smoking or periodontal treatment.

The mean values of PD, CAL and BOP (%) of all sampling sites were shown in Table 2. These results revealed that there were no statistical differences in these parameters between CP and AgP groups, while there were significant differences between either CP group or AgP group and periodontal healthy subjects.

Occurrence and distribution of *A.actinomycetemcomitans*

Using real-time PCR, we examined 255 subgingival plaque samples to evaluate the levels of *A.actinomycetemcomitans* in different status of periodontal sites. Among all of the 255 subgingival samples, *A.actinomycetemcomitans* was

Table 2 Clinical characteristics of periodontitis patients and healthy controls

	H	CP	AgP
Subjects	10	10	10
Sampling sites	71	79	105
Age(years-old, mean ± SD)	27.5 ± 2.1	32.5 ± 1.8	29.7 ± 2.1
Gender(male/female)	5/5	5/5	5/5
PD(mm, mean ± SD)	2.4 ± 0.8	4.1 ± 2.0 *	4.9 ± 1.9 *
CAL(mm, mean ± SD)	0	4.8 ± 2.0 *	4.5 ± 1.9 *
BOP(%, mean ± SD)	15.8 ± 0.9	30.8 ± 5.5 *	30.6 ± 7.2 *

AgP, CP and H stand for aggressive periodontitis, chronic periodontitis and periodontal healthy, respectively.
The mean probing depth, clinical attachment loss and bleeding on probing (%) were recorded in 255 sampling sites from 30 patients. * There were significant differences for mean probing depth, clinical attachment loss and bleeding on probing (%) between AgP or CP and H ($P < 0.05$), but there was no significant difference between AgP and CP.

detected from 170 (66.7%) samples. Only 5 samples (7.0%) out of the 71 subgingival samples from the periodontally healthy individuals were *A.actinomycetemcomitans* 16 s rDNA positive (Table 3). 73 of the 79 subgingival plaque samples (92.4%) from CP patients were *A.actinomycetemcomitans* 16 s rDNA positive. And among the 105 subgingival plaque samples from AgP patients, 92 (87.6%) were *A.actinomycetemcomitans* 16 s rDNA positive. There was no statistical difference of *A.actinomycetemcomitans* positive rates between CP (92.4%) and AgP (87.6%) ($P > 0.05$) (Table 3).

Mean log-transformed numbers of *A.actinomycetemcomitans* 16 s rDNA in healthy subjects, CP patients and AgP patients were 0.8 ± 0.5, 2.1 ± 0.9 and 2.3 ± 1.1 (Table 3), respectively. Higher numbers of *A.actinomycetemcomitans* were detected in samples from periodontitis sites in contrast to those from healthy subjects ($P < 0.05$). However, there were no significant differences between these two periodontitis groups ($P > 0.05$).

Prevalence and distribution of *A.actinomycetemcomitans cdtB* gene

Out of 255 subgingival samples, *A.actinomycetemcomitans cdtB* was detected in 126 (49.4%) samples. In the 71 subgingival samples from periodontal healthy individuals, none of the samples were *cdtB* positive (Table 3). While 54 of the 73 (74.0%) *A.actinomycetemcomitans* positive subgingival plaque samples from CP patients were *cdtB* positive. Among the 92 *A.actinomycetemcomitans* positive subgingival plaque samples from AgP patients, 72 (78.3%) samples were *A.actinomycetemcomitans cdtB* positive. There was no statistical difference for the *A.actinomycetemcomitans cdtB* positive rates between CP (74.0%) and AgP (78.4%) ($P > 0.05$). None of the *A.actinomycetemcomitans* 16 s rDNA negative samples were positive for *cdtB.*

Table 3 Prevalence of *A.actinomycetemcomitans* and *cdt* in all sampling sites

	AgP	CP	H
Total	105	79	71
Aa(+)	92	73	5
Aa(+)/Total(%)	87.6	92.4	7.0
cdt(+)	72	54	0
cdt(+)/*Aa*(+)(%)	78.3	74.0	0
Quantity-*Aa*	2.3 ± 1.1*	2.1 ± 0.9*	0.8 ± 0.5
Quantity-*cdt*	1.6 ± 0.8**	1.3 ± 0.8***	0.4 ± 0.3

Aa, AgP, CP and H stand for *A.actinomycetemcomitans*, aggressive periodontitis samples, chronic periodontitis samples and periodontal healthy samples, respectively.
*There were significant differences for quantity of *A.actinomycetemcomitans* between AgP or CP and H ($P < 0.05$) and no significant differences between AgP and CP ($P > 0.05$). **Quantity of *cdt* genotype of *A.actinomycetemcomitans* from AgP samples were significantly higher than that of CP and H ($P < 0.05$). ***Quantity of *cdt* genotype of *A.actinomycetemcomitans* from CP samples were significantly higher than H ($P < 0.05$).

Levels of *cdtB* gene were also detected in 255 samples. Mean log-transformed numbers of *cdtB* in CP and AgP were 1.3 ± 0.8 and 1.6 ± 0.8 (Table 3). Samples of AgP group showed the higher numbers of *cdtB* genotype *A. actinomycetemcomitans* contrast to CP group and periodontal healthy subjects ($P < 0.05$).

Association of *A.actinomycetemcomitans* and *cdt* distribution with different periodontal status

Mean log-transformed numbers of *A.actinomycetemcomitans* and *cdtB* gene in shallow, moderate and deep pockets were 1.8 ± 0.9, 2.2 ± 0.8, 3.1 ± 1.2 and 1.3 ± 0.8, 1.5 ± 0.8, 2.1 ± 1.1 (Table 4), respectively. Higher quantity of *A.actinomycetemcomitans* and *cdtB* gene in deep periodontal pockets were detected than moderate and shallow pockets ($P < 0.05$).

With respect to the various periodontal statuses, mean log-transformed numbers of *A.actinomycetemcomitans* and *cdtB* gene from CP and AgP in shallow, moderate and deep pockets were shown in Figure 1 respectively. The quantity of *A.actinomycetemcomitans* and *cdtB* gene in deep periodontal pockets from AgP was higher than CP ($P < 0.05$).

Discussion

This research we employed TaqMan real-time PCR to direct target quantification *A.actinomycetemcomitans* and *cdt*B gene in different severity of periodontitis sites of AgP or CP patients.and it is effective for detecting pathogen species that are extremely difficult to culture. Therefore, we chose different periodontal sites as targets to explore the occurrence and quantity of A.actinomycetemcomitans and its CDT encoding gene in Chinese subjects and different periodontal status. *A.actinomycetemcomitans* might not be present in all oral sites in an untreated periodontitis patient [18]. Taking subgingival samples from all teeth would be the most reliable way to detect *A.actinomycetemcomitans*. However, this method is money- consuming and time-consuming which is not suitable to be used in daily practice. Sampling from the deepest pocket of each quadrant has been demonstrated

Table 4 Prevalence and distribution of *A. actinomycetemcomitans* and *cdt* in periodontitis sampling sites by probing depth

	Shallow	Moderate	Deep
Total	100	49	35
Quantity-*Aa*	1.8 ± 0.9	2.2 ± 0.8*	3.1 ± 1.2**
Quantity-*cdt*	1.3 ± 0.8	1.5 ± 0.8	2.1 ± 1.1**

*Mean log-transformed numbers of *A.actinomycetemcomitans* 16 s rDNA from moderate pockets samples were significantly higher than that from shallow pockets ($P < 0.05$).
**Mean log-transformed numbers of *A.actinomycetemcomitans* 16 s rDNA and *cdt* genotype of *A.actinomycetemcomitans* from deep pockets samples were significantly higher than that from moderate and shallow pockets ($P < 0.05$).

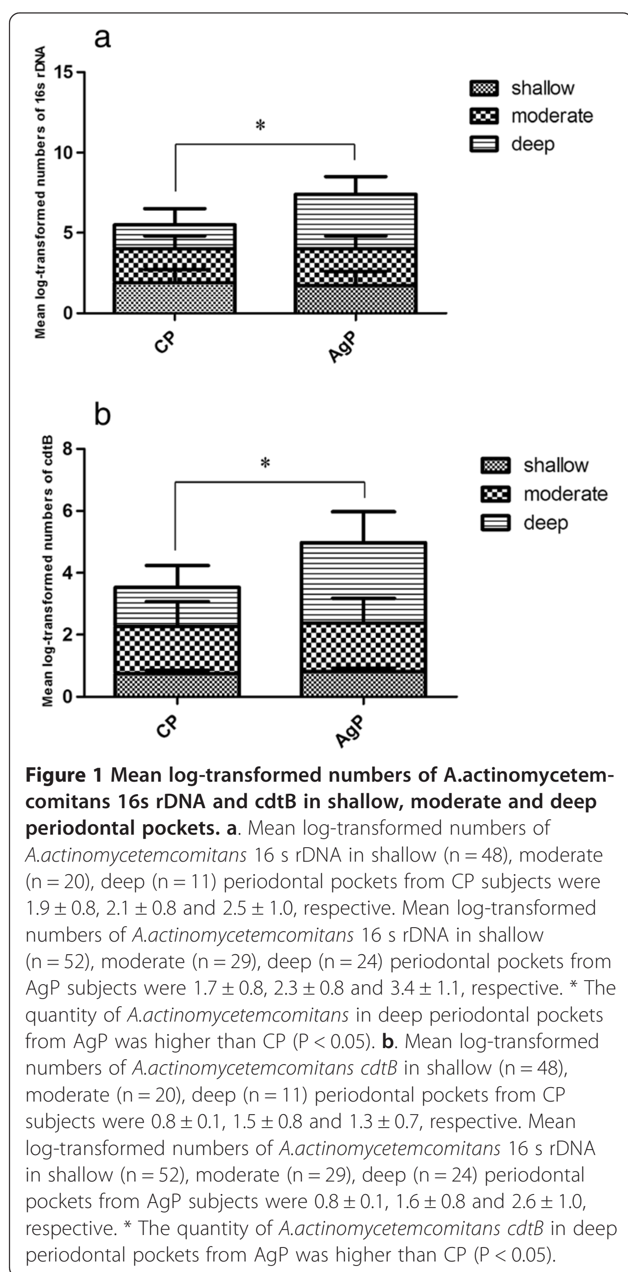

Figure 1 Mean log-transformed numbers of A.actinomycetemcomitans 16s rDNA and cdtB in shallow, moderate and deep periodontal pockets. a. Mean log-transformed numbers of *A.actinomycetemcomitans* 16 s rDNA in shallow (n = 48), moderate (n = 20), deep (n = 11) periodontal pockets from CP subjects were 1.9 ± 0.8, 2.1 ± 0.8 and 2.5 ± 1.0, respective. Mean log-transformed numbers of *A.actinomycetemcomitans* 16 s rDNA in shallow (n = 52), moderate (n = 29), deep (n = 24) periodontal pockets from AgP subjects were 1.7 ± 0.8, 2.3 ± 0.8 and 3.4 ± 1.1, respective. * The quantity of *A.actinomycetemcomitans* in deep periodontal pockets from AgP was higher than CP (P < 0.05). **b**. Mean log-transformed numbers of *A.actinomycetemcomitans cdtB* in shallow (n = 48), moderate (n = 20), deep (n = 11) periodontal pockets from CP subjects were 0.8 ± 0.1, 1.5 ± 0.8 and 1.3 ± 0.7, respective. Mean log-transformed numbers of *A.actinomycetemcomitans* 16 s rDNA in shallow (n = 52), moderate (n = 29), deep (n = 24) periodontal pockets from AgP subjects were 0.8 ± 0.1, 1.6 ± 0.8 and 2.6 ± 1.0, respective. * The quantity of *A.actinomycetemcomitans cdtB* in deep periodontal pockets from AgP was higher than CP (P < 0.05).

to be quite reliable for detecting the subgingival presence of periodontal pathogens in untreated patients [18]. This technique was based on individual level. While one suffered from periodontitis, aggressive or chronic mode, severity of periodontal disease might differ from site to site. So we evaluated relevance of *A.actinomycetemcomitans* and its CDT encoding gene with periodontitis stuates base on "site".

The primers chosen for detection of *A.actinomycetemcomitans* and *cdt* gene were based on *A.actinomycetemcomitans* 16 s rDNA gene and *cdtB* gene respectively. The 16 s rDNA gene has been reported to be highly conserved and this pattern for detection of *A.actinomycetemcomitans* has been well preserved [19,20]. Moreover, recent data have shown that CdtB is the main component and indispensable for the expression of CDT holotoxin activity [6,8], and the sequence of *cdtB* was also highly conserved among *A.actinomycetemcomitans* species [21]. Thus, the prevalence of CDT encoding genes was evaluated using *cdtB* specific primers and probes.

Standard curves were used in this study to evaluate the absolute quantification of *A.actinomycetemcomitans* and *cdt* gene in samples. Plasmids containing cloned target sequences were used as standard substances in quantitative PCR, which enabled the measurements more precise and steady compared to using PCR amplicon as standard substances directly [22].

Some studies have examined the prevalence of *A.actinomycetemcomitan*s in some different populations [4,13,18]. Our work is the first report on the analysis of both positive rate and absolute quantity of *A.actinomycetemcomitans* and its *cdt*B gene in Chinese periodontitis patients, and the association of the distribution of *A.actinomycetemcomitans* and *cdtB* with various periodontal status.

We found that *A.actinomycetemcomitans* and its *cdtB* gene were significantly more prevalent and with higher quantity in samples from patients suffering from AgP or CP than periodontal healthy subjects, and *A.actinomycetemcomitans* and its *cdt* gene were also more prevalent and with higher quantity in deep periodontal pockets than in moderate and shallow periodontal pockets.

Although *A.actinomycetemcomitans* is linked to the etiology of AgP, this bacterium is also found in subjects who are healthy or have other forms of periodontal disease [8]. Our results showed that only 7.0% periodontal healthy sites were *A.actinomycetemcomitans* positive; 92.4% CP samples and 87.6% AgP samples exhibited *A. actinomycetemcomitans* positive, demonstrating that the presence of *A.actinomycetemcomitans* was correlated with periodontitis.

Quantitative data showed that the amount of *A.actinomycetemcomitans* in CP and AgP samples were observably higher than in healthy samples, while no difference between CP and AgP. But the amount of *cdtB* genotype strain of *A.actinomycetemcomitans* in AgP samples were remarkablly higher than in CP and healthy samples (Table 3). These data may indicate quantitative results were more suitable to analyse the distribution of *A.actinomycetemcomitans* and its *cdtB* genotype strain*s*. The *cdtB* genotype strain of *A. actinomycetemcomitans* may be more relevant with aggressive periodontitis.

Tan and his coworkers found *A.actinomycetemcomitans* with the *cdt* genotype were at a higher frequency from sites obtained from patients diagnosed with aggressive periodontitis [23]. Our study showed the *cdtB* genotype *A.actinomycetemcomitans* were at a higher quantity

from sites obtained from deep pockets. The higher occurrence and amount of this bacterium in samples with severe periodontitis status was not a surprising observation. *A.actinomycetemcomitans* CDT toxin may be similar to *H.ducreyi* CDT toxin, which may contribute to the pathogenicity of bacteria at a higher concentration [7]. In vivo studies are still needed to explore the exact pathogenic role of *A.actinomycetemcomitans* CDT in the future. Our data showed *A.actinomycetemcomitans* and its *cdt* genotype strain were prevalent in deep and moderate periodontal pockets. To move forward a single step, quantitative analysis showed *cdtB* genotype strain of *A.actinomycetemcomitans* were more prevalent in deep periodontal pockets. The quantities of *cdt* genotype strain of *A.actinomycetemcomitans* were correlated with severe forms of periodontitis (CP or AgP). Numerous studies have considered *A.actinomycetemcomitans* as an important etiological microorganism involved in AgP [24-26], however, from a new perspective, our data showed that *cdtB* genotype strain of *A.actinomycetemcomitans* was mainly found among sites with severe forms of periodontitis. *A.actinomycetemcomitans* with *cdtB* genotype may be more virulent to human periodontium.

As expected, none of the *A.actinomycetemcomitans* 16 s rDNA negative samples were positive for *cdtB*. This result confirmed that *A.actinomycetemcomitans* was the exclusive member in the oral microbial flora identified to carry and express the cytolethal distending toxin locus. Besides, there were 5 of healthy samples in our study which were positive for *A.actinomycetemcomitans* while negative for *cdt*. This may be results of minute quantity of *cdt* genotype strain of *A. actinomycetemcomitans* in this 5 subgingival plaque samples, while may be a new supporting proof for the exist of *cdt*-negative genotype strain of *A.actinomycetemcomitans* in oral cavity.

The *cdt* gene as well as *lktA* (leukotoxin A) of *A.actinomycetemcomitans* is a single copy gene [21]. However, 16 s rDNA gene may generally have 4 to 6 copies per cell (e.g., 6 copies for *E. coli*) [22]. Our results showed that within the same sample the absolute quantity of *A. actinomycetemcomitans* 16 s rDNA were 1–10 times over that of *cdtB* gene, which indicated that one periodontal site might be infected with two or even more genotypes of *A.actinomycetemcomitans* simultaneously. Some genotypes of *A.actinomycetemcomitans* possess *cdt* gene, which could express CDT activity; while other genotypes of *A.actinomycetemcomitans* are *cdt*-negative, which would be less virulent than *cdt*-positive strains. Yamano [9] reported that 89% of *A.actinomycetemcomitans* strains possessed the *cdt* gene. Another study [4] discovered that 86% of *A.actinomycetemcomitans* isolates presented complete operon of *cdt* gene and its characteristic cytotoxic activity. Tan and his coworkers showed a close association between AgP and *cdt*-positive genotype *A.actinomycetemcomitans* strains [23]. These findings suggested that not all the strains of *A.actinomycetemcomitans* possessed *cdt* gene, in other words, not every *A.actinomycetemcomitans* strains presented cytotoxic CDT activity. Similar to *A.actinomycetemcomitans* strains, *Campylobacter spp*., *C.jejuni*, *H.ducreyi* and other CDT-producing bacteria don't express CDT activity or contain all of the *cdtABC* genes in all strains [4].

Conclusion

Our study investigated the prevalence and distribution of *A.actinomycetemcomitans* and its *cdt* gene in subgingival plaque from Chinese periodontitis patients. The significantly increased quantities of *cdt*-positive genotype *A.actinomycetemcomitans* were found in AgP periodontal sites and in deep pockets of both CP and AgP patients, which indicated that *cdt* gene might be a potential virulence-associated gene involved in the pathogenesis of AgP and severe periodontal destruction. Our results suggested the importance of obtaining both *A.actinomycetemcomitans* titer and genotype identification in periodontitis microbiological diagnosis. Extensive studies are necessary for providing more information about the molecular pathophysiological role of *A.actinomycetemcomitans* and its CDT.

Transparency declaration

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XW and LL carried out the samples collection, periodontal examination, participated in the DNA extraction and Real-time PCR and drafted the manuscript. YG and HC participated in the sequence alignment. YX, YS participated in the design of the study. MY participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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