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Enhancement of cariogenic virulence properties of dental plaque in asthmatics

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ABSTRACT

Objective: The aim of this study was to investigate the caries risk of asthmatics in relation to acidogenicity and the expression of caries-related genes in dental plaque.

Methods: A case-control study composed of 38 asthmatics (cases) and 22 controls with an age range from 6 to 60 years. Characteristics of asthma, use of medications, oral hygiene practices and dietary habits assessed by questionnaires and interviews. The dental plaque maturity evaluated using GC Tri Plaque ID Gel TM. The expression of *brpA*, *gtfB*, *gbpB*, *ldh*, *luxS* and *spaP* genes analyzed using real-time PCR.

Results: Asthmatics had a higher percentage of mature and acidogenic plaque than immature plaque. In contrast, immature plaque was more evident in controls. Acidogenic plaque commonly occurred in patients using 1 or a combination of two medications. High frequency in meals and sweets were found in asthmatics. Real-time PCR revealed that the expression of *spaP*, *gtfB*, *gbpB*, *ldh*, *brpA* and *luxS* were enhanced in asthmatics compared with the control group.

Conclusion: An increase in acidogenic and mature plaque is found in asthmatics. The expression of *spaP*, *gtfB*, *gbpB*, *ldh*, *brpA* and *luxS* in dental plaque are upregulated in asthmatics.

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Introduction

Untreated caries in permanent teeth was the most prevalent health condition in 2010, affecting 2.4 billion people worldwide (1). Likewise, asthma is a chronic disease affecting 272.68 million people worldwide (2).

Recently, a meta-analysis of 3[5] studies (2011–2017) have revealed that individuals with asthma have 1.5 times higher likelihood of dental caries (3). This may be attributable to β_2-agonists administration, which causes reduced saliva secretion (4), a decrease in buffer capacity (5) and higher salivary levels of *Streptococcus mutans* and lactobacilli (6,7). However, other studies have not found this effect (8,9).

Dental caries has risen as a substrate disruption of the bacterial community in terms of the "ecological plaque hypothesis" (10). Therefore, attention should not be on specific bacteria but on the virulence, function and output of the dental plaque (11,12). Presently, some reports indicate a correlation between asthma and plaque accumulation (13) while others have found no such connection (14,15).

Various methods are used to assess caries risk including plaque staining and bacterial culture, however, recent studies show that the polymerase chain reaction (PCR) technique is a reliable, fast and sensitive method in determining bacterial virulence genes (16,17).

Streptococcus mutans plays roles in dental biofilm [3]d dental caries. Its virulence genes are classified as the genes involved in bacterial adhesion, extracellular polysaccharide formation, biofilm formation, sugar uptake and metabolism, acid formation, and acid tolerance (17). SpaP is an adhesion protein mediating early bacterial attachment in plaque. Glucan binding proteins (GBP) and glucosyl transferases (Gtfs) facilitate bacterial adherence and biofilm accumulation. LDH contributes to lactic acid production in plaque. BrpA plays roles in acid tolerance and biofilm development. LuxS-mediated signaling pathway contributes in plaque formation (17–21). Despite these findings, research and studies on cariogenicity of plaque in asthma is scarce.

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Table 1. Primers used in this study.

Primer	Forward primers (5′–3′)	Reverse primers (5′–3′)	Size (bp)
brpA	CGTGAGGTCATCAGCAAGGTC	CGCTGTACCCCAAAAGTTTAGG	148
gtfB	AGCAATGCAGCCATCTACAAAT	ACGAACTTTGCCGTTATTGTCA	98
gbpB	CGTGTTTCGGCTATTCGTGAAG	TGCTGCTTGATTTTCTTGTTGC	108
ldh	TTGGCGACGCTCTTGATCTTAG	GTCAGCATCCGCACAGTCTTC	92
luxS	ACTGTTCCCCTTTTGGCTGTC	AACTTGCTTTGATGACTGTGGC	93
spaP	TCCGCTTATACAGGTCAAGTTG	GAGAAGCTACTGATAGAAGGGC	121
16SrRNA	TCCACGCCGTAAACGATGA	TTGTGCGGCCCCCGT	119

Thus the aim of this study is to assess and compare the acidogenic properties and virulence genes of plaque between asthmatic and control groups. Pronounced differences in the cariogenicity of plaque between the two groups would support the hypothesis that asthma is a risk factor of dental caries.

Methods

Participants and background

A case-control study was designed in which 38 patients aged 6–60 years old with asthma (cases) were compared with 22 healthy volunteers (controls) aged 6–32 years old without asthmatic conditions. Ethical approval from the Institutional Review Board (IRB), Thammasat University, Pathum Thani, Thailand (IRB number 022/2557) was given before selecting the sample. Sample size was calculated using the PS software version 3.1.2 by Dupont and Plummer (Vanderbilt University, Tennessee). In order to detect a standardized difference of 0.5 in mean gene expression between two groups, with 80% power of detection and an alpha error of 0.05, at least 38 asthmatic patients and 19 control subjects were required (22). To classify asthma, we used the GINA guideline (23). The participants were given appointments at an allergy clinic of Thammasat University hospital. A consent form was given to the controls, parents and/or patients before plaque staining and collection.

Questionnaire

Interview questionnaires were used to obtain demographic data and/or asthma characteristics; and a 3-day dietary history. Dietary analysis was performed as described by Wongkamhaeng et al. (24).

Dental plaque staining

A plaque maturity assessment was done using GC Tri Plaque ID Gel™ (GC Corporation, Tokyo, Japan). All tooth surfaces except occlusal surfaces were stained. The original dark blue color changed to light blue when the pH in the plaque was less than 5. Immature and mature plaque turned pink and purple, respectively (25). Based on the color changes on tooth surfaces, the plaque maturing staining (PMS) was obtained by using the formula:

$$\%\ \mathrm{PMS} = (\text{number of tooth with each colored plaque}) \times 100/\text{total number of teeth examined}$$

Dental plaque collection

Plaque samples were collected with a sterile dental explorer. Each sample picked was then placed in a sterile 1.5 ml micro-centrifuge tube. The plaque sample was stored at −80° C until use.

Determination of gene expression by real time PCR

Total RNA of plaque samples was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc., Japan). Real-time PCR was performed using a KAPA SYBR® FAST qPCR Kit Master Mix (Kapa Biosystems, Wilmington, MA) on a Bio-Rad IQ5 cycler (Bio-Rad, Hercules, CA). The PCR primers for *brpA, gtfB, gbpB, ldh, luxS* and *spaP* genes were retrieved from Wen et al. 2010 (26). The *16SrRNA* was served as the internal control (27) (Table 1). The gene expressions were evaluated using the $2^{-\Delta Ct}$ equation. In this case, $\Delta Ct = Ct$ (*interested gene*) − Ct (*16SrRNA*).

Statistical analyses

The statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad software Inc., La Jolla, CA, USA). Normality of data was assessed by the Shapiro–Wilk normality test. Two tailed unpaired student's *t*-test or one-way analysis of variance was used when appropriated for parametric data. Otherwise, the non-parametric Mann–Whitney *U*, Fisher's exact test, Kruskal-Wallis tests and Spearman's rank correlation

Table 2. Characteristic of the subjects included in this study.

	Case ($n = 38$)	Control ($n = 22$)	p-value
Age (years), (median, range)	10 (6–60)	11 (6–32)	0.42*
Sex (% male)	22 (52.89)	6 (27.27)	0.03*
(% female)	16 (42.10)	16 (72.72)	
Asthma Onset (Duration) (%)			
Less than 2 years	11 (28.95)	–	–
Duration 2–4 years	14 (36.84)	–	–
More than 4 years	13 (34.21)	–	–
Asthma Severity (%)			
Control asthma	28 (73.68)	–	–
Partial asthma	6 (15.79)	–	–
Uncontrolled asthma	4 (10.53)	–	–
Comorbidities (%)			
Asthma	12 (31.58)	–	–
Asthma and allergic rhinitis	23 (60.53)	–	–
Asthma and sinusitis	1 (2.63)	–	–
Others	2 (5.26)	–	–
Mouth cleaning after used the drug (mouth rinse with water) (%)			
Yes	23 (60.53)	–	–
No	15 (39.47)	–	–
Medicine use (%)			
1 drug	5 (13.16)	–	–
2 drugs	12 (31.58)	–	–
3 drugs	11 (28.95)	–	–
4 drugs	3 (7.89)	–	–
5 drugs	5 (13.16)	–	–
6 drugs	2 (5.26)	–	–
Mode of administration of the drug, (%)			
Inhalation	1 (2.63)	–	–
Oral	4 (10.53)	–	–
Inhalation and oral	33 (86.84)	–	–

*Mann–Whitney *U*-test.

Table 3. Distribution of asthmatic patients and controls according to oral hygiene practice and dietary habits.

Oral hygiene practice	Case	Control	p-value
Tooth brushing			
1 time/day, *n* (%)	5 (13.16)	1 (4.54)	
$\geq$ 2 times, *n* (%)	33 (86.84)	21 (95.45)	0.39*
Fluoride toothpaste			
Use, *n* (%)	38 (100)	1 (4.54)	
Not use, *n* (%)	0 (0)	21 (95.45)	0.37*
Dental checkups frequency			
Never, *n* (%)	7 (18.42)	6 (27.27)	
At least 1 time, *n* (%)	31 (81.58)	16 (72.72)	0.52*
Dietary history			
Meals per day, (median, range)	5.33 (1.66–7.66)	4.00 (3.00–6.33)	0.03#
Sweet snacks, (median, range)	3.00 (0.00–4.66)	1.00 (0.66–3.33)	0.01#
Sugary drink, (median, range)	2.67 (0.00–5.00)	2.66 (0.33–3.66)	0.63#

*Fisher's exact test.
#Mann-Whitney-*U*-test.

coefficient (ρ) were conducted. P values ≤ 0.05 was considered statistically significant.

Results

Studied samples

Demographic and clinical characteristics of subjects are presented in Table 2. Cases and controls had a similar age, ranging from 6 to 60. However, there were more male subjects with asthma than female subjects. In contrast, female subjects were more common in the controls ($p < 0.05$).

Oral hygiene practice and dietary habit

Table 3 shows no significant difference between asthmatic and control groups with regards to oral hygiene practices. However, frequencies of meals and sweets in asthmatics were higher than in the controls ($P < 0.05$).

Medications

The data presented in Tables 4 and 2 demonstrated that most patients (12, 31.58%) used corticosteroids with the addition of antihistamine or leukotriene. Eleven patients (28.95%) used a combination of 3

Table 4. The number and names of medication use.

The number of medication	Name of medication
1 drug	Leukotriene; Oral corticosteroid; Inhaled corticosteroid (Nasonex); or Antihistamine
2 drugs	Antihistamine + inhaled corticosteroid (Nasonex); Oral corticosteroid + inhaled corticosteroid (Avamys); Leukotriene + inhaled corticosteroid (Avamys); or Antihistamine + inhaled corticosteroid (Avamys)
3 drugs	ICS + LABA + oral corticosteroid; ICS + LABA + antihistamine; Leukotriene + antibiotics + inhaled corticosteroid (Avamys); Leukotriene + antihistamine + inhaled corticosteroid (Avamys); Leukotriene + antihistamine + inhaled corticosteroid (Nasonex); or Oral corticosteroid + leukotriene + inhaled corticosteroid (Avamys)
4 drugs	ICS + LABA + leukotriene + SABA; or ICS + LABA + leukotriene + antihistamine
5 drugs	ICS + LABA + leukotriene + SABA + antihistamine; ICS + LABA + leukotriene + antihistamine + antibiotics; ICS + LABA + xanthine + leukotriene + antihistamine; ICS + LABA + xanthine + leukotriene + antihistamine; or ICS + LABA + anti-Ig E + leukotriene + antihistamine
6 drugs	ICS + LABA + xanthine + anti-Ig E + leukotriene + antihistamine

ICS: inhaled corticosteroids, LABA: long-acting β_2 agonists, SABA: short -acting β_2 agonists.

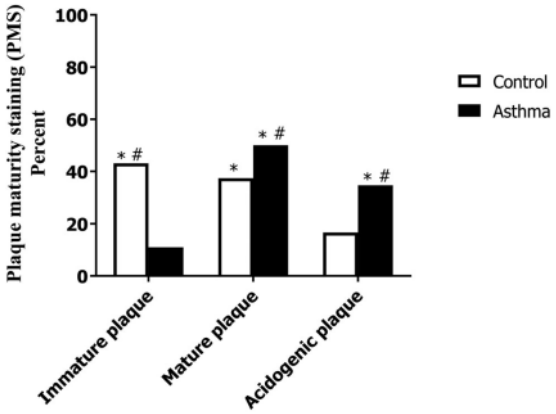

Figure 1. Dental plaque maturity was assessed by staining with a three-tone disclosing agent. Data express as percent of tooth stained with each color. The bar graph represented the median values. *Symbols* indicate significant difference ($p < 0.05$) between colored plaque (*Kruskal–Wallis test and Dunn's multiple comparisons *post-hoc* test) in each group and between groups (#, Mann-Whitney *U*- test).

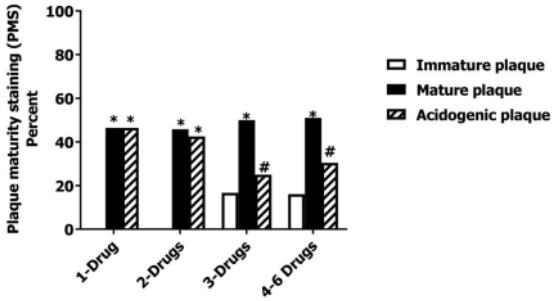

Figure 2. The difference in plaque maturity in respective numbers of drug use. The bar graph represented the median values. *Symbols* indicate significant difference in each group (Kruskal–Wallis test followed by Dunn's multiple comparison *post- hoc test*, $p < 0.05$).

drugs which were corticosteroid and long acting β_2 antagonist (LABA) with the addition of leukotriene, antihistamine or antibiotics.

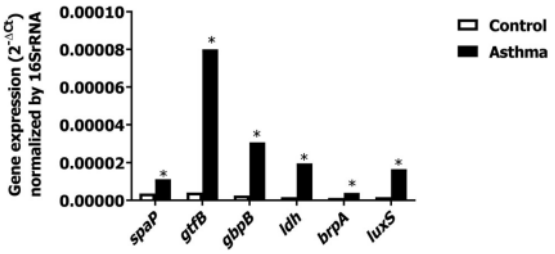

Figure 3. The difference in selected gene expression between asthmatic and control groups. Real- time PCR of *spaP*, *gtfB*, *gbpB*, *ldh*, *brpA* and *luxS* was carried out in triplicate. Different expression is shown as Delta Ct values. Data presented here was generated from at least three independent sets of determination. The bar graph represents the median value ($n = 3$), with * illustrating statistical difference in each gene at $p < 0.05$ when compared between asthmatic and control groups (Mann-Whitney *U*-test).

Plaque maturity

Figure 1 shows the lowest percentage of immature plaque (pink) staining in asthmatics. There was no statistical significance between the percentage of mature (purple) and acidogenic (light blue) plaque in the asthmatic group. In the controls, percentage of acidogenic plaque was significantly lower than that of immature and mature plaque. There was 34.67% acidogenic plaque in asthmatics compared to controls (16.67%) ($p < 0.05$).

Figure 2 shows that mature plaque was found in patients using 1, 2, 3 or 4–6 medications. However, the highest percentage of acid producing plaque was found in patients using 1 or 2 drugs.

Virulence gene expression

As compared to the controls, the expression of *gtfB*, *gbpB*, *ldh*, *luxS*, *brpA* and *spaP* was increased by 19.57, 11.96, 11.29, 9.73, 3.08 and 3.07 folds, respectively in asthmatics (Figure 3). Spearman's rank correlation indicated that there was a negative correlation

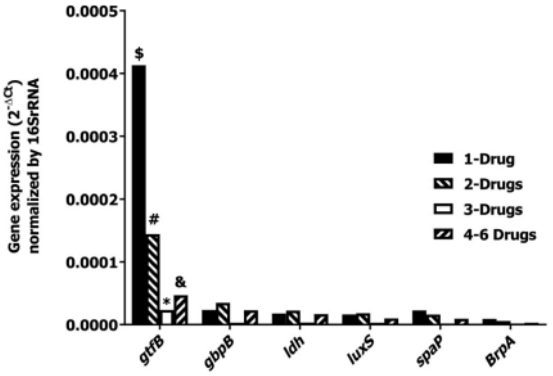

Figure 4. The distribution of selected gene expression according to the numbers of drug use. Real-time PCR of *gtfB*, *gbpB*, *ldh*, *luxS spaP* and *brpA* was carried out in triplicate. The gene expression is shown as Delta Ct values. The bar graph represents the median value ($n = 3$), with *symbols* illustrating statistical difference in the numbers of drug use in the respective genes at $p < 0.05$ (Kruskal–Wallis test followed by Dunn's multiple comparison *post-hoc* test).

between the percentage of immature plaque and the expression of *ldh*, ($\rho = -0.474$, $p < 0.05$), *spaP* ($\rho = -0.432$, $p < 0.05$), *luxS* ($\rho = -0.430$, $p < 0.05$), *gbpB*, ($\rho = -0.404$, $p < 0.05$), *brpA* ($\rho = -0.367$, $p < 0.05$), and *gtfB* ($\rho = -0.356$, $p < 0.05$) in our samples. We found a positive correlation between acidogenic plaque prevalence and the expression of *ldh*, ($\rho = 0.379$, $p < 0.05$), *spaP* ($\rho = 0.333$, $p < 0.05$), *luxS* ($\rho = 0.321$, $p < 0.05$), *brpA* ($\rho = 0.310$, $p < 0.05$), and *gbpB* ($\rho = 0.306$, $p < 0.05$). There was no association between *gtfB* expression and acidogenic plaque ($\rho = 0.183$, $p > 0.05$). In contrast, we could not demonstrate the relationship between studied genes expression and mature plaque ($p > 0.05$). Figure 4 demonstrated that *gtfB* expression was markedly observed in patients with 1, 2, 3 or 4–6 medications ($p < 0.05$). Conversely, we could not demonstrate the differences in the numbers of drug use according to the expression of *spaP*, *gbpB*, *ldh*, *brpA*, and *luxS*.

Discussion

Currently, the association between plaque accumulation and asthma is still controversial (9,13,14). Previous studies on asthma and dental caries determined plaque formation using plaque index (PI) (28), simplified oral hygiene index (OHI-S) (29) or plaque control record by O' Leary et al. (30) which may fail in detecting small plaque areas (31). Toolta et al. (32) demonstrated the differences in plaque pH between asthma and control groups. However, Stensson, et al. (15) reported that there was no difference as such. Here we used GC Tri Plaque ID Gel™, which recognizes plaque maturity based on pH response of the dyes (33). Our findings show high percentage of acidogenic and mature plaque in asthmatics. Therefore, inconsistent results may be explained by differences in plaque staining. No difference in oral hygiene practices between cases and controls in this study suggests that the acidogenicity of plaque may be attributed to the disease itself and/or medication used rather than local factors (34). However, we found an increase in meals and sweets in asthmatics which is in agreement with a previous study (35). Therefore, it is possible that an increase in sweet intake plays a role in cariogenic plaque development in asthma (33). Another explanation may be the effect of medications on plaque properties. The number and types of medications used in this study were, to a certain extent, the same as previous reports (36,37). However, we found that the patients who used corticosteroid alone or in combination with β_2-antagonists had high levels of acidogenic and mature plaque. The result is expected because it is known that β2-antagonists is associated with changes in salivary and plaque pH below the critical pH for enamel demineralization of 5.5 (32,38).

We found *gtfB*, *gbpB*, *ldh*, *luxS*, *brpA* and *spaP* were markedly expressed in dental plaque of asthmatics. In addition, a positive correlation between the acidogenic plaque and the expression of *ldh, brpA, luxS, spaP* and *gbpB* but not *gtfB* was found in the study. This indicates that the up-regulation of these virulence genes might be a contributing factor to the formation and development, acid production and acid tolerance of dental plaque (39,40). Up-regulation of *ldh*, may lead to an increase of acid production in plaque (41). Acid tolerance and plaque development might occur due to an increase of *brpA* expression (21,39). Increased *luxS* might contribute to dental plaque accumulation (42). The *spaP* plays an important role in early bacterial adherence to teeth via interaction with the salivary pellicle (20) whereas *gbpB* involves in the adhesion of *S. mutans* to glucans, extracellular polysaccharides of plaque matrix, in the late bacterial adherence (43). Surprisingly, our findings suggest that *gtfB* involves in initial plaque formation, however, it may not play a role in dental plaque metabolism (44).

This study bears some limitations. Although a decrease in salivary quantity was commonly observed in asthmatic patients, salivary flow was not measured in this study because collecting sufficient saliva samples posed a challenge especially in young children

with low compliance. Therefore, the influence of saliva in caries risk must be interpreted with caution. However, the strength of our study is the analysis of multifactor affecting dental caries using genetic identification of putative virulence genes in cariogenic bacteria in combination with acidogenic potential of dental plaque analyses, dietary intake assessment and medications to help shed light on dental caries risk in asthma patients.

Conclusion/key findings

Our data suggests that asthmatic conditions and/or medication induce the virulence properties of plaque. An increase in acidogenic and mature plaque is observed in asthmatics. Furthermore, the expression of *ldh, brpA, luxS, spaP*, *gbpB* and *gtfB* in dental plaque is enhanced in asthma.

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Disclosure statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article. The authors have no financial, consulting or personal relationship(s) disclosures to report.

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