Original Article

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Association Between Periodontal Pathogens and Severity of Periodontal Diseases Among Adolescents in Kunming City: China

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Abstract:

Objective: To investigate the association between periodontal pathogens and periodontal status among adolescents in Kunming City.

Material and Methods: A total of 560 adolescents from five high schools within five urban districts in Kunming participated in this study. Clinical periodontal examination was assessed, and the levels of *Porphyromonas gingivalis (P. gingivalis), Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), Prevotella intermedia (P. intermedia),* and *Fusobacterium nucleatum* (*F. nucleatum*) in saliva samples were examined using a real-time polymerase chain reaction (PCR). The relationship between periodontal pathogens and the severity of periodontal disease was investigated using multivariate logistic regression analysis.

Results: The presence of *P. gingivalis, A. actinomycetemcomitans,* and *P. intermedia* was positively correlated with the extent of probing depth, clinical attachment level (CAL), and bleeding on probing. *P. gingivalis* and *A. actinomycetemcomitans* were detected at approximately 55–58% among adolescents with healthy periodontal status. *P. gingivalis* and *A. actinomycetemcomitans* were detected in all adolescents diagnosed with periodontitis (stage I or II), which was an increased number compared to healthy adolescents. It was found that *P. gingivalis* (odd ratio (OR)=7.03, 95% confidence interval (CI) 3.77–13.11) and *A. actinomycetemcomitans* (OR=5.37, 95% CI 2.73–10.57) were strongly associated with stage II periodontitis. *F. nucleatum* had no significant relationship with the occurrence of gingivitis and periodontitis.

Conclusion: *P. gingivalis*, *A. actinomycetemcomitans,* and *P. intermedia* are important microbiological risk factors for periodontal diseases in adolescents.

Keywords: periodontal diseases, periodontal pathogens, *P. gingivalis,* risk factors

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Introduction

Periodontal diseases are a group of diseases; including gingivitis and periodontitis, that are related to the inflammatory process. They are prevalent in many countries, and their prevalence and severity increase with age[1]. Clinically, gingivitis often precedes periodontitis, with the detection of periodontal attachment loss[2,3]. An epidemiological study reported that periodontal diseases could be found starting at a young age, 5–14 years old, with a high prevalence of gingivitis (84.4%)[4]. Another study showed that 31.3% of adolescents aged 12–15 in Shandong, China have gingivitis[5]. This recent study in Kunming, China also reported a high prevalence of gingivitis (80.4%) and periodontitis (7.3%) among adolescents aged 17–19[6].

Childhood and adolescence are crucial periods for the development of periodontitis; therefore, it is crucial to comprehend the colonization and attachment of periodontal pathogens and their association with disease development. Many studies have identified risk factors for periodontal diseases; including an increase in the amount of certain periodontal microbiota[7–11]. *Porphyromonas gingivalis (P. gingivalis)*, *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), Prevotella intermedia (P. intermedia),* and *Fusobacterium nucleatum (F. nucleatum)* have been reported to play an important role in periodontal diseases occurrence and progression[8,12]. However, the information relating to periodontopathogens among the Chinese population, particularly among adolescents, is scarce. Therefore, this study aimed to investigate the association between periodontal pathogens; including *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia,* and *F. nucleatum,* and the periodontal status among adolescents in Kunming City.

Material and Methods

Study setting and participants

This was the second phase of a cross-sectional study regarding the prevalence and associated factors of dental caries and periodontal diseases among adolescents (aged 17–19) in Kunming High School. It was carried out from the academic year of July 2019 to November 2020. The study setting and participant recruitment have been published in our previous report[12], which has been approved by the Ethics Committee of Prince of Songkla University (EC6208–033) and Kunming Medical University (KY2020MEC019).

In brief, one public school from each of the five urban districts in Kunming was selected. High school students having lived in Kunming for more than a year were invited, and a total of 560 adolescents (17.3±0.5 years old) with an equal gender ratio gave consent to participate in this study. A self-administered questionnaire was then used in the survey to collect sociodemographic characteristics, health status, and oral health behaviors related to dental caries and periodontal diseases. Unstimulated whole saliva samples were collected before the periodontal examination.

Clinical periodontal examination

A full mouth clinical periodontal examination of all participants was carried out using a manual rigid periodontal probe (PCPUNC15, Hu Friedy, Chicago, IL, USA). Two calibrated examiners (Kappa value >0.8) assessed bleeding on probing (BOP), the probing depth (PD), and the clinical attachment level (CAL) at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual). Clinical periodontal status was presented according to the 2018 AAP/EEP classification of periodontal disease into healthy, localized gingivitis, generalized gingivitis, stage I and stage II periodontitis[13].

Following the clinical examination, the children were informed of their oral health status. Anyone who had gingivitis or periodontitis was advised to see a dentist.

Determination of periodontal pathogens using quantitative real-time polymerase chain reaction (PCR) assay

The unstimulated saliva from individual subjects was collected, transferred into a sterile container, and kept at -80°C until used for Deoxyribonucleic acid (DNA) extraction. Then, the saliva samples were extracted using the PureDirex Genomic DNA Isolation Kit (Bio-Helix Co., Ltd., Keelung, Taiwan); according to the manufacturer's instructions. The number of periodontal pathogens; including *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum, P. intermedia,* were determined using a 16S rRNA-based real-time polymerase chain reaction with specific primers (Table 1), and sterile deionized water was used as control.

The real-time PCR was performed in a 20-µL aliquot of real-time PCR master mix; including 5 µL of a purified DNA template, 5 pM of forward and reverse specific primers (Table 1), nuclease-free water, and 10 µL of 2x SensiFAST SYBR® No-ROX Mix (Bioline Reagent Ltd., California, USA), for PCR amplification. The real-time PCR conditions were as follows: denaturation at 95 °C for 10 min and 50 °C for 2 min, followed by 40 cycles at 95 °C for 20s, 60 °C for 20s, and 72 °C for 25s. All samples were run in duplicate by a QuantStudio3 & QuantStudio5™ Flex Real-Time PCR System (Thermo Fisher Scientific, USA). The number of targeting bacteria (*A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum, P. intermedia*) in the saliva samples was calculated with a standard curve. For standard curve construction, bacterial cells were diluted in two-fold dilution and were aliquot into two tubes. The first aliquot counted the bacterial number using the plate count method (10^1-10^9 CFU/mL) and the second aliquot extracted DNA to measure the Ct value using QuantStudio3 & QuantStudio5™ Flex Real-Time PCR System. A linear standard curve was plotted for each bacterial species from log CFU/ mL against the corresponding Ct (R^2 >0.99). The bacterial number was reported as log CFU/mL[14].

Statistical methods

For overall analyses, the normal distribution and homogeneity of variance of continuous variables were examined. The median values of all log-transformed periodontal pathogen counts were calculated. As the distribution of the four periodontal pathogens' numbers was not normal, Spearman's rank correlation coefficient (r_s) was used to evaluate the correlation between the number of periodontal pathogens and the extent of periodontal clinical parameters (probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP)). The bacteria detection frequency was estimated by dividing the number of subjects that tested positive for a specific bacterium, using real-time PCR by the total number of subjects. In addition, the correlation of the number of periodontal pathogens with different periodontal diseases statuses was analyzed by one-way analysis of variance (ANOVA) or nonparametric tests (Kruskal-Wallis test). Post-hoc pairwise comparisons with Bonferroni adjustment were conducted to determine significant differences between the periodontal diagnostic groups. Multivariate logistic regression analysis was used to investigate the relationship between periodontal pathogens and the severity of periodontal disease. All p-value<0.05 were considered statistically significant.

Results

Spearman's rank correlation coefficients revealed a statistically significant positive correlation between the number of *P. gingivalis*, *A. actinomycetemcomitans*, and *P. intermedia* and three clinical periodontal parameters (BOP, PD, and CAL), indicating the level of gingival inflammation and periodontal destruction, as well as between the number of *F. nucleatum* and CAL. However, the correlations were weak (r_s=0.10-0.36) (Table 2).

The prevalence and number of targeted periodontal pathogens in the saliva samples of 560 adolescents with different periodontal statuses, are demonstrated in

Table 3. Generally, the prevalence of all target periodontal pathogens; except *A. actinomycetemcomitans*, was relatively high among the Chinese adolescents; being 94.1%, 100%, 100%, and 59.6% for *P. gingivalis, P. intermedia, F. nucleatum,* and *A. actinomycetemcomitans,* respectively. It was found that the detection frequency and number of *P. gingivalis* and *A. actinomycetemcomitans* increased with the severity of periodontal status. They were substantially higher in adolescents with periodontitis and generalized gingivitis compared to those who were healthy or had localized gingivitis. In contrast, *P. intermedia* and *F. nucleatum* could be detected in all adolescents (100%); they were found to be higher in number than *P. gingivalis* and *A. actinomycetemcomitans* (Table 3).

In the multivariate logistic regression analysis for the association between periodontal pathogens and severity of periodontitis (Table 4), it was found that *P. gingivalis* was significantly associated with the occurrence of gingivitis (odd ratio (OR)=3.38, 95% confidence interval (CI) 2.40–4.75 for localized gingivitis, and OR=4.50, 95% CI 3.03–6.68 for generalized gingivitis and periodontitis (OR=3.20, 95% CI 1.75–5.84 for stage I, and OR=7.03, 95% CI 3.77–13.11 for stage II). An increased number of *A. actinomycetemcomitans* was associated with the increased odds of having generalized gingivitis (OR=3.32, 95% CI 1.91–5.77) and periodontitis (stage I and II). The odds ratio varied between 5.83–5.37. *P. intermedia* was associated only with the occurrence of periodontitis, with an odds ratio of 2.84–2.44. However, *F. nucleatum* had no significant relationship with the occurrence of gingivitis and periodontitis.

Table 1 Bacterial species and the real-time PCR primers used in this study

Species		Sequence (5′–3′)	Product size
P. gingivalis	Forward primer	TGCAACTTGCCTTACAGAGGG	344 bp
	Reverse primer	ACTCGTATCGCCCGTTATTC	
A. actinomycetemcomitans	Forward primer	GAACCTTACCTACTCTTGACATCCGAA	80 bp
	Reverse primer	TGCAGCACCTGTCTCAAAGC	
P. intermedia	Forward primer	CCACATATGGCATCTGACGTG	232 bp
	Reverse primer	CACGCTACTTGGCTGGTTCA	
F. nucleatum	Forward primer	GGATTTATTGGGCGTAAAGC	163 bp
	Reverse primer	GGCATTCCTACAAATATCTACGAA	

PCR=polymerase chain reaction

Table 2 Correlation between periodontal pathogens and clinical parameters

Clinical parameters	Spearman's rho (p-value)			
	P. gingivalis	***A. actinomycetemcomitans***	***P. intermeidia***	***F. nucleatum***
PD	0.20 (<0.001)	0.24 (<0.001)	0.14 (0.001)	0.08 (0.063)
CAL	0.20 (<0.001)	0.30 (<0.001)	0.20 (<0.001)	0.10 (0.024)
BOP	0.36 (<0.001)	0.36 (<0.001)	0.13 (0.003)	0.02 (0.691)

PD=probing depth, CAL=clinical attachment level, BOP=bleeding on probing

Table 3 Prevalence and number of periodontal pathogens among 560 adolescents having different periodontal statuses

Periodontal pathogens	Total n=560	Periodontal status				
		Healthy n=69	Gingivitis		Periodontitis	
			Localized n=295	Generalized n=155	Stage I n=24	Stage II n=17
P. gingivalis						
Frequency of detection, %	94.10	55.10	99.30	100.00	100.00	100.00
Log CFU/ml, Median	2.04	0.36 ***	1.89 **	2.45 *	2.34 *,**	2.85 *
(min, max)	(0.00, 7.17)	(0.00, 0.27)	(0.00, 5.59)	(0.07, 7.17)	(0.04, 4.25)	(1.35, 4.13)
A. actinomycetemcomitans						
Frequency of detection, %	59.60	58.00	46.80	74.20	100.00	100.00
Log CFU/ml, Median	0.40	0.13 **	0.00 **	1.12 *	2.24 *	2.01 *
(min, max)	(0.00, 9.00)	(0.00, 9.00)	(0.00, 3.07)	(0.00, 3.16)	(0.52, 8.09)	(0.11, 3.24)
P. intermedia						
Frequency of detection, %	100.00	100.00	100.00	100.00	100.00	100.00
Log CFU/ml, Median	4.79	4.67 **	4.77 **	4.72 **	5.87 *	5.26 *,**
(min, max)	(0.84, 7.19)	(0.91, 6.40)	(0.84,7.12)	(1.40, 7.12)	(3.10, 7.19)	(2.58, 6.76)
F. nucleatum						
Frequency of detection, %	100.00	100.00	100.00	100.00	100.00	100.00
Log CFU/ml, Median	5.32	5.36 *	5.33 *	5.24 *	5.73 *	5.72 *
(min, max)	(0.12, 7.10)	(0.63, 6.47)	(0.13, 7.10)	(0.24, 6.66)	(2.75, 6.65)	(0.12, 6.47)

*,**,*** indicated the statistical difference between groups. One-way analysis of variance (ANOVA) was performed on variables with homogeneity of variance (*P. gingivalis, P. intermedia,* and *F. nucleatum*), and non-parametric test analysis (Kruskal-Wallis test) was performed on variables with unequal variance (*A. actinomycetemcomitans*). Post-hoc pairwise comparisons with Bonferroni adjustment were conducted to determine significant differences between the periodontal diagnostic groups.

Table 4 Multivariate logistic regression analysis of periodontal pathogens and different periodontal statuses (n=560)

Periodontal pathogens	Localized gingivitis n=295		Generalized gingivitis n=155		Stage I periodontitis n=24		Stage I periodontitis n=17	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
P. gingivalis	3.38 (2.40, 4.75)	<0.001	4.50 (3.03, 6.68)	<0.001	3.20 (1.75, 5.84)	<0.001	7.03 (3.77, 13.11)	<0.001
A. actinomycetemcomitans	1.26 (0.75, 2.12)	0.385	3.32 (1.91, 5.77)	<0.001	5.83 (3.07, 11.07)	<0.001	5.37 (2.73, 10.57)	<0.001
P. intermedia	1.00 (0.74, 1.36)	0.994	1.15 (0.81, 1.63)	0.428	2.84 (1.45, 5.56)	0.002	2.44 (1.24, 4.82)	0.010
F. nucleatum	1.02 (0.66-1.55)	0.942	1.23 (0.76, 2.00)	0.404	1.08 (0.52, 2.23)	0.834	0.73 (0.40, 1.33)	0.297

The reference category is "Healthy" (n=69).
OR=odds ratio, 95% (CI)=95% confidence intervals

Discussion

Even though there are many studies investigating microbial species associated with periodontal diseases, most studies have been performed in adults with chronic periodontitis. This research examined the rate and number of periodontal pathogens in relation to the 2018 classification of periodontal diseases among Chinese adolescents. According to this study, the prevalence of gingivitis was 80.4% and periodontitis was 7.3%. The presence of *P. gingivalis* and *A. actinomycetemcomitans* is an important microbiological risk factor for periodontitis in adolescents.

Gingivitis and periodontitis are a result of gradually inflammatory processes, and certain bacteria play an important role in its initiation. In spite of a thousand species in the oral cavity, only some species; for example, *P. gingivalis* and *A. actinomycetemcomitans*, exhibited the potential to cause periodontal tissue destruction[15]. The main periodontal pathogens in this study included *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *F. nucleatum.* Overall, the bacterial prevalence obtained showed that most target periodontal pathogens except *A. actinomycetemcomitans*, were very high in prevalence among the Chinese adolescents; the higher prevalence of *P. gingivalis* and *A. actinomycetemcomitans* were found in the diseased stage (Periodontitis stage I and II) compared to the healthy. Furthermore, an increased severity of periodontitis was associated with an increase in *P. gingivalis* and *A. actinomycetemcomitans* levels (log CFU/mL). These results were very similar to a previous report;[15] however, some discrepancies were noted in detail. In a study among 255 Indonesian adolescents with untreated periodontal disease, 57% had *A. actinomycetemcomitans,* 87% had *P. gingivalis*, and 89% had *P. intermedia*. The overall prevalence profile of periodontal pathogens was similar to this study. Furthermore, in that study, at site level *P. gingivalis* was more prevalent in sites with attachment loss, which is consistent with this study's findings; in that *P. gingivalis* was more prevalent in subjects with stage I to stage II periodontitis. However, there was no significant association between the clinical periodontal parameters and the prevalence of microorganisms at the subject level in Indonesian adolescents[16]. Another study of periodontal pathogenic bacteria among high school children aged 15–18 years old in Saudi Arabia reported a relatively low prevalence of *A. actinomycetemcomitans* (21.7%), *P. gingivalis* (21.3%), *P. intermedia* (12.3%), and red complex bacteria (2.9%); however, there was no significance related to any clinical characters[17].

Previous studies of periodontal microbiota and the clinical periodontal status among Thai and Chinese adults showed that the 'red complex' bacteria *(P. gingivalis*, *Tannerella forsythia,* and *Treponema denticola*) revealed a strong association between both pocketing and the extent of attachment loss. In addition, *P. gingivalis* was the one species that conferred the highest odds ratio[18,19]. This is in agreement with a number of previous studies that reported *P. gingivalis* prevalence and levels significantly increased in adults with gingivitis and periodontitis[20–23]. A study carried out among 116 Chinese with chronic periodontitis aged 20–75 years old reported that *A. actinomycetemcomitans* was more frequently detected in diseased sites than in periodontally healthy sites; its higher prevalence was more associated with severe sites than moderate and mild sites[24].

This study examined the association of periodontal pathogens in saliva and periodontal status among younger aged subjects (17–19 years old) using a 16S RNA real-time PCR. It should be stated that saliva can be used as a method for the detection of periodontal pathogens, which is in agreement with previous reports[25,26]. Saliva sampling is non-invasive, painless, and easy to perform. It has been found that the presence and number of periodontal pathogens in saliva are correlated with those in dental

plaque[27]. Results in this study showed that there was a strong, positive correlation between the levels of individual periodontal pathogens in saliva. The quantitative analysis of the correlation between periodontal pathogens and clinical periodontal status revealed that the increased levels of *P. gingivalis* and *A. actinomycetemcomitans* increased the severity of clinically periodontal diseases. Odds ratio analysis, according to the high levels of both those species, revealed a strong association with clinical periodontal status, and it became stronger in subjects with a higher degree of periodontitis. This suggests that *P. gingivalis* and *A. actinomycetemcomitans* may be suitable variables for the risk/causative role of the salivary microbiota in adolescents with periodontitis. The prevalence and level of *F. nucleatum* were high in all subjects; however, there was no association with any clinical parameters and status. As known, *F. nucleatum* acts as a bridge organism between early and late colonizers of dental plaque formation[28] although its specific role in periodontal diseases may need clarification. Based on the findings of this study, it is reasonable to monitor the levels of certain salivary microbiota related to periodontal conditions at an early age, because it can provide timely preventive and/or therapeutic measures for periodontal diseases at an early stage. In this study, *P. gingivalis* and *A. actinomycetemcomitans* could be found in healthy children; however, they were in low levels of frequency and numbers compared to children with diseases. Due to the high sensitivity of the real-time PCR used, a low number of microorganisms could be detected. However, this may not necessarily result in the onset of periodontal diseases since the occurrence of diseases involves multifactors such as immunity, personnel habits, oral hygiene and so forth. However, those children should be monitored.

Within the limitations of this cross-sectional study, it is difficult to explain any differences that may result from different facturs, for example, ethnicity, age, a technique used for microbial detection, clinical parameters and the classification system. A longitudinal study on the role of periodontal pathogens in an adolescent population will provide more understanding of the development of periodontitis and could provide new insights for periodontitis monitoring and prevention strategies.

Conclusion

In conclusion, levels of *P. gingivalis*, *A. actinomycetemcomitan,* and *P. intermedia*, but not *F. nucleatum*, were significantly correlated with clinical periodontal conditions. *P. gingivalis, A. actinomycetemcomitans,* and *P. intermedia* are significant risk factors for the development of periodontal disorders in adolescents based on their higher odds ratios.

Ethical approval of research

The research protocol was approved by the Ethics Committee of Research Prince of Songkla University (EC6208-033) and Kunming Medical University (KY2020MEC019)

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Conflicts of interest

The authors have no conflicts of interest.

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