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ORIGINAL ARTICLE

Red bacterial complex is associated with the severity of chronic periodontitis in a Thai population

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BACKGROUND: The distribution of periodontal pathogens differs in various geographic locations and racial/ethnic groups. This study investigated the microbiological features of chronic periodontitis (CP) patients in Thailand.

METHODS: Subgingival plaque samples from 20 non-periodontitis subjects, 20 patients with mild CP, and 20 patients with moderate to severe CP were examined using polymerase chain reaction (PCR) to identify *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans*.

RESULTS: In the moderate to severe CP patients, there was high prevalence of *P. gingivalis* (95%), *T. forsythia* (95%), *T. denticola* (80%), as well as the red complex (coexistence of all three species at the same lesion) (75%). *A. actinomycetemcomitans* was detected in only 35% of the patients in this study group. *P. gingivalis* was detected in as high as 45% of the non-periodontitis controls. CP and disease severity were significantly related to the presence of *T. forsythia* together with *T. denticola* and the red complex. The red complex was not found in any non-periodontitis site.

CONCLUSION: Red complex bacteria were predominant periodontal pathogens of the moderate to severe form of CP in this Thai population. The presence of *T. forsythia* together with *T. denticola*, and the red complex species at the same site were significantly associated with the disease severity.

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Introduction

Chronic periodontitis (CP) is an inflammatory disease caused by gram-negative periodontopathic bacteria. *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans* have been shown to be among the major etiologic agents (Zambon, 1996; Slots and Ting, 1999; Rylev and Kilian, 2008). These organisms express a number of potential virulence factors and induce host inflammatory mediators, eventually leading to connective tissue breakdown and alveolar bone resorption (Offenbacher, 1996; Tatakis and Kumar, 2005).

Several studies have suggested that the distribution and prevalence of periodontal pathogens vary in different geographic locations as well as racial/ethnic groups (Choi *et al*, 2000; Dogan *et al*, 2003; Haffajee *et al*, 2004, 2005; Lopez *et al*, 2004; Ximenez-Fyvie *et al*, 2006). It is well accepted that the most appropriate method to arrest the progression of periodontal diseases involves effective control of microorganisms composing dental plaque. Therefore, a better understanding of the composition of the subgingival plaque and the association of periodontal pathogens with periodontal status in a particular population are crucial to carry out the most effective periodontal treatment for this population.

To our knowledge, very few studies have investigated the periodontal microbiota in adults from Thailand (Dahlen *et al*, 2002; Papapanou *et al*, 2002). Both studies were conducted in southern Thailand using culture and checkerboard hybridizations. In this study, we investigated the microbiological features of Thai patients with CP using a 16S rRNA-based polymerase chain reaction (PCR) to identify *P. gingivalis*, *T. forsythia*, *Treponema denticola*, and *A. actinomycetemcomitans*. The PCR offers a highly sensitive and specific detection method for bacteria in subgingival plaque without cross-reactivity with other oral microorganisms (Ashimoto *et al*, 1996). It is particularly valuable for detection of periodontal pathogens that cannot be cultivated or easily distinguished in culture.

The presence of these individual periodontal pathogens and the coexistence of *P. gingivalis*, *T. forsythia*, and *T. denticola* as the red complex (Socransky *et al*, 1998) were evaluated for association with periodontal status. The relationships among the periodontopathic bacteria were also studied.

Materials and methods

Subjects and clinical examination

Subjects were a convenient sample of apparently healthy Thai adults who sought dental treatment at the Faculty of Dentistry, Khon Kaen University, Khon Kaen, Thailand. To be eligible for the study, a subject must have at least 20 natural teeth. Exclusion criteria included smoking, use of dental prostheses/orthodontic appliances, pregnancy, nursing, periodontal therapy in the past six months, use of antibiotics or mouthrinse in the past three months, and any systemic condition that might affect the progression of periodontitis. The Khon Kaen University ethical review committee approved the study protocol. Informed consent was obtained from all subjects.

Periodontal examinations were performed by a trained and calibrated examiner (LC). Probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) were evaluated using a periodontal probe (PCPUNC 15, Hu Friedy, Leimen, Germany). The measurements were determined on six sites of all present teeth except third molars. Subjects were divided into three groups according to their periodontal diagnosis: non-periodontitis controls (PD $\leq$ 4 mm, $n = 20$), mild CP (at least 10% of sites with PD of 5 mm [diseased sites] but no diseased sites $>$ 5 mm, $n = 20$), and moderate to severe CP (at least 10% of sites with PD $\geq$ 6 mm [diseased sites], $n = 20$). CP patients were diagnosed according to the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions (Armitage, 1999). Table 1 summarizes the demographic data (age and gender) and clinical characteristics of the study population. Race/ethnicity and socio-economic status of the subjects were homogeneous without a considerable difference among the study groups.

Sampling of subgingival plaque

In both CP groups, subgingival plaque samples were collected from six diseased sites (deepest PD in each sextant) and from four non-periodontitis sites (PD $\leq$ 4 mm in each quadrant) of the same subject. Sampling sites of non-periodontitis subjects were on the mesio-buccal site of teeth numbers 16, 11, 24, 36, 31, and 44. Teeth were gently dried with sterile cotton swabs. After removing supragingival plaque by cotton pellets and air-drying, subgingival plaque was collected with two sterile paper points inserted into the bottom of the periodontal pocket or gingival crevice for 20 s. The sample from each site was stored in a separate tube at −80°C until DNA extraction.

PCR detection

Plaque samples were suspended in 1 ml sterile double-distilled water, pelleted, and resuspended in 200 μl of DNA isolation reagent (InstaGene Matrix, Bio-Rad Lab, Hercules, CA, USA). Total DNA was extracted according to the manufacturer's instructions. The suspension was centrifuged and 5 μl of resulting supernatant was used for PCR. The PCR reactions (Taq PCR Core Kit, Qiagen, Valencia, CA, USA) were carried out as previously described using oligonucleotide primers specific for *P. gingivalis*, sense 5′AGG CAG CTT GCC ATA CTG CG 3′, antisense 5′ACT GTT AGC AAC TAC CGA TGT 3′; *T. forsythia*, sense 5′GCG TAT GTA ACC TGC CCG CA 3′, antisense 5′TGC TTC AGT GTC AGT TAT ACC T 3′; *T. denticola*, sense 5′TAA TAC CGA ATG TGC TCA TTT ACA T 3′, antisense 5′TCA AAG AAG CAT TCC CTC TTC TTC TTA 3′; and *A. actinomycetemcomitans*, sense 5′AAA CCC ATC TCT GAG TTC TTC TTC 3′, antisense 5′ATG CCA ACT TGA CGT TAA AT 3′ (Ashimoto *et al*, 1996). The PCR product was analyzed by 1% agarose gel electrophoresis.

Statistical analysis

Characteristics of the three study groups were compared using the chi-square test for categorical data, and analysis of variance for continuous data. We determined the differences in bacterial detection frequencies among groups using multivariate logistic regression. Multiple linear regression and generalized estimated equation analyses were performed to compare the differences in the mean percentages of bacterial species among study groups, and the difference between non-periodontitis sites and CP diseased sites within the same group, respectively. Odds ratios (ORs) and 95% confidence intervals (CIS) were calculated by logistic regression to examine the associations between any two bacterial

Table 1 Characteristics of the study population

	Control (n = *20*)	*Mild CP* (n = *20*)	*Moderate to severe CP* (n = *20*)
Age (years), mean ± s.d.**	23.6 ± 6.1	35.2 ± 11.7	46.5 ± 10.1
Male, %*	6 (30.0)	13 (65.0)	15 (75.0)
Probing depth (mm), mean ± s.d.**	2.41 ± 0.29	2.72 ± 0.30	3.57 ± 0.41
Clinical attachment level (mm), mean ± s.d.**	1.63 ± 0.57	2.32 ± 0.51	4.19 ± 0.83
% sites with bleeding on probing, mean ± s.d.	47.9 ± 28.1	61.7 ± 27.1	68.0 ± 28.6

CP denotes chronic periodontitis.
Significantly different among the study groups at *$P = 0.01$, **$P < 0.001$.

species. As there were differences in age and gender among the comparison groups, statistical adjustment by multivariate regression analysis was therefore used to control for the effect of age and gender on the association between the presence of bacteria and periodontal conditions in Table 2, and the association between different bacterial species in Table 3. All statistical tests were two-sided. *P*-value <0.05 was considered significant.

Results

In general, the prevalences of each bacterium and the combinations of different species detected at the same site were the highest in the moderate to severe CP group, followed by the mild CP group, and the lowest in the non-periodontitis control group (Table 2). The exception was the occurrence of *A. actinomycetemcomitans*, which was lower in the moderate to severe CP group than in the mild CP group. The prevalence of *P. gingivalis* was as high as 45% in the non-periodontitis controls, which was not significantly different from the CP groups. The prevalences of *T. forsythia* and *P. gingivalis* + *T. forsythia* were clearly higher in the mild CP and moderate to severe CP groups when compared to the non-periodontitis controls, but the difference between the two CP groups was not statistically significant. The prevalences of *T. denticola* and *P. gingivalis* + *T. denticola* were significantly higher in the moderate to severe CP group than the mild CP and control groups. The presence of *A. actinomycetemcomitans* was only associated with mild CP. The presence of *T. forsythia* + *T. denticola* and the coexistence of the red complex species at the same site in the subject were markedly different among the three study groups.

Table 2 Bacterial detection frequencies (% of subjects)

Bacteria	*Control* (n = *20*)	*Mild CP* (n = *20*)	*Moderate to severe CP* (n = *20*)
Porphyromonas gingivalis	45.0	85.0	95.0
		***	
Tannerella forsythia	0	75.0	95.0
		***	
			**
Treponema denticola	10.0	30.0	80.0
		**	
		*	
Aggregatibacter actinomycetemcomitans	5.0	45.0	35.0
		***	
P. gingivalis + *T. forsythia*[a]	0	60.0	90.0
		***	
			**
P. gingivalis + *T. denticola*[a]	5.0	20.0	75.0
		**	
		***	**
T. forsythia + *T. denticola*[a]	0	20.0	80.0
		***	
		***	**
Red complex[b]	0	15.0	75.0
		***	

Statistical differences were calculated from multivariate logistic regression adjusted for age and gender: *$P = 0.01$, **$P < 0.01$, ***Could not calculate *P*-value due to the zero cell problem.
[a]Presence of these bacteria at the same site/having at least 1 site in the subject harboring these bacteria.
[b]Presence of *P. gingivalis, T. forsythia,* and *T. denticola* at the same site/having at least 1 site in the subject harboring these bacteria.

Detection frequencies of the periodontopathic bacteria at the site level of each study group are summarized in Figure 1. In general, the non-periodontitis sites of CP patients had markedly lower detection frequencies of each individual pathogen and the red complex than the diseased sites and appeared to be similar to the sites of non-periodontitis control subjects. The coexistence of *P. gingivalis*, *T. forsythia*, and *T. denticola* (red complex) was not found in any non-periodontitis site (Figure 1d). The highest percentage of sites harboring *P. gingivalis* was observed in the diseased sites of the moderate to severe CP subjects (79.2%) (Figure 1a), which was significantly higher than that in the mild CP subjects (35.8%). This tendency was also observed for *T. forsythia* (Figure 1b), *T. denticola* (Figure 1c), and the red complex (Figure 1d). The detection of *P. gingivalis* and *T. forsythia* at the site level was significantly associated with CP as well as the disease severity. The detection of *T. denticola* and the red complex at the site level was specifically associated with the moderate to severe form of CP. *A. actinomycetemcomitans* was detected in 16.7% of the moderate to severe CP diseased sites and 10% of the mild CP diseased sites (Figure 1e), but this difference was not statistically significant ($P = 0.48$). The presence of *A. actinomycetemcomitans* at the site level was not related to periodontal disease status.

The OR analyses showed a significant positive association between any two bacteria members of the red complex (Table 3). The strongest relationship was found between *P. gingivalis* and *T. denticola* (OR = 9.15, $P = 0.048$). The association between *P. gingivalis* and *T. forsythia* was also strong (OR = 8.15, $P = 0.02$), while the association between *T. forsythia* and *T. denticola* was weaker (OR = 5.59, $P = 0.02$). No

Table 3 Odds ratios (ORs) and 95% confidence intervals (CIs) of the association among bacteria adjusted for age and gender ($n = 60$)

	Porphyromonas gingivalis		*Tannerella forsythia*		*Treponema denticola*		*Aggregatibacter actinomycetemcomitans*	
	OR (95% CI)	P-*value*	*OR (95% CI)*	P-*value*	*OR (95% CI)*	P-*value*	*OR (95% CI)*	P-*value*
P. gingivalis	–	–	8.15 (1.38–48.13)	0.02	9.15 (1.02–82.07)	0.05	1.34 (0.27–6.65)	0.72
T. forsythia			–	–	5.59 (1.29–24.20)	0.02	1.70 (0.39–7.29)	0.48
T. denticola					–	–	2.57 (0.73–9.02)	0.14
A. actinomycetemcomitans							–	–

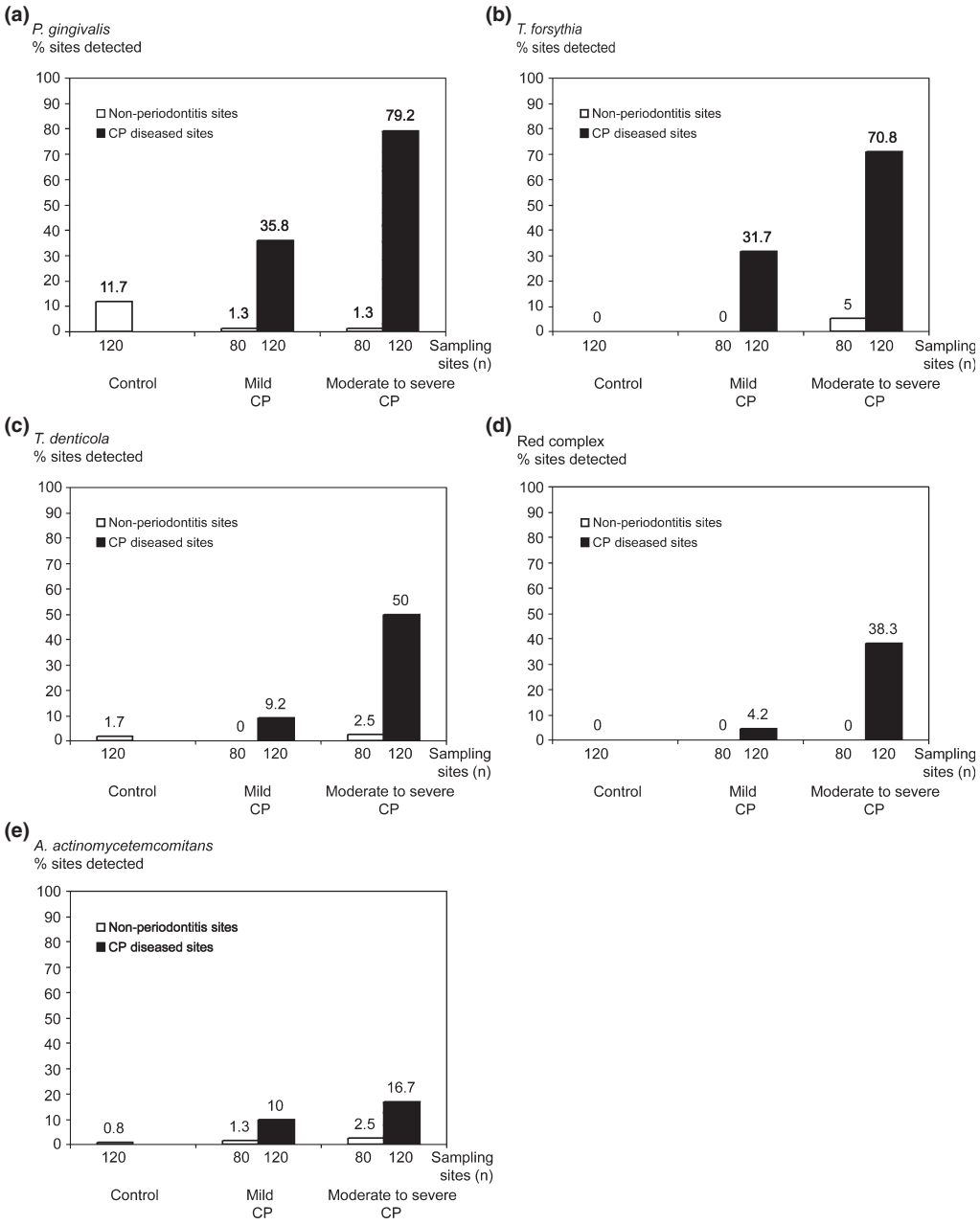

Figure 1 Percentage of sites positive for (**a**) *Porphyromonas gingivalis*, (**b**) *Tannerella forsythia*, (**c**) *Treponema denticola*, (**d**) red complex, and (**e**) *Aggregatibacter actinomycetemcomitans*

statistically significant association was observed between the presence of *A. actinomycetemcomitans* and any other periodontal pathogens.

Discussion

Several studies have examined the distribution of periodontal pathogens in Asian populations (Choi *et al*, 2000; Takeuchi *et al*, 2001, 2003; Dahlen *et al*, 2002; Papapanou *et al*, 2002; Dogan *et al*, 2003; Yang *et al*, 2004). In general, the periodontitis subjects showed significantly higher prevalence of *P. gingivalis*, *T. forsythia*, and *T. denticola* than the non-periodontitis individuals. Previous studies in southern Thai subjects also reported high prevalence of *A. actinomycetemcomitans* (88.0–92.7%) (Dahlen *et al*, 2002; Papapanou *et al*, 2002). Our study showed high prevalence of *P. gingivalis*, *T. forsythia*, *T. denticola*, and the red complex, but low prevalence of *A. actinomycetemcomitans* (35%), in subjects with moderate to severe CP.

A wide range of the prevalence of *A. actinomycetemcomitans* has been reported in Asian populations with CP, ranging from lower than 10% in Japanese to more than 85% in Korean populations (Choi *et al*, 2000; Dogan *et al*, 2003; Takeuchi *et al*, 2003). This study was conducted in northeastern Thailand. We observed a difference in the prevalence of *A. actinomycetemcomitans* compared with previous reports conducted in

southern Thailand (*Dahlen et al,* 2002; Papapanou *et al*, 2002). This apparent discrepancy may be the result of methodological differences as well as the study populations derived from varied geographic locations and socioeconomic status. In our study population, the moderate to severe CP group demonstrated lower prevalence of *A. actinomycetemcomitans* (35%) than the mild CP group (45%). This may be partly explained by the observation that the prevalence of *A. actinomycetemcomitans* decreased as probing depth increased (Hamlet *et al*, 2001).

Our findings showed that in the non-periodontitis control group, the prevalence of *P. gingivalis* was as high as 45%. High prevalence of *P. gingivalis* in healthy sites and non-periodontitis subjects has been previously reported in other populations (Teanpaisan *et al*, 1996; Dogan *et al*, 2003). It has been suggested that some specific genotypes of *P. gingivalis* are associated with periodontitis (Teanpaisan *et al*, 1996; Ozmeric *et al*, 2000). The PCR method used in this study is unable to either quantify the number or identify the subspecies of bacteria. In this study, the presence of *P. gingivalis* in a subject was not significantly related to periodontal disease status.

T. forsythia was not detected in any non-periodontitis subjects in our study, suggesting that in this population, the presence of *T. forsythia* and *P. gingivalis* + *T. forsythia* was specifically associated with CP, but might not be related to the disease severity. *T. denticola* and *P. gingivalis* + *T. denticola* were associated with the moderate to severe form of CP. *T. forsythia* + *T. denticola* and the coexistence of the red complex species detected at the same site in an individual were strongly associated with CP as well as the disease severity. Our findings suggested a major role of these bacteria in CP in this study population. Nonetheless, further studies with a larger sample size are warranted to confirm our findings.

The detection frequencies of periodontopathic bacteria at the site level were also investigated in this study (Figure 1). In general, the detection figures of these periodontal pathogens and the red complex in non-periodontitis sites of CP patients were markedly low and appeared to be similar to those of the non-periodontitis control subjects. This is in contrast to some studies demonstrating that the prevalence of these pathogens in the healthy sites of periodontitis patients was higher than in the sites of periodontally healthy subjects (Haffajee *et al*, 1998; Choi *et al*, 2000). *P. gingivalis* was detected in 11.7% of sites in non-periodontitis controls (Figure 1a). The detection frequency of *P. gingivalis* was reported to be positively correlated with BOP (Kojima *et al*, 1993). As the control group in our study had 47.9% of sites with BOP, it is possible that these sites were included in this study group and contributed to the high prevalence of *P. gingivalis*.

It is noteworthy that coexistence of *P. gingivalis*, *T. forsythia*, and *T. denticola* at the same site (red complex) was not found in any non-periodontitis sites of all study groups (Figure 1d), confirming the specific association of this bacterial complex with CP in our study population. This is in agreement with the previous reports in other populations, demonstrating that the members of the red complex were found together in diseased lesions, and that the sites harboring all 3 species showed the deepest mean pocket depth (Umeda *et al*, 1996; Socransky *et al*, 1998). However, it is possible that the red complex could not be detected in non-periodontitis site in our study because of the small number of sampling sites.

In our study population, the presence of *A. actinomycetemcomitans* at the site level was rather low and was not markedly related to periodontal disease status (Figure 1e). This is in accordance with a study in a Japanese population reporting that less than 10% of the CP diseased sites was positive for *A. actinomycetemcomitans* (Takeuchi *et al*, 2003). In contrast, studies in Chinese, Korean and southern Thai populations have shown high detection frequencies of *A. actinomycetemcomitans* ranging approximately from 40% to 75% of the sampling sites (Papapanou *et al*, 1997, 2002; Choi *et al*, 2000). Nonetheless, our results did not completely rule out the role of *A. actinomycetemcomitans* in CP in our study population. Further study on the genetic diversity of *A. actinomycetemcomitans* in this population is needed to elucidate an association of specific genotypic profiles with health or disease.

Although the bacterial composition in periodontal pockets appears to be mixed, certain combinations of bacterial species have been implicated in periodontal disease (Haffajee *et al*, 1998; Socransky *et al*, 1998). We found significantly positive association between *P. gingivalis* and *T. denticola* (OR = 9.15), *P. gingivalis* and *T. forsythia* (OR = 8.15), as well as *T. forsythia* and *T. denticola* (OR = 5.59) (Table 3). These pathogens were shown to co-aggregate strongly *in vitro* (Grenier, 1992a; Onagawa *et al*, 1994; Yao *et al*, 1996) and were frequently found together in periodontitis lesions of various study populations, in particular, in sites with deeper pockets or more advanced lesions (Umeda *et al*, 1996; Socransky *et al*, 1998). The biological basis of the association among these pathogens is not well understood. One species of the complex may produce growth factors required by another in that complex. This has been implicated in the interaction between *P. gingivalis* and *T. denticola* (Grenier, 1992b). The association of *A. actinomycetemcomitans* with any test bacteria was not demonstrated in this study, which was in accordance with a previous study in a Turkish population (Dogan *et al*, 2003).

In conclusion, our findings in a Thai population demonstrated the occurrence and interspecies relationships of certain bacteria in CP. Our results suggested that red complex bacteria were predominant periodontal pathogens of the moderate to severe form of CP in this study population. The presence of *T. forsythia* together with *T. denticola* at the same site and the red complex (coexistence of three species) were significantly associated with the disease severity. Therefore, these pathogens should be considered as targets for the prevention and treatment of periodontal disease in this population. When developing strategies for prevention and treatment of periodontal disease, clinicians should take into

account the potential differences in the subginigival microbiota and their association with periodontal status in a particular population.

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Author contributions

Drs. N Wara-aswapati and W Pitiphat contributed to the study design, data analysis and manuscript drafting. Dr. L Chanchaimongkon contributed to the study design, data collection, PCR analysis and critically reviewed the manuscript. Drs. S Taweechaisupapong, JA Boch and I Ishikawa contributed to the study design and critically reviewed the manuscript.

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