CARIOGENICITY ACTIVITY AND RELATED GENE EXPRESSION OF CARIOGENIC BACTERIA IN DENTAL BIOFILM OF ASTHMATIC PATIENTS

BY

MRS. DHYANI WIDHIANINGSIH

A DISSERTATION SUBMITTED IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
FACULTY OF DENTISTRY
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ENTITLED

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was approved as partial fulfillment of the requirements for

the degree of Doctor of Philosophy (Oral Health Science)

on January 3, 2020

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Dissertation Title	CARIOGENICITY ACTIVITY AND RELATED GENE EXPRESSION OF CARIOGENIC BACTERIA IN DENTAL BIOFILM OF ASTHMATIC PATIENTS
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Academic Years	2019

ABSTRACT

Background: Both of dental caries and asthma are multifactorial diseases, this highlights the importance for dental practitioners to evaluate the risk of caries in asthma patients so that prevention can be anticipated. To our knowledge, this study is the first report on evaluating cariogenic dental plaque using three-tone disclosing agent and expression of caries virulence gene in dental plaque samples of asthma patients.

Material and methods: In the cross-sectional study, subjects were divided into 2 groups, including group of 38 asthma patients and group of 22 healthy subjects with an age range between 6-60 years. Data collection was included demographic data, asthma characteristic, use of medication, oral health behavior, and dietary habits using questionnaire and interview. The dental plaque maturity in the asthma and healthy group were assessed using GC Tri Plaque ID gel. The stained plaque was collected and used to determine the expression of caries virulence genes including *spaP*, *gtfB*, *gbpB*, *ldh*, *brpA*, and *luxS* genes by real-time PCR. The expression level of gene was normalized with *16S rRNA* as an internal control gene.

Result: Asthmatic had a higher percentage of mature and acidogenic plaque compared to immature plaque. In contrast immature plaque was obviously found in controls. Acidogenic plaque was obviously found in the patients who used 1 or combination of 2 medications. High frequency in food and sugar intakes were found in asthmatic. Real-time PCR revealed that the expression of *spaP*, *gtfB*, *gbpB*, *ldh*, *brpA*, and *luxS* genes increased in asthmatic compared to healthy group.
Conclusion: An increase in acidogenic and mature plaque was found in asthma patients. The expression of *spaP*, *gtfB*, *gbpB*, *ldh*, *brpA*, and *luxS* genes in detal plaque were upregulated in asthmatics

Keyword: Asthma, caries, GC Tri Plaque ID Gel, *spaP*, *gtfB*, *gbpB*, *ldh*, *brpA, luxS*

ACKNOWLEDGEMENTS

First, I would like to thank Allah SWT who the giver of all grace for me. He continued to give me strength and health to do my doctoral studies.

I would like to express my deep and sincere thanks to my advisor, Prof. Dr. Sittichai Koontongkaew, B.Sc., DDS., Ph.D., Dip. DPH, for his kindness, patience, and give me the opportunity, advice, great guidance, and support for study at The Faculty of Dentistry Thammasat University.

I am also very thankful to my co-advisor, Asst. Prof. Dr. Kusumawadee Utispan, Ph.D, for advice, knowledge, comments and precise information have contributed significantly to this research

Special thanks to Prof. Dr. Orapan Poachanukoon from Pediatric OPD Clinic Thammasat University Hospital for imparting her knowledge and expertise in asthma

I would also like to thank all my friend which help me for work in laboratory and supporting (Dr. Suppanut, Mrs. Paupanga K, Dr. Nattisa) and thanks to past students of Faculty of Dentistry Thammasat University (Dr. Watcharee Kliangkaeo, Dr. Sirirat Reungsuwat, Dr. Pornwatcharin Limwattanachai) and nurse for helping in data collection.

I am also very grateful to Dean of Faculty of Dentistry Trisakti University Prof. Dr. drg. Erri Astoeti, M. Kes and former Dean Prof. Dr. drg. Melanie Sadono Djamil, M. Biomed for their give support me to continue my studies in the Faculty of Dentistry Thammasat University. I also special thanks to all staff members of Pediatric Dentistry Department Faculty of Dentistry Trisakti University (Prof. Dr. drg. Arlia Boedijanti, SU, SpKGA, Prof. Dr. drg. F. Loes D Sjahruddin, M.Kes, Dr.drg. Fatimah Boenjamin, SpKGA, drg. Liane Andajani T, Dr.drg. Sri Ratna Laksmiastuti, SpKGA, Dr.drg. Jeddy, SpKGA, drg. Tri Putriany Agustin, SpKGA, drg Enrita Dian Rahmadini, SpKGA, drg. Arianne Dwimega,

SpKGA, Dr. drg. Armelia Sari Widyarman, M.Kes, drg. Labiba Idzni Marjani, drg. Idham Tegar Badruzzaman) for their support and encouraged me to complete this study.

Finally, I want to express my love and thanks to my family. My beloved husband, Ir. Rachmat Prijatna, for your wise counsel and sympathetic ear, you are always there for me. My beloved children Annisa Widhiyanti, Aurelia Chairunisa, and Dimas Naufal Maheswara, who served as my inspiration to pursue this undertaking. My late dear parents and my late parent-in-law thanks for your love and prayer when we were together, may Allah SWT provide the best place for you by His side. My brother, my sister in law and brother in law and all my family, all my friends, thank you very much, your help and support will never be forgotten.

Mrs. Dhyani Widhianingsih

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statistical difference in the numbers of drug use in the respective genes at $p < 0.05$ (Kruskal-Wallis test follow by Dunn's multiple comparison *post-hoc* test).

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
ß	Beta
µg	Microgram
µl	Microliter
AR	Allergic Rhinitis
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
Brp A	*Biofilm regulatory protein A*
°C	Degree Celsius
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DexA	*Exodeoxyribonuclease*
EPS	Extracelullar polysaccharides
g	Gram
GbpB	*Glucan Binding Protein B*
GtfB	*Glucosyltransferase B*
GTF	Glucosyltransferase
h	Hour
ICS	Inhaled corticosteroid
IPS	Intracellular polysaccharides
mg	Milligram
ng	Nanogram
Ldh	*Lactate dehydrogenase*
LABA	Long acting ß2-agonist

Lux S	*S-ribosylhomocysteine lyase*
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
SABA	Short acting ß2-agonist
S.mutans	*Streptococcus mutans*
SpaP	*Surface protein adhesin*
RNA	Ribonucleic acid
RT	Room temperature

CHAPTER 1
INTRODUCTION

1.1 Dental caries

Dental caries is a multifactorial multi-processes disease that involves interaction between causative agent (cariogenic bacteria), host, diet (fermentable carbohydrate) and time.[1] Missing of any of these, lesion would not develop into dental caries.[2] Salivary dysfunction which affects flow rate of saliva would increase caries risk through encourages the growth of aciduric organisms such as *Streptococcus mutans* (*S. mutans*) and *Lactobacilli.*[3] *Streptococcus* which are the earliest genii of bacteria which colonize on tooth surfaces and initiates the formation of plaque for further colonization of cariogenic bacteria including *S. mutans*, *Streptococcus sobrinus* (*S. sobrinus*) and *Lactobacillus.* These cariogenic bacteria exhibit cariogenic properties including adherability, acidogenicity, aciduricity, and adaptability.[4]

1.2 Asthma

Asthma is a chronic inflammatory disease which affects more than 300 million people worldwide. There was forecasted that it will increase further as much as one hundred million people in 2025.[5] According to report from Global Asthma network in 2018, Indonesia has prevalence of childhood asthma range from 4-11% in 6-7 years old and 6-13% in 13-14 years old, while prevalence in children of Thailand is still over 10%.[6] Although previous studies have shown asthma is associated with some oral problems, particularly dental caries.[7,8] Some studies on dental caries in asthmatic patients have shown some controversial results. Several studies had reported that the treatment of asthma can lead to the reduction of salivary flow, changing in salivary composition, including changes in pH, and increasing of the number of cariogenic bacteria in dental plaque that can contribute to an increased risk of caries,[5,8] but some other studies found no correlation between asthma with drugs usage and increased caries risk.[7]

1.3 Statement of the problem

Asthma is a chronic inflammatory disorder of the airway involving inflammatory cells and multiple mediators that can lead to changes in pathophysiological characteristics. Several studies have reported that asthma possibly associated with oral problem, particularly dental caries. Previous reports have shown that the treatment of asthma can lead to reduction of salivary flow, changing of saliva composition, including changes in pH, increases of cariogenic bacteria in dental plaque, and saliva that can contribute to an increased risk of caries.[5,8] Asthmatic patients who received treatment with the drug can decrease salivary flow rate, decrease pH, alter composition of saliva, xerostomia and altered behavior (mouth breathing) lead to occurrence of dental caries. Several studies found individuals with asthma appear to accumulate higher amounts of dental biofilm, such *S. mutans*, and lactobacilli. *S. mutans* known as cariogenic bacteria that have virulence factor and resides on the tooth surface along the oral biofilm. The main virulence factor from cariogenic bacteria are adhesiveness, acidogenicity, aciduricity, and adaptativeness. Each of these factors work coordinately to alter dental plaque ecology.

The characteristic of dental plaques can be determined by using GC Tri Plaque ID gel. Cariogenic bacteria in dental biofilms can be seen by the presence of color staining on dental plaque.

The presence of genes from cariogenic bacteria *S. mutans* in dental biofilm can be detected by PCR. The target gene for the PCR may be related to virulence factors such as *Glucosyltranferases B (gtfB), Glucan binding protein B (gbpB), Surface associated protein P (spaP), Lactate dehydrogenase (ldh), Biofilm regulatory protein A (brpA)* and *S-ribosylhpmocysteine lyase* (*luxS)* genes. The function of these genes are very important in the formation of biofilm and further on the development of dental caries. This study will investigate the expression of the genes and what they can be done on caries prevention efforts for asthma patients.

1.4 Objective

1. To study the behavior Oral Health Instruction (OHI) and diet consumption of asthmatic patients and healthy subjects
2. To determine the differences of the cariogenic dental plaque quality in asthmatic patient and healthy subjects
3. To investigate cariogenic-associated gene expression in microorganism in dental plaque collected from asthmatic patient and healthy subjects

1.5 Conceptual framework

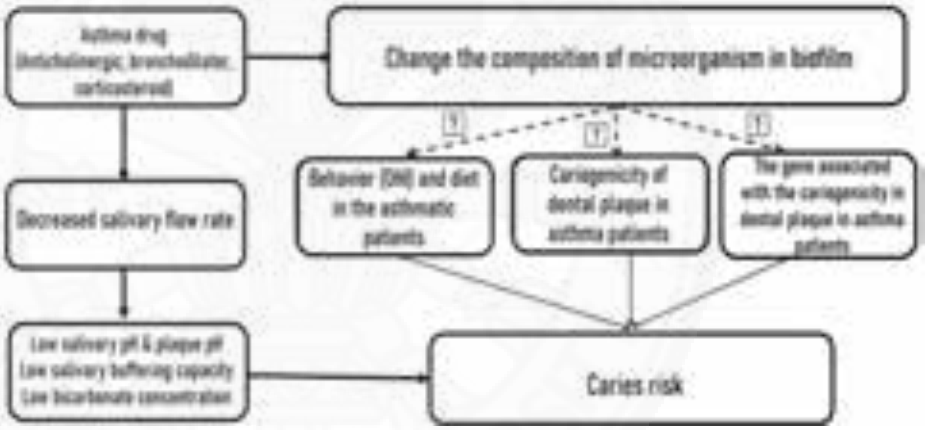


Figure 1.1 Conceptual framework

CHAPTER 2
LITERATURE REVIEW

2.1 Dental caries

Dental caries is a multifactorial multi-processes disease that involves interaction between causative agent (cariogenic bacteria), host, diet (fermentable carbohydrate) and time[1]. Missing of any of these, there would not be dental caries.[2]

Dental caries is the destruction on the tooth surface caused by bacteria fermentation of diet carbohydrate. This is the result of the interaction of cariogenic bacteria with fermentable dietary carbohydrate on the tooth surface overtime. Four major factors which directly contributed to occurrence of the dental caries are susceptible teeth, cariogenic bacteria, diet with fermentable carbohydrate, and time. Caries could occur only when bacteria present. If the oral cavity free of bacteria, then caries would not occur. Mutans bacterial group are very suitable for the role as causative agents because they have their ability to adhere well to the tooth surface produce a high amount of acid and extracellular polysaccharides from sucrose and can survive at low pH environment. The result acids from bacteria, particularly lactic acid, can demineralize the tooth enamel.[2] Sucrose is the most cariogenic carbohydrate due to dietary sucrose serves as an energy source for cariogenic bacteria and helps the bacteria in their attempts to adhere to the teeth. The virulence level of bacteria depends on quantity of sucrose consumption. Acid formation occurs in thick plaque also increases survivability of *S. mutans* and destructiveness toward the tooth. The external hard surface of tooth enamel is the part of the tooth where the caries progression process starts. Enamel mainly contain mineral in the form of hydroxyapatite, and also contains other organic and inorganic components.[2] Time means duration and frequency of exposure. When the acid challenge occurs repeatedly it may result in collapse enough of enamel crystals to produce a visible cavity. This process must occur over a sufficiently long period of time. Previous studies by Stephan (1944) show the

metabolic potential by severe pH drop at plaque-enamel interface after glucose rinse. Plaque bacteria will ferment carbohydrates, particularly sucrose and produce acids, causing plaque pH to drop within 1-3 minutes to pH 4.5-5.0. The pH will return to normal at a pH 7 in 30-60 minutes.[9] Stephan suggested the concept of ‘‘critical pH’’ a value close to 5.0.[10] Repeated and frequent sugar consumption will keep the pH of the plaque depressed and cause tooth demineralization. Remineralization on enamel can occurs if the pH has risen, and repaired with calcium, phosphate and bicarbonate ions from the saliva. [9,11]

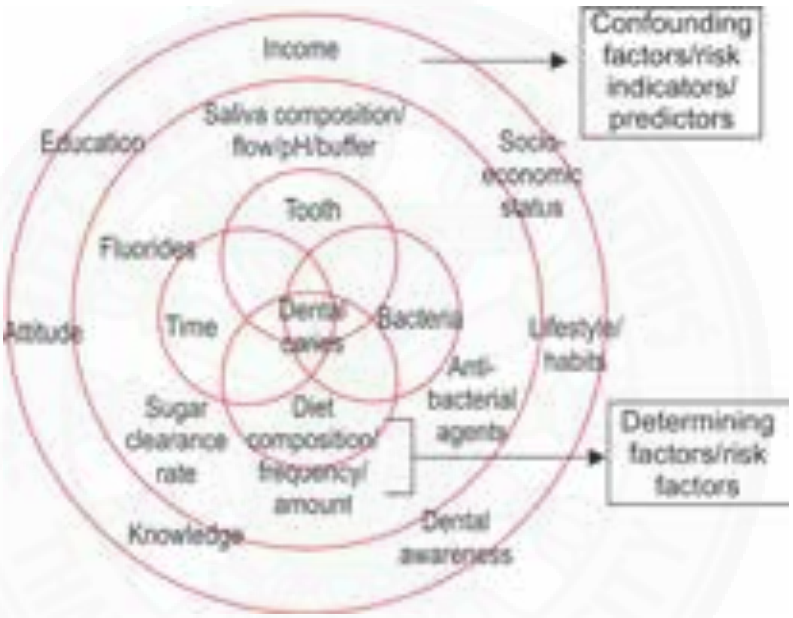


Figure 2.1 Modified Keyes - Jordan diagrams [12,13]

Dental caries onset and activity are complex with several modifying factors and protective factors influence the dental caries process (Fig. 2.1).[12] The second ring is primary modifying factors directly and significantly affect in susceptibility to caries contains tooth anatomy such as tooth surface, shape pit, grooves, and fissures on the crowns of the teeth can have complex and varied anatomy. The pits and grooves provide favorable mechanical shelter for organism and harbor a community dominated by streptococci. Saliva is the first and main defense against caries. Saliva protecting the teeth and provide mechanical cleansing of the teeth. Salivary flow is an important

protective factor from caries. Caries increased in the human with salivary flow disruption. Saliva buffer, that is important for maintaining neutral pH in plaque and in the oral cavity after eating, thus reducing the time for demineralization.[9] Other factors are including diet such as protein, sugar (clearance rate, frequency), calcium ion, phosphate ion, biofilm pH, microbial species, fluoride ion, immune system, and genetic factors. The last ring is secondary modifying factors which influence positively or negatively the likelihood of getting caries such sociodemographic status, education, life style, environment and occupation.[12,14,15] Caries is related to one's lifestyle and behavioral factors. These factors including poor oral hygiene, bad diet habits, frequent consumption of refined carbohydrates and frequent use of oral medications containing sugar. Other factors associated with caries risks include poverty, social status, number of years in education; dental, insurance coverage, and use of dental sealants.[14] In the oral cavity under modified physical environmental conditions such as poor oral hygiene, frequent sugar intake, low salivary flow rate, and decreasing of pH after meals, lead to increased acidification of plaque environment, and consequently alter the composition of bacterial flora.[16] Previous studies have shown that mutans streptococci, especially *S. mutans* and *S. sobrinus*, are more endurable on acidic environment at pH 4.0 than non-mutans streptococci, such as *Streptococcus sanguinis*, *Streptococcus oralis* and *Streptococcus gordonii)*.[16,17]

Ecological change is characterized by an increase in the proportion of *S. mutans* and other species that are both acidogenic and aciduric.[4]

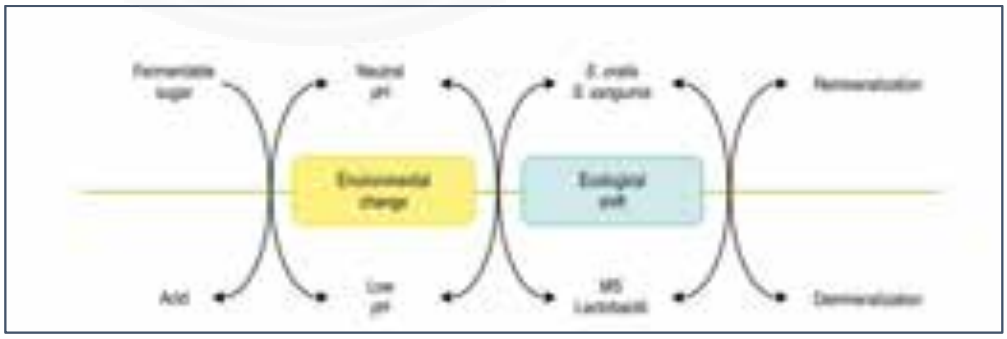


Figure 2.2. Scheme Ecological Plaque Hypothesis.[18]

Caries is the result of change in microflora balance due to changes in the surrounding environment, namely repeated consumption of sugar. Sugar metabolism that can be fermented in dental plaque results in low pH conditions and environmental changes in plaque causing the growth of bacterial species that can survive in acids and produce acids, such as Mutans Streptococcus (MS) and Lactobacilli (Fig. 2.2.). If decrease of pH continues, demineralization will occur.[18]

2.1.1 Cariogenic bacteria

Streptococcus mutans and *Streptococcus sobrinus* are grouped as Mutans Streptococcus (MS) known as a group of oral microorganisms which have virulence factors and residing the tooth surface along the oral biofilm. The key virulence factors are synthesized water insoluble glucan from sucrose, acidogenicity and acid tolerance.[19] The main virulence factors associated with cariogenicity are adherability, acidogenicity, aciduricity, and another properties such adaptation.[4] Each of these properties work coordinately to alter dental plaque ecology.[4,20] The ecological changes are characterized by increase proportions of *S. mutans* and other species those are similarly acidogenic and aciduric.[4] Acidogenicity from *S. mutans* contains a complete glycolytic pathway and can produce lactate, formate, acetate, and ethanol as fermentation products.[4,21]

Lactobacilli are also known as cariogenic bacteria. The taxonomy of lactobacilli is complex. The main characterized into 2 groups, namely homofermenters and heterofementers. Lactic acid (65%) from glucose fermentation *Lactobacillus casei* (*L. casei*) is the main production of homofermenters, while heterofermenters besides producing lactic acid also produce acetate, ethanol, and carbondioxide *Lactobacillus fermentum (L. fermentum)*. The ability of Lactobacilli is produce lactic acid, to grow in low environment, and synthesize extracellular and IPSs from sucrose. Lactobacilli plays a role in the continuity of dentin caries.[22]

Takahashi and colleagues[16] showed that non-mutans streptococci can increase acid tolerance and acidogenicity in response to environmental acidification.[17,23] Non-

mutans streptococci strains are pioneering bacteria in the formation of dental plaque and are the dominant part of the microflora plaque[24], so that it can play an important role in shifting the dental plaque environment towards the acidic environment and consequently increasing bacterial colonization of acidogenic and acidic bacteria such as MS and lactobacilli.[17]

2.1.2 Properties of *Streptococcus mutans* as cariogenic bacteria

The properties of *S. mutans* include adhesion, acidogenicity, aciduricity, and adaptation.

2.1.2.1 Adhesion

The adhesion of *S. mutans* in dental plaque can be mediated by sucrose-independent and sucrose-dependent. The attachment process may be initiated from sucrose-independent adhesion to the salivary component in the acquired enamel peliclicle.[4,25] The adherence of oral bacteria to the tooth surface is the first step for colonization. P1 or antigen I/II and also called SpaP in *S. mutans* as initial adhesion is mediated by variety of surface-associated proteins, which binds to salivary proteins present in the pellicle on the tooth surface.[26] The mechanism of *S. mutans* adheres to the tooth surfaces is important because the initial step in the biofilm formation is adhesion (Fig. 2.3) which promoted by genes *spaP*. [27] Sucrose intake is one of the factors that correlates with the level of infant colonization. Sucrose-dependent adhesion may be primarily responsible for establishing colonization to the tooth surface.[25]Adhesion to pre-formed glucan on the tooth surface may also facilitate colonization.[28] The ability of *S. mutans* to synthesize glucans from sucrose increases the effectiveness of adhesion and enhances the proportion of *S. mutans* within dental plaque. Thus, sucrose-dependent adhesion plays a prominent role in initiating the changes in plaque ecology that can lead to dental caries.[4]

Glucosyltransferases (Gtfs) enzymes that plays a role in the production of water insoluble glucan, that is glucose polymer with an α-1,3 linkage, namely mutan and water-soluble glucan, that is glucose polymer with an α-1,6 linkage called dextran through the use of sucrose as the only substrate. Sticky mutan is involved in increasing

of the plaque mass and enhances adhesion of the cariogenic microorganisms on the tooth surface, and also responsible for facilitating biofilm formation.[29,30] *S. mutans* produces 3 types of Gtfs, namely GtfB, GtfC, and GtfD.[29-31] Function of GtfB is to produce extracellular polysaccharide (EPS) which provide binding sites that promote accumulation of microorganisms on the tooth surface and further establishment of pathogenic biofilms strengthening *S. mutans* attachment on tooth surfaces. EPS also provides the energy source for biofilm microorganisms, biofilm matrix stability and protecting biofilm-forming microorganisms from unfavorable environmental influences, limiting the diffusion of biofilm substances, and assisting the absorption of other metal and nutrient.[32] *S. mutans* have the ability to store excess carbohydrates available as intracellular polysaccharides (IPS), which acts as a source of energy reserves in a period of "starvation".[33]

S. mutans also produces several glucan binding proteins (Gbps) which are suspected to promote adhesion.[31] The binding of *S. mutans* to glucans is mediated by the presence of cell-related GTF enzymes and non-GTF (Gbps) *glucan binding proteins*. At least *S. mutans* produces 4 Gbps that is GbpA, GbpB, GbpC, and GbpD.[4,31,34] Function binding of glucans produce by gtf, adherence to the teeth and biofilm accumulation. Together with GtfB and D extracellular glucans, constitute the sucrose dependent pathway mechanism for *S. mutans* to establish on the tooth surface and are of central importance in plaque formation and development of caries. Gbp C/B associated with the bacterial cell wall acts as a specific receptor for glucans and play a role in microorganism adhesion and biofilm formation. GbpB has been shown to participate in cell wall construction and cell separation.[35,36] GbpB including bacterial components associated with adhesion phase of *S. mutans.* Adhesion phase is important from biofilm formation.[31]

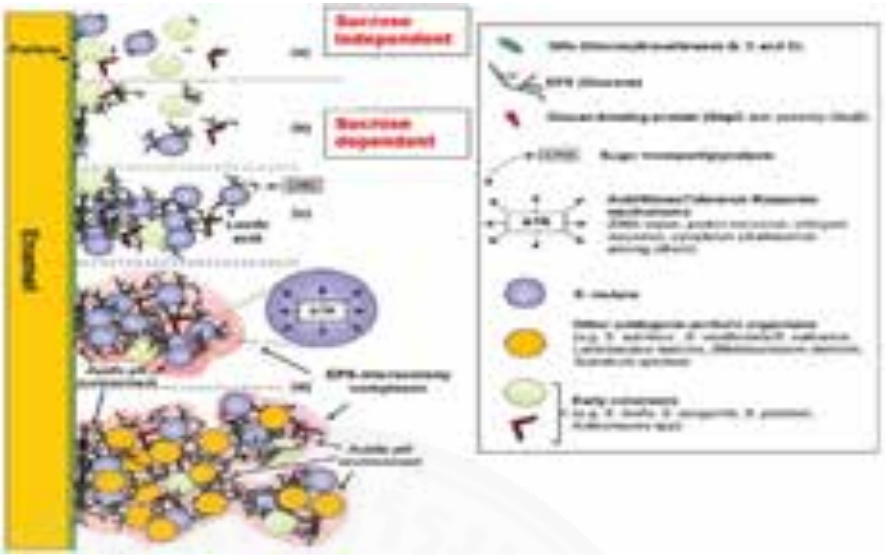


Figure 2.3. Represent the adhesion of bacteria, including independent and dependent adhesion (Modified from Koo and colleagues, 2013)[27]

a. The early attachment of *S. mutans* on the tooth surface is done by sucrose independent mechanism involves the activity of adhesion spaP (produce by *S. mutans* and recognized glycoproteins present in saliva as a receptor molecules)
b. *S. mutans* produce (Gtfs) and joined into the pellicle by GtfC and mainly GtfB absorbed to bacterial surface. Although the microorganism such as *Actinomyces* do not produces (Gtfs), GtfB can absorb this microorganism. Beside that *Actinomyces* can express GbpB and can bind *S. mutans.*
c. In the next colony mix between *Actinomyces* and *Streptococci*. In the presence of sucrose GtfB and GtfC can rapidly utilize dietary sucrose. Insoluble and soluble glucan a result the synthesized in situ. GtfD produced soluble glucan as primary for GtfB to increase total insoluble EPS synthesis.
d. The population of *S. mutans* more than another microorganism. *S. mutans* express *Gbps* more than *Actinomyces.* This process occurs primarily glucan-glucan and glucan-glucan binding protein

interaction. Simultaneously, dietary carbohydrate metabolized into acid by acidogenic organism. Here occurs the process of glycolysis which produced lactic acid. When the EPS rich matrix and biofilm have been formed then ecological pressured community selection with only bacteria which can survive in acidic pH environment. Changes in conditions and structures that support high survivability and resilience bacteria to build a new type of cariogenic biofilm.

2.1.2.2 Acidogenicity

S. mutans contains a complete glycolytic pathway and can produce lactate, formate, acetate, and ethanol as fermentation products. The precise distribution of fermentation products will depend on growth conditions with lactate being the major product when glucose is abundant.[4] Lactic acid produced in the presence of high sugar concentrations, while low sugar concentration lead to the production of acetic acid, formic acid, and ethanol (Fig. 2.4).[37]

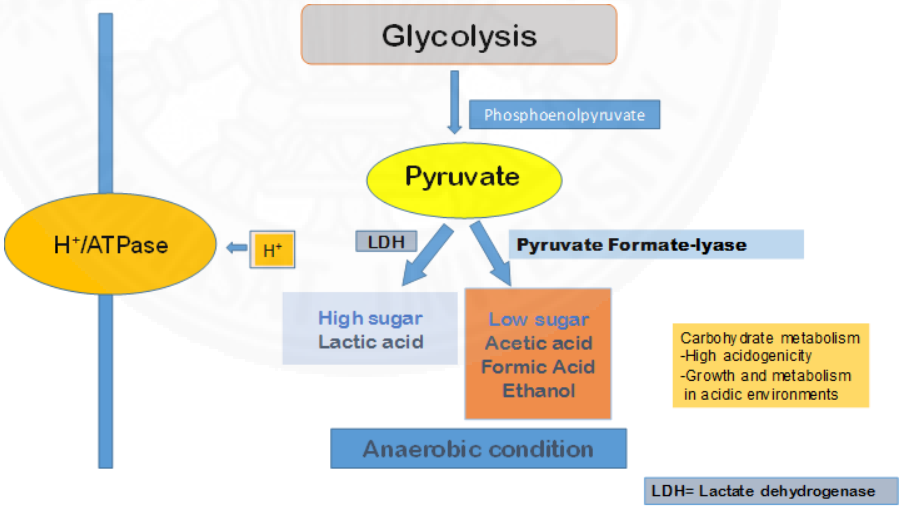


Figure 2.4. Sugar metabolism and acid formation in cariogenic bacteria.[37] (Modified from Nishimura et.al, Advances in Microbiology, 2012)

Strains which deficient in lactate dehydrogenase (LDH) show to be reduced in cariogenicity and the absence of lactate dehydrogenase (LDH*)* is lethal.[4] The absence of LDH on *streptococcus mutans* biofilm can affect acid production by *streptococococcus mutans* and can reduce their cariogenicity. Indeed, a genetically modified strain deficient in *lactate dehydrogenase* is being considered for replacement therapy as a means of out-competing more cariogenic strains of *S. mutans.* The rate which *S. mutans* produces acid when tested at a pH in the range from 7.0 to 5.0 exceeds that of other oral streptococci in most instances. The relative acidogenicity of *S. mutans* can vary from one isolate to another, and strict correlations between acidogencity and caries experience is lacking. Nonetheless it is generally thought that the acidogenicity of *S. mutans* leads to ecological changes in the plaque flora that includes an elevation in the proportion of *S. mutans* and other acidogenic and acid-tolerant species. This cariogenic flora will reduce plaque pH to lower levels than will a healthy plaque flora upon the ingestion of fermentable carbohydrate, and the recovery to a neutral pH will be prolonged.[4]

Lactic acid produced in the presence of high sugar concentrations, while low sugar concentration lead to the production of acetic acid, formic acid, and ethanol. Cariogenic bacteria such *S. mutans* survive and grow at low pH. Survival in acidic environment depends on the ability of *S. mutans* and other acid tolerance to maintain intracellular pH homeostasis using H^+/ATPase (proton pump).[37]

2.1.2.3 Aciduricity and adaptability

S. mutans have ability to tolerate with acid to survive from the low pH of 4.4. Acid tolerance from *S. mutans* can be mediated by proton pump F1F0-ATPase proton pump. Evidently, acid tolerance can increase synthesis of water insoluble glucan and biofilm formation. *S. mutans* cells in dental plaque can survive acidity better than cells grow from plankton or another bacteria. Adaptation to acidic environment of *S. mutans* may be related to the system

of the efficient quorum sensing to induction acid tolerance response (ATR) and characteristic physic biofilm. Hata dan Mayanagi investigated the speed of diffusion of hidronium ions related to amount *S. mutans* production water insoluble glucan.[4]

BrpA plays a major role in acid and oxidative stress tolerance and biofilm formation by *S. mutans*.[38] The *brpA* gene codes for a predicted surface associated protein with apparent roles in biofilm formation, autolysis, cell division and function on biofilm as regulatory protein. This gene plays critical roles in environment stress responses and biofilm formation. BrpA regulates genes that are required for stable biofilm formation by *S. mutans* and if deficient can cause the severe defect in biofilm formation.[20,39]

In the supragingival area, *S. mutans* and *Actinomyces* species are predominant bacteria. The bacteria can adhere to the surface of the tooth coated with saliva by adhesin and receptor attachments and are known to use the salivary component as a nutrient. Saccharolytic derivative bacteria are broken down into glucose and fructose. Glucose is broken down through the mechanism of glycolysis through the Embden-Meyerhof-Parnas pathway which will produce two pyruvate molecules.[40] Under conditions of low carbohydrate, pyruvate is converted to ethanol, acetate and formate. Conversely when carbohydrates are excessive, pyruvate will be changed to lactic acid.[37,40]

Result from Takahashi's study from year 1999 and 2005 on the acidification process showed that non-mutans streptococci were able to increase acid tolerance and acidogenicity in response to environmental acidification.[17,40] The acidification can produce damage to non-mutans streptococci bacteria after exposure to pH 4 for 60 minutes. However, these bacteria can respond to acid pressure and become more tolerant of acids. In weak acidification, exposure to pH 5.5 for 30 to 60 minutes indicated the

ability to adapt to acidic conditions. Furthermore, through acidic adaptation can increase their cariogenic potential.[40]

The adaptive response to this acid stress comes from proton-translocated ATPase induction (H+-ATPase), increases alkali production, stress protein, and increases permeability of cell membranes against proton (Fig. 2.5).[40]

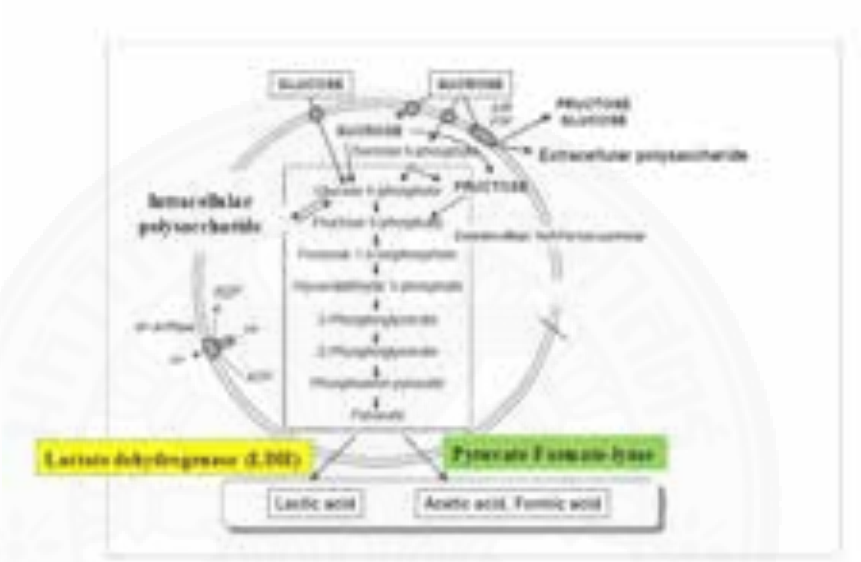


Figure 2.5. Streptococcal carbohydrate metabolism and adaptation.[40]
(Modified from Takahashi, International Congress Series, 2005)

The adaptation for controlled the population uses the quorum sensing mechanism. Quorum sensing (QS) is an intercellular signaling mechanism by which bacteria monitor their own population density or that of other populations by recognizing local concentrations of chemical molecules, referred to as autoinducers. The autoinducer-2 signal (AI-2) produced by the LuxS protein mediates interspecies communication among Gram-positive and Gram-negative bacteria.[41] The LuxS enzyme, which is responsible for the production of autoinducer signal molecules those are involved in interspecies communication in a wide range of bacteria, affects

acid and oxidative stress tolerance, as well as biofilm accumulation and biofilm structure.[41]

The *luxS* gene has function for regulatory role in controlling the expression of the genes related to early stage of biofilm formation. For adherence and biofilm formation, bacterial properties such as cell hydrophobicity, autoaggregation, and coaggregation are important during biofilm formation. Cell surface hydrophobicity is one of important factors involved in oral bacterial adherence to the tooth surface. Cell surface hydrophobicity can decrease if has the deletion of *luxS* gene.[42]

2.2 Asthma

Asthma is a chronic inflammatory disturbance of the airways and the lung in which many cells play their roles, especially mast cells and eosinophils. Chronic airway inflammation from hyperresponsiveness probably a result of the increase in the number of mast cells in the airway smooth muscle.[43] It is characterized by recurrent and repetitive episodes of wheezing, breathlessness, chest tightness mostly at night or in the early morning and often accompanied by a cough.[43] Asthma that occurs in childhood could influence function and pathological severity of symptoms from lungs in the future. Severe asthma has influence on children growth and development, reduced education outcome, and reduce quality of life of patients both present and future.[6]

2.2.1 Prevalence

Asthma affects more than 300 million people in worldwide. The prevalence of asthma is increasing particularly in preschool children.[5] According to Global Asthma network in 2018, Indonesia has prevalence of childhood asthma range from 4-11% in 6-7 years old and 6-13% in 13-14 years old. While Thailand asthma prevalence in children is still more than 10%.[6,44]

2.2.2 Asthma medication

Asthma medication can be classified as controller and relievers. Controllers are medications taken daily on a long-term basis to keep asthma under clinical control

chiefly through their anti-inflammatory effects.[43] They include inhaled and systemic glucoccorticosteroids, leukotriene modifiers, long-acting inhaled β_2-agonist in combination with inhaled glucocorticosteroids, sustained-release theophylline, chromones, and anti IgE.[43] Relievers are medications used on acute needed basis that act quickly to reverse bronchoconstriction and relieve its symptoms.[43] They include rapid acting inhaled β_2-agonist, inhaled anticholinergics, short-acting theophylline, and short-acting oral β_2-agonist[43], while the other classification according to Kaur and Singh, (2017) drug used for asthma can be categorized into three categories (1) reliever or rescue medications, used on 'as needed basis' and include short-acting ß2 agonist (SABA) and anticholinergics. (2) Controller medications used for long term management to reduce airway inflammation, control symptoms and minimize risk of exacerbation and development of fixed airflow limitation, include inhaled corticosteroids (ICS), combination ICS/LABA (long-acting ß2 agonist), leukotriene receptor antagonist (LTRA), and chromones. (3) Add on therapies for severe asthma, used to treat truly refractory severe asthma that remains uncontrolled despite optimized treatment with high dose ICS controller medications. These include Anti IgE (Omalizumab), Anti IL-4, Anti IL-5. Theophylline, etc.[45]

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Research Design

Research design in this present study was designed as observational study (cross sectional)

3.2 Variables

This study is an observational study that should include predictable variables and outcomes.

Predictable variables are as follow: diet consumption, fluoride use, and oral hygiene

Outcomes are as follow: plaque color and gene expression

Variables

The Variables are:

1. Predictable Variable: asthma status from asthma patient from the Allergic Clinic in Thammasat University Hospital
2. Outcome Variable: staining the biofilm and gene expression in both groups

3.3 Subjects

Ethical approval was given by the Institutional Review Board (IRB), Thammasat University, Pratum Thani Thailand (IRB number 022/2557) before selecting the sample.

Data was taken from 2 groups of asthmatic and healthy or control group. The information of the asthmatic group from the recruited and examined by a specialist from Allergy Clinics, Thammasat University, according to Global Initiative for Asthma Management (GINA, 2012).[43]

The sample size for the study was calculated using Power and Sample Size Calculation software version 3.1.2 by Dupont and Plummer (Vanderbilt University, Tennessee), based on the comparison between means. Standardized effect size with

Cohen d value of 0.5 was used in this study.[46] In order to detect a difference of 0.5 in mean gene expression between the control and asthmatic groups, with 80% power of detection and an alpha error of 0.05, at least 38 asthmatic patients and 19 control subjects would be required. The selected subject (38 asthmatic patients and 22 controls) were given appointments at an allergy clinic of Thammasat University hospital. At each appointment, a consent form was given to the controls, parents and/or patients before plaque staining and collection.

Inclusion criteria of asthmatics patients have been examined, diagnosed, and recruited by a specialist from Allergic Clinic, Thammasat University Hospital. The asthma cases selected were children and adults, having previous asthma disease and physical examination according to the Global Initiative for Asthma (GINA), (2012) and Asthma Diagnosis and Treatment Guidelines in Thailand.[43,47] The healthy subjects group randomly selected from the volunteer dental students in dental clinic of Thammasat University Hospital, age equal or older than 6 years. gender male and female. The exclusion criteria for selection of study sample, in both groups, have any other systemic diseases and using any other medications that can affect the saliva, such as hypertension and diabetes, physical & mental condition such as depression, patients who were uncooperative and not willing to participate.

3.4 Materials and methods

3.4.1 Data collections

Questionnaires for data collection which includes a list of questions regarding asthma information obtained from an asthma specialist doctor, general information, dental health behavior, and diet data. (Tab.3.1)

3.4.1.1 Asthma information

The duration, severity, comorbidity, and medications used by asthma patients are based on the reports made by asthma specialists. The diagnosis of asthma can be made through history taking and physical examination, special tests include lung function tests with spirometry, measurement of respiratory sensitivity to stimuli measurement of allergic status.[43,47] (Tab. 3.1). Data

including duration, severity, comorbidities, mode of administration of the drug [43,48], mouth cleaning after using the drug (with water)[5], and medication.[43,48] Duration the patients who have been suffering from asthma. The duration was less than 2 years, 2-4 years, and more than 4 years designed for the questionnaire form. In the previous study of Ersin N.K and colleagues (1987) they found significant difference in the salivary level of *S. mutans* of the children who had been suffering from asthma for more than 2 years.[49] Assessment of severity or the level of asthma control was used in all patients. To assess the ability to control the symptoms of the disease by considering drug use and cooperation in care for 3 levels, controlled asthma, partially controlled, and uncontrolled shared care.[43] The present of comorbidities that can may worsen asthma, such as chronic sinusitis. The comorbidities are asthma, asthma and allergic rhinitis, asthma and sinusitis, and others. Comorbidities should be addressed and treated appropriately.[43] Mode of administration of the drug in asthma treatment can be administered in different way, inhaled, oral, injection and other ways.[43] Mouth cleaning with water can cleanses the residue in the mouth such as food debris, cell, and non-adherent bacteria.[5] Asthma medication are classified into controller or reliever. Controllers are medicines taken daily for long term controlling of asthma. They include oral corticosteroid, long acting ß2 agonist (LABA), ICS + LABA, and leukotriene modifier. Reliever are medication which act quickly to reverse bronchoconstriction and relieve symptoms, such as short acting ß2 agonist (SABA).[43] Another medication usage was recorded including antihistamine, antibiotics, and other medicine those are used simultaneously in the treatment of asthma such as intranasal glucocorticosteroids for allergic rhinitis.[43]

3.4.1.2 Demographic data

General information recorded from asthmatic patients and healthy group included age, sex, income per month, and level of education. In this study we conduct interviews and questionnaires were filled out by parents of the children for child patients.

The questionnaire included contact information, age, sex, income, and level of education. For income data we divide into low income for income less than 15000 bath per month, medium 15,001-50,000 bath per month, and high for income more than 50,001 baht per month. Level of education including never studied, Elementary school were primary, Junior highschool and Senior high school were Secondary. Vocational course, Bachelor degree, Diploma or Master degree certificate, and Post Graduate were diploma or high.

Data about oral hygiene behavior, including tooth brushing, fluoride toothpaste, and visit dentist[50] were also collected. Oral Hygiene behaviors are including oral hygiene instruction tooth brushing frequency per day, use of fluoride toothpaste,[48] and dental clinic visits in the past year are in the question form.

To assess dietary habits, parents were filled out survey forms at home. Dietary diary part would be collected information of 3 days of their child's diet. Written instructions were attached to the diary. They were asked to record everything that their child eats or drinks over a period of 3 days (including weekends), along with meal times. Rugg-Gunn and colleagues (1984) describe the calculation of the frequency of diets including sugary snacks and consumption of drinks between meals.[51,52] Intake was considered separate if the consumption time at least 30 minutes after the previous intake, or at least 15 minutes after eating a previous snack. These separate intakes were called events. The number of events per day was calculated with all foods, snack consumption between meals and sugar consumption those were recorded in diary (total event). Fill out the forms would be done at home and sent back to the researchers after

3 days of filling out the forms including weekends. Envelopes including stamps and addresses were prepared and the subjects or the parents must send the form back after the form is completed.[52]

Table. 3.1 Data collections.[43,48,50-54]

Topic	Data record
Asthma Information (filled by an asthma specialist doctor)	Duration, severity, comorbidities, mode of administration of the drug, mouth cleaning after using the drug (with water), and medication
General information	Age, gender, income per month, education, and disease
Dental health behavior	How many times do tooth brushing per day, type of toothpaste use Fluor or not, and how many times visit dental clinic
Diet data for intakes food	How many times intakes food frequency, snack, and sugar per day

3.4.2 Plaque staining and acidogenicity of dental plaque

After the interview, materials and instrument which would be used in This study was prepared, composed of mouth mirror, dental explorer, cotton pellet, cotton plier, sterilized cotton bud, three-tone plaque disclosing GC Tri Plaque ID Gel™ (G C Corporation, Tokyo, Japan), Eppendorf tubes 1.5 ml (Eppendorf AG, Hamburg, Germany), and box with dry ice. The patients were prepared and provided them with information before researchers collect plaque from the surface of their tooth. First, the three-tone disclosing gel (GC Tri Plaque ID Gel™) was applying

with cotton buds on all the tooth surfaces. Subjects were left undisturbed for 2 min and then the subjects were asked to rinse for remove excess gel. The score recorded was based on the color that would be revealed after applying the gel. Colors from this plaque at the teeth provided some information about the cariogenic conditions.

Plaque, as revealed by GC Tri Plaque ID Gel™, contains 3 colors of pink, blue or purple, and light blue. Pink areas were where the plaque is thin recently developed within 24 hours prior testing. The blue or purple areas were where the plaque is thicker and older with development more than 48 hours and light blue areas were extra high-risk plaque because the plaque was starting to produce acids[55](Fig. 3.1) and (Table. 3.2).

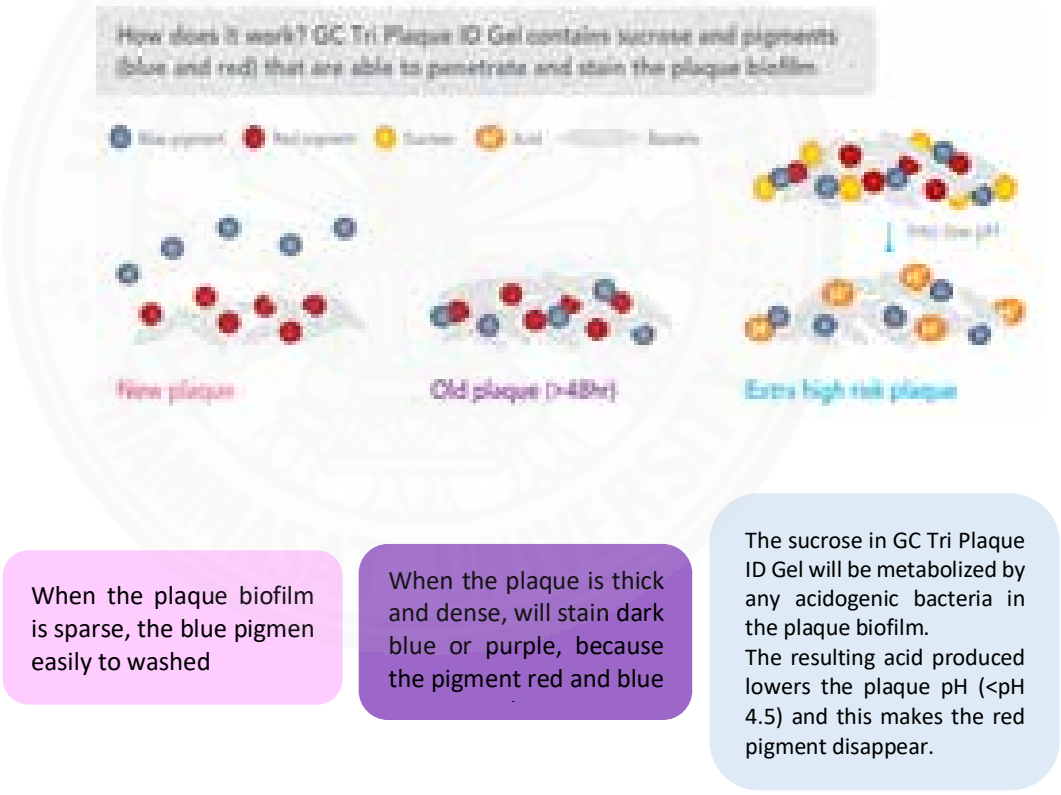


Figure. 3.1 The staining plaque color criteria and GC Tri Plaque ID Gel mechanism[55]

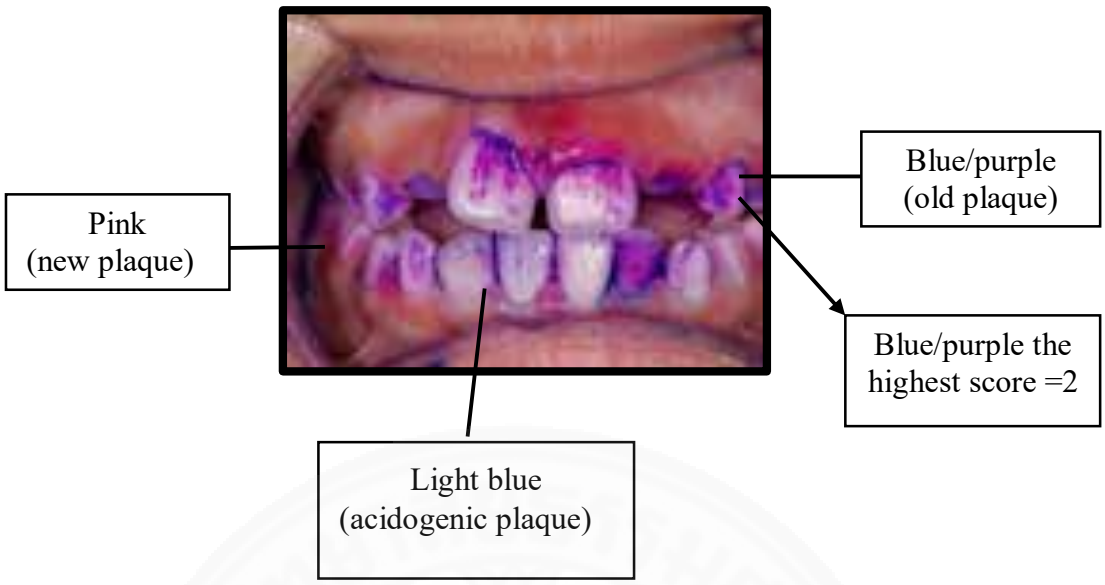


Figure. 3.2. Characteristic plaque sample

Table. 3. 2. Color indication

Color	Indication	Clinical examination
No color	No plaque	0
Pink	Immature plaque	1
Blue or purple	Mature plaque	2
Light blue	Mature plaque and acid producing plaque	3

The plaque in the supragingival was assessed, if a tooth stain only one color, such as only pink score 1, only blue/purple score 2, and only light blue score 3. In case if a tooth stained more than one color, only the highest score was counted. For example, the lower left lateral incisor contains only two colors (pink, blue/purple). Thus, this tooth score 2 was given, because blue/purple is the highest severity. (Figure. 3.2).

Based on all the changes of color on the total tooth surfaces, the plaque maturity staining (PMS) was obtained by using this formula:

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

3.4.3 Dental plaque collection

Dental plaque biofilm from all surfaces in all samples was collected by dental explorer and put into Eppendorf tube and then put into dry ice box and kept in a refrigerator at -80^0C at Oral Biology Research, Faculty of Dentistry Thammasat University until used.

3.4.4 *Streptococcus mutans* preparation

S. mutans (ATCC 25175) was used in this study as positive control, growth in the tube containing brain heart infusion (BHI) broth (BD Difco; San Jose, CA, USA) at 37^0C for 24 hours. The planktonic of *S. mutans* was grew up until they reached the exponential growth phase at an optical density of 600 nm that was determined by Spectrophotometer Smart Pac (Bio-Rad Smart TM Hercules, CA, USA). The exponentially grown *S. mutans* was then used as positive control. Both positive control and dental plaque samples were carried out with the same methods. The pellets can quickly be resuspended and then treat with Trizol Reagent (Life Technologies Corporation, Carls, CA, USA) by using the procedures recommended from the manufacturer.

3.4.5 Determination of cariogenic related genes

To do the extraction of RNA, dental plaque samples were prepared, homogenized, and washed twice using 1 ml PBS and then centrifuge at 4^0C at 12000 x g for 15 minutes. 1 ml of Trizol reagents (Invitrogen, Carlsbad CA) were added to the cell pellet and mixed with a vortex. After incubating sample at room temperature for 5 minutes, the sample was pipetted up and down to mix the lysate several times to homogenize the sample. Chloroform was added as much as 0.2 ml to the sample per 1 ml of Trizol, then sample tube was shaken vigorously by hand for 15 seconds.

Samples were centrifuged and then incubated for 2-3 minutes at room temperature and centrifuged again at 12000 x g for 15 minutes at 4^0C. The aqueous phase was collected from the sample by angling the tube at 45^0 and pipetted the solution into a new tube, then 0.5 ml of 100% isopropanol was added to the aqueous phase per 1 ml of Trizol and incubated at room temperature for 10 minutes. The incubated aqueous phase was centrifuged at 12000 x g for 10 minutes at 4^0C then removed supernatant from the tube to leaving only RNA pellet. Then the RNA pellet was washed with 1 ml of 75% ethanol per 1 ml of Trizole, vortex and centrifuged at 7500 x g for 5 minutes at 4^0C. The supernatant was removed and the remaining RNA pellets were dried in the air for 5-10 minutes. The RNA pellet was dissolved with RNase-free water 10 µl and resuspend bypassing the solution up and down several times through pipetting tip. Resuspended RNA was incubated in ice bath and detected for protein contamination by using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA) calibrated with 2 µl RNase-free water, cleaned with a special tissue, closed and measured green blanch (set 0) solution 2 µl, measured, set black, clean tissue. The synthesis of cDNA from RNA was done by using a PrimeScript strand 1 st strand Cdna Synthesis Kit (Takara Bio Inc., Japan). Reagent 5x PrimeScript buffer 2 µl was mixed with PrimeScript enzyme mix 0.5 µl, Oligo dT Primer (50 µM) 0.5 µl, random 6 mers (100 µM) 1 0.5 µl, total RNA (calculate each sample), RNase Free $_dH_2O$ (calculated each sample) to the total amount of 10 µl. RT reaction was scaled up as needed and 10 µl of the reaction mixture could be reverse transcribed up to 500 ng of total RNA. The reaction mixture was incubated under the following condition; 37°C in 15 minutes for reverse transcription, 85°C in 5 seconds for inactivation of reverse transcriptase with heat treatment, and back to 40^0C. This stage was done using Thermo Scientific Hybaid PX2 thermal cycler.

After synthesis of cDNA from RNA, concentration was calculated and diluted to a concentration of 20 ng /ml and keep at refrigerator -20^0C until used.

3.4.6 Real-Time RT-PCR.

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). PCR primers were based on the previous studies with the *brpA, gtfB, gbpB, ldh, luxS* and *spaP* gene primer sequences were taken from Wen and colleagues, (2010)[20] and The *16S rRNA* was served as the internal control.[56] The PCR master mix was prepared according to the instruction from manufacturer and DNA Sequence primers for Real Time PCR[20,56] (Table. 3.3). The reaction mixture was added with the following components according to the concentrations: PCR grade water 7.2 µl; 2X KAPA SYBR®FAST qPCR Kit Master Mix (2X) Universal 10 µl, 10 µM Forward Primer 0.4 µl, 10 µM Reverse Primer 0.4 µl, and template 2 µl. Total volume for each tube was 20 µl. Analyses of the gene expressions were evaluated using the $2^{-\Delta Ct}$ equation.[57] In this case, ΔCT= CT (*interested gene*) – CT (*16S rRNA*).

Table. 3.3 Primers for Real Time PCR[20,56]

Primers	**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
16S rRNA	TCCACGCCGTAAACGATGA	TTGTGCGGCCCCCGT	119

The absolute quantification programme was used to determine the bacterial load in the samples of the same condition. *ldh* and *brpA* genes that were conditioned at 59^0C. *gbpB*, *SpaP* and *luxS* genes were conditioned at 60^0C. *gtfB* was conditioned at 55.1^0C. The *16S rRNA* condition at 58^0C was used for the internal control.

3.5 Statistical Analysis

The statistical analysis was done using GraphPad Prism Version 6.0. (GraphPad Software Inc., La Jolla, CA, USA). Normality of continuous data was analyzed by Shapiro-Wilk normality test.

Demographic data, age and gender in asthmatics group and healthy subjects group were analyzed using Mann-Whitney *U*-test.

Chi-square for trend was used for analyze education and income per month.

Fisher's exact test was used to analyze the comparison of tooth brushing behavior, fluoridated toothpaste, and visited dental clinics in asthma and healthy group.

The diet consumption was analyzed using Mann-Whitney *U*-test.

The differences in oral biofilm staining in asthmatics group and healthy group was analyzed using Kruskal-Wallis test.

The comparison of gene expression between asthma and control was analyzed using Mann-Whitney *U*-test.

Spearman's rank correlation coefficient (ρ) was used to measure the association between two non-parametric variables with p- *value* ≤ 0.05 was considered statistically significant.

CHAPTER 4
RESULT AND DISCUSSION

4.1 Demographic, asthma characteristics and medicines

The study included 38 asthma patients and 22 non asthmatic or healthy subjects. Median asthma patients 10 years (range 6-60 years) and median healthy 11 years (range 6-32 years). Result from Shapiro-Wilk test $p < 0.05$, data is not normally distributed. There were no significant differences in age between asthma and healthy groups (p>0.05, Mann-Whitney U-test). Asthma and healthy group had a similar age.

The asthma group consisted of 22 males and 16 females and the healthy group consisted of 6 males and 16 females, there was a significant difference in sex between asthma and healthy group ($p < 0.05$, Mann-Whitney U-test). There were more male subjects in asthma group, in contrast in the healthy group female more than male.

No significant difference between asthma and healthy groups were found with regard to income and education, respectively (p>0.05, Chi-square test for trend). (Table 4.1)

Asthma characteristic and use of medicine of the asthma patients are summarized in table 4.2. Among 38 asthma patients the onset (duration) less than 2 years in 11 (28.95%), duration 2-4 years in 14 (36.84%), and more than 4 years in 13 (34.21%). Asthma severity in this study we found that 28 (73.68%) asthma controls, 6 (15.79%) partial asthma, and 4 (10.53%) uncontrolled asthma. The comorbidities of asthma found in this study for asthma 12 (31.58%), asthma and allergic rhinitis 23 (60.53 %), asthma and sinusitis 1 (2.63 %) and others 2 (5.26%).

Number of subjects with asthma onset duration more than 2 years in this study was high. The possibility for the use of the drug Inhaled Corticosteroid (ICS) + long acting beta agonist (LABA) has also taken place since the onset of the disease. Previous study by Totlaa and colleagues (2004) said that children who use long-term inhalers have lower oral pH than healthy populations. Inhalers in the form of manually actuated

metered dose inhalers (MDI) either Corticosteroids or ß2 agonists have the potential to be acidogenic.[58]

Table 4.1 Education and Income

	Asthma	Healthy	*p* – value
Educational level, n (%)[a]			0.79*
Primary	2 (5.26)	0(0)	
Secondary	6 (15.79)	6 (30)	
Diploma or higher	30 (78.94)	14 (70)	
Income, n (%)[b]			0.62*
Low	8 (21.05)	8 (36.36)	
Medium	20 (52.63)	6 (27.27)	
High	10 (26.31)	7 (31.81)	

*Chi-square test for trend
[a] Data education healthy subjects available for 20
[b] Data income healthy subjects available for 21

Stenson and colleagues (2010) concluded, adolescents with long term asthma had a higher total DFS and caries risk, more gingival bleeding and lower plaque pH than adolescents without asthma.[59] Differs from a study by Meldrum and colleagues 2001, who found no association between long-standing asthma sufferers and caries.[60] Ellot and colleagues (2004) also stated there was no significant regarding the duration of asthma, prolonged use of anti-asthmatic medication or severity of the asthma on caries risk [61], and Ryberg's study (1987), in the asthma group, the DFS scores was not statistically different than the control group.[49]

The comorbidities asthma and allergic rhinitis in this study were relatively high in accordance with previous research in Thailand. [52,62]

In this study in asthma group, we found 15 (39.47%) did not wash their mouth with water after taking medication and as many as 23 (60.53%) did oral cleansing with water. Rinse with water is helpful to avoid food waste and prevent dental caries and also oral candidiasis.[5]

The usual treatment for asthma is a combination of regular anti- inflammatory medication to suppress the inflammatory response (controller medication) and drugs used to reverse bronchospasm (reliever medication). Latest study by Fang-yi Wu and collegues (2019), they revealed the children taking asthma medication including bronchodilator and quick relieve agents contain ß2 agonist in asthma patients had a higher rate of caries than the children without asthma.[63] Asthma treatment that uses ß2 agonist affects the saliva secretory rate.[54] Furthermore, children with asthma receiving ß2 agonist had *S. mutan* and lactobacilli counts increased compared with the control group.[53]

Total medicine usage in the asthma group, in this study patient which use one drug 5 (13.16%), two drugs 12 (31.8%), three drugs 11 (28.95%), four drugs 3 (7.89%), five drugs 5 (13.16%), and six drugs 2 (5.26%). The use of drugs in the treatment in our results are in agreement with a number of studies which have investigated combination of two drugs in the medication asthma treatment.[8,48] Alaki and colleagues (2013) reported result of combination of 2 types of drugs anti-asthmatic ß2 agonist inhalers combined with corticosteroid. They found level of MS and lactobacilli was high and showed a significant association between MS and lactobacilli level and the type of anti-asthmatic medication with those taking combination 2 drug.[48]

According to the study of Konde and colleagues (2018) who stated that asthma treatment uses many types of drugs and they found that there was a significant difference among children who were using combination drugs (corticosteroid + long β2-agonist [salmeterol] inhaler) than those who were using Salbutamol or ICSs (Corticosteroid + Salbutamol).[64]

For mode administration of the drug, inhalation 1 (2.63%), oral 4 (10.53%), inhalation and oral medication and other is high 33 (86.84%)

Table 4.2 The characteristic 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(57.89)	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)	…	…
Total medicine usage, %			
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 medication	33 (86.84)	…	…

*Mann-Whitney *U*-test

4.2 Oral health behavior

In this study, oral health behavior, including tooth brushing, frequency, use of fluoride toothpaste, dental clinic visit, and plaque staining were measured

Table 4.3 Oral health behavior

	Asthma	Healthy	*p*-value
Tooth brushing			
1 time/day, n (%)	5 (13.16)	1 (4.54)	
≥ 2 times/day, n (%)	33 (86.84)	21 (95.5)	0.39[#]
Fluoride toothpaste			
Use Fluoride toothpaste, n (%)	38 (100)	21 (95.5	
Use non Fluoride toothpaste, n (%)	0 (0)	1 (4.5)	0.37[#]
Visit dental clinic			
Never, n (%)	7 (18.42)	6 (27,27)	
At least 1 time/ year, n (%)	31 (81.58)	16 (72.72)	0.52[#]

[#]Fisher's exact test

Table 4.3 showed that the majority of asthma and healthy group brushed their teeth more than or equal 2 times/day, that 33 (86.84%) of asthma patients and 21 (95.5%) of healthy. Both the groups showed similar tooth brushing frequency and not significantly different ((p>0.05, Fisher's exact test). those reported by Chala and colleagues (2017).

All of the asthma group used fluoride toothpaste and only 1 from healthy group not use fluoride toothpaste and not significantly different (p>0.05, Fisher's exact test)

Both the groups have at least 1 time/year visited a dental clinic, that 31 (81.58%) of asthma group and 16 (72.72%) of healthy group. For never visit dental clinic that 7 (18.42%) of asthma and 6 (27.27%) of healthy group. There are and not significantly different (p>0.05, Fisher's exact test). No significant difference was found with regard to oral health behavior between asthma and healthy group (p>0.05, Fisher's exact test). The present results are in line with those reported by Chala and colleagues (2017). There

was no significant difference between asthmatic and control groups was found with regard to oral hygiene practices. [65]

4.3 Diet

For diet, we measured the frequency of food, sweet between meals (snack) and sugar intakes. (Table 4.4)

Table 4.4 Diet

	Asthma	Healthy	*p*-value
Food			
Food/day, (median, range)	5.33 (1.66-7.66)	4.00(3.00-6.33)	0.03*
Snack			
Snack/day, (median, range)	3.00 (0.00-4.66)	1.00 (0.66-3.33)	0.01*
Sugar			
Sugar/day, (median, range)	2.67 (0.00-5.00)	2.66 (0.33-3.66)	0.63

*Mann-Whitney *U*-test
Data diet available for 30

Table 4.4 Described food and snack intakes in asthmatic showed significantly higher compared to the healthy group, but there were no significant differences in sugar intake. Asthmatic patients had food intake per day 5.33 (1.66-7.66) compared to healthy group 4.00 (3.00-6.33) ($p = 0.03 < 0.05$, Mann-Whitney *U*-test). Snacks intake per day in asthmatic patients 3.00 (0.00-4.66) compared to the healthy group 1.00 (0.66-3.33) ($p = 0.01 < 0.05$, Mann-Whitney *U*-test). There were significant differences in food and snacks intake. In asthmatic group, sugar intakes per day 2.67 (0.00-5.00) compared to healthy group 2.66 (0.33-3.66). There were no significant differences in sugar intakes ($p = 0.63 > 0.05$, Mann-Whitney *U*-test).

Our results regarding snacks or sweet between meals are in accordance with Tootla and colleagues (2004) which argues that frequent of consumption of sweet and sweets drink between meals can also be one of the reasons for the increase in caries rate

in asthmatic children.[58] Beside that, Ryberg and colleagues (1987) found at their studies were no differences in sugar consumption and the nutritional value of the diet.[49] However, this finding was limited because not all of the asthma patients and healthy or control group sent their questionnaire back to our address by post.

4.4 Plaque staining

In healthy group we used non-parametric Kruskal-Wallis analysis test to compare among three groups of oral biofilm staining. As for the plaque maturity, color stains consist of pink color as immature plaque, blue/purple color as mature plaque, and light blue color as acidogenic plaque. Followed by Dunn's multiple comparisons *post-hoc* test. In our study we found the high percentage of immature dental plaque in control group (Fig. 4. 1).

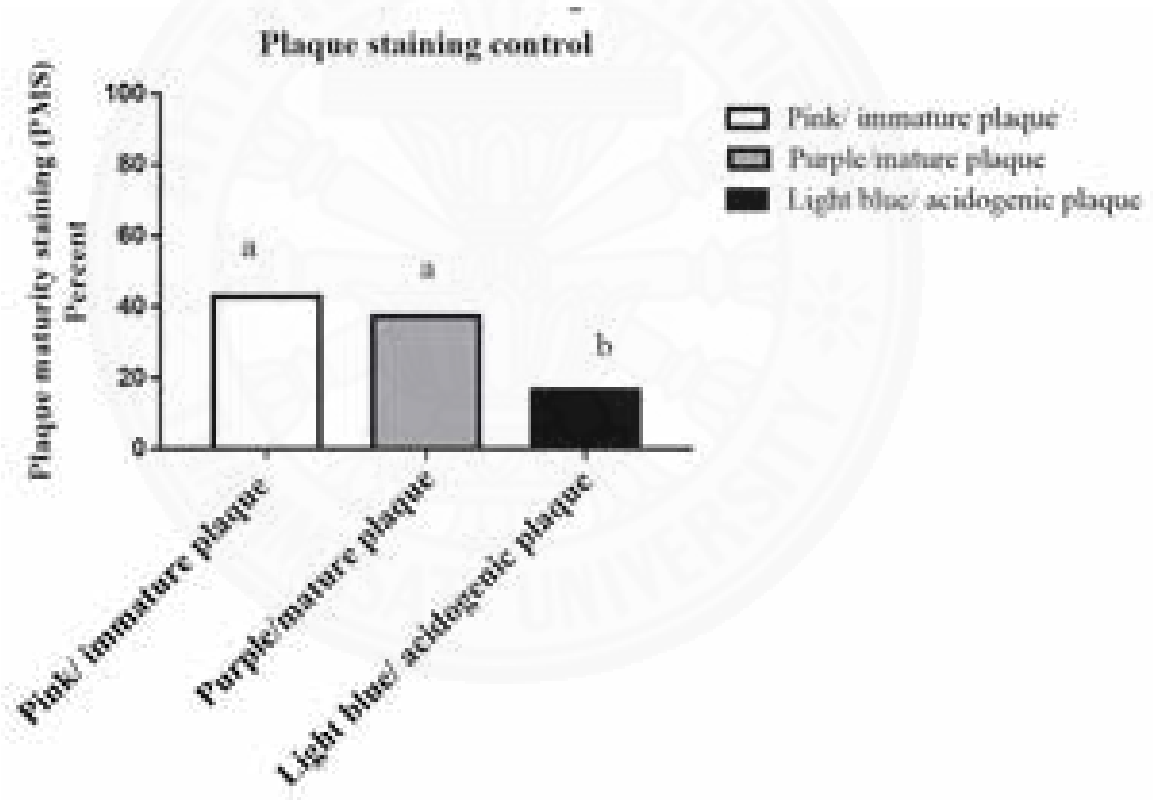


Figure 4.1 Plaque staining in healthy subjects/controls. Bars with different superscript letters indicate a significant difference (Kruskal-Wallis test $p < 0.05$ and followed by Dunn's multiple comparisons *post-hoc* test)

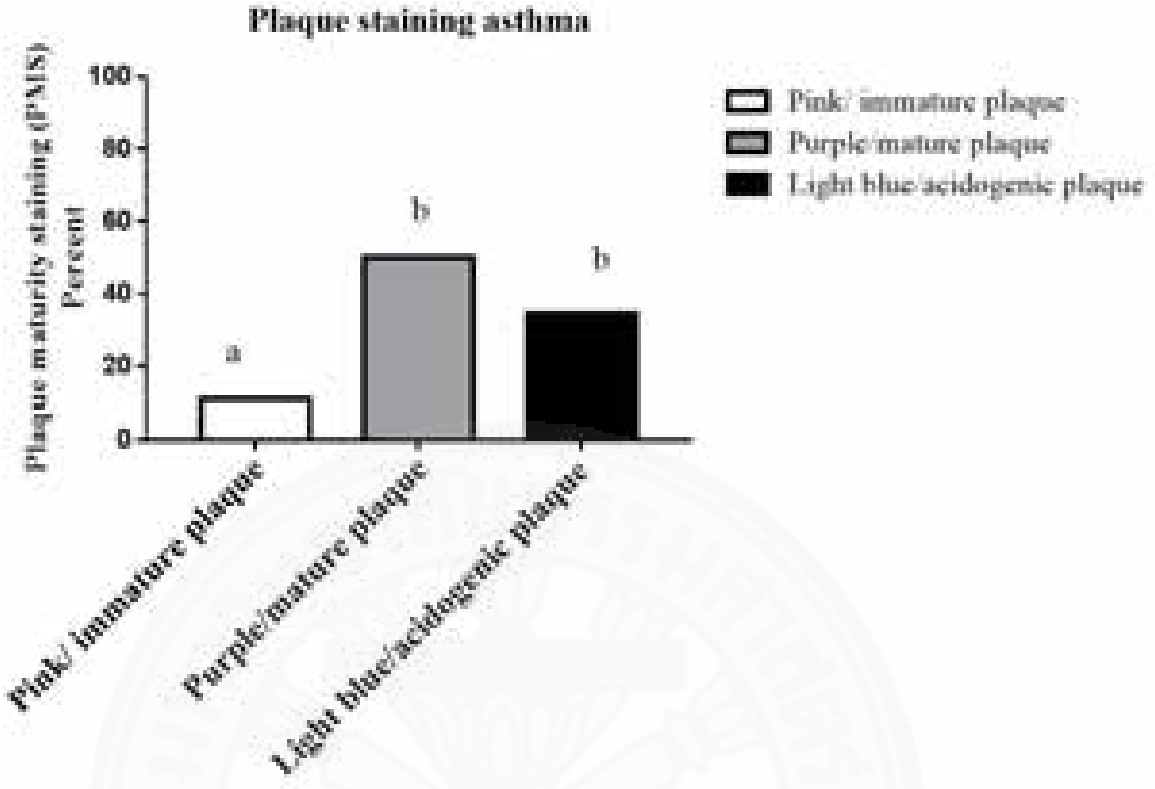


Figure 4.2 Plaque staining in asthma patients. Bars with different superscript letters indicate a significant difference (Kruskal-Wallis test $p < 0.05$ and followed by Dunn's multiple comparisons *post hoc* test)

In contrast with the control group, our study found the high percentage of mature and acidogenic dental plaque in asthmatic group. (Fig. 4.2).

In the asthmatic group, we found the lowest percentage of immature plaque (pink) staining. There was no statistical significance between the percent of mature (purple) and acidogenic plaque (light blue). In the control group, percent of acidogenic plaque was significantly lower. There were 34.67 % acidogenic plaque in asthmatic subjects compared to controls (16.67 %) ($p < 0.05$).

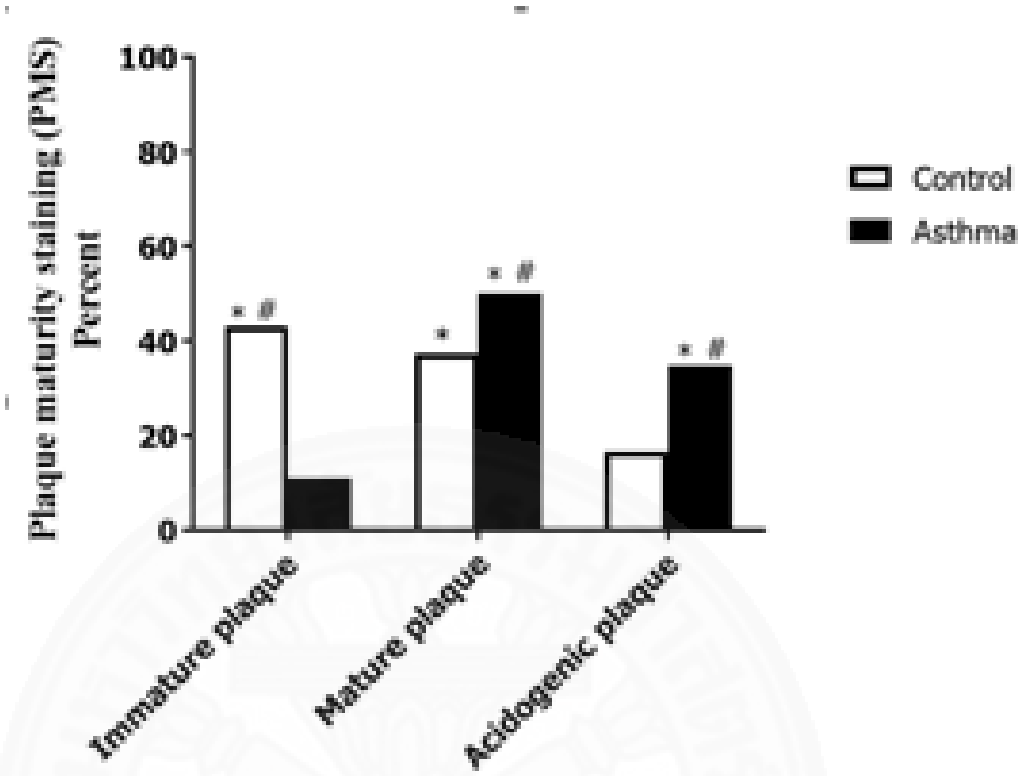


Figure 4.3. Dental plaque maturity was evaluated by staining with GC Tri Plaque ID Gel. Data express as percent of tooth stained with each colors. The bar graph represented the median values. *Symbols* indicate significant difference ($p < 0.05$) between colored plaque (*, Kruskal -Wallis test and Dunn's multiple comparisons *post-hoc* test) in each group and between groups (#, Mann-Whitney *U*- test).

Our study found high percentage of acidogenic and mature plaques in asthmatic group. In consistent results may be explained by differences in plaque index use. GC Tri Plaque ID Gel which rapidly detected acidogenicity of dental plaque was used in our studies. Furthermore, analysis of our questionnaires data revealed an increase in food and sugar intakes in asthmatic subjects which is in agreement with previous studies.[66] Therefore it is possible that an increase in sugar intake may play role in cariogenic plaque development in asthma.[67] Even though it is suggested that 4-5 meals or snacks per day as found in our asthmatic patients appear to be "safe" when their teeth are cleaned with fluoride tooth pastes twice or more daily.[52]

By using GC Tri Plaque ID Gel shows that plaque biofilm in patients with asthma have high-risk plaque. The acidogenic bacteria in the plaque biofilm will metabolize sucrose in GC Tri Plaque ID Gel so that the pH is low (<4.5) and causes the red pigment to disappear and leave a light blue color. The technology used by GC Tri Plaque ID Gel relies on the pH response of different dyes included sucrose to show the age of plaque and the production of acidogenic plaque within 2 minutes.[68]

Another explanation may be the complex nature of asthma. Patients with asthma are affected by both the disease and the medications. We found that asthmatic patients who were taking corticosteroid alone or in combination with β2-antagonists have a high level of acidogenic and mature. The result is expected because it is known that β2-antagonists is associated with changes in salivary and plaque pH to below the critical value of 5.5.[58,69]

For the difference in plaque maturity in each categories of drug use, matured plaque was obviously found in patients who used 1, 2, 3 or 4-6 medications. However, the highest percent of acid producing plaque was found in patient with only 1 or 2 drugs. Surprisingly, the lowest percent of immature plaque was also occurred in patient who used 1 or 2 drugs as well. (Fig. 4.4)

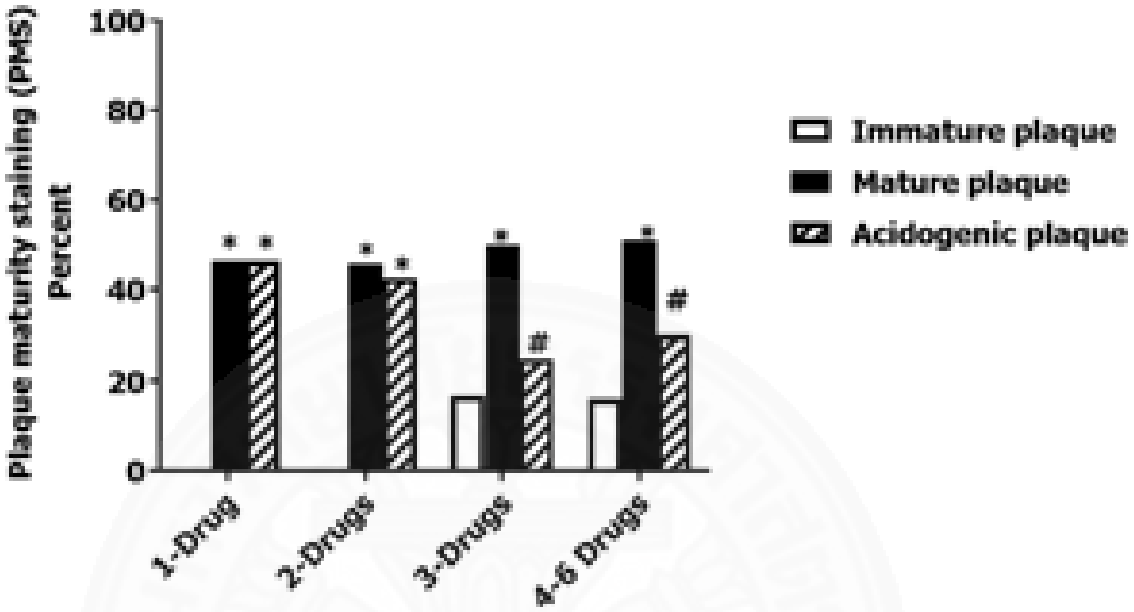


Figure 4.4 The difference in plaque maturity in respective numbers of drug use. The difference in plaque maturity related to respective numbers of drug use. The bar graph represented the median values. *Symbols* indicate significant difference in each groups (Kruskal-Wallis test followed by Dunn's multiple comparison *post-hoc test,* $p < 0.05$).

Table 4.5 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); 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 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

4.5 Virulence gene expression

Real Time PCR for specific genes was carried out in triplicate. The selected genes based on the properties of cariogenic bacteria in dental plaque biofilm, including adhesion, acidogenic, aciduric, and adaptation.[4] Data presented here were normalized by *16S rRNA* and further analyzed using nonparametric (Mann-Whitney *U*-test).

Several genes involved in biofilm formation have been identified in previous studies.[20] Real Time PCR is used to analyze the expression of several genes that have an important role in bacterial adherence and biofilm accumulation by *S.mutans*, including *spaP, gtfB* and *gbpB* genes. [4]

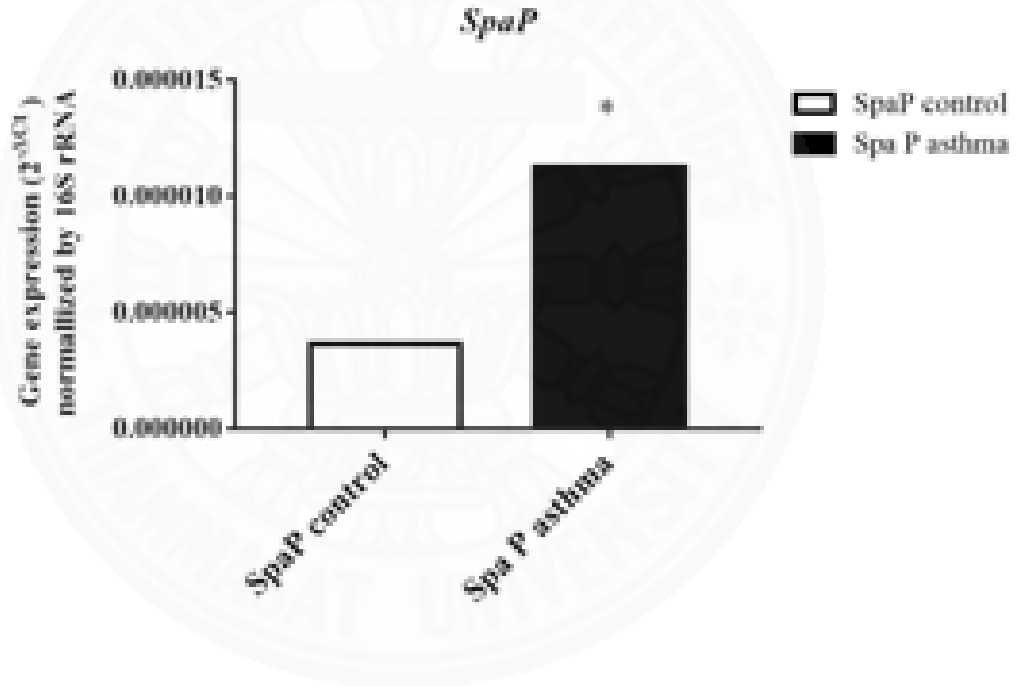


Figure 4.5 The result of Real Time PCR from selected genes based on sucrose independent adhesion properties of *S. mutans.* The bar graph represents with * illustrating statistical difference at $p< 0.05$ (Mann-Whitney *U*-test). The bar graph showing the expression of *spa P* gene in dental plaque samples of asthmatics was significantly higher when compared to controls ($p< 0.05$).

Specific primers were designed and selected according to the properties of *S.mutans.* In our study for adhesion with dental plaque by sucrose independent, *spaP* gene was used to design PCR primers. Multi-functional adhesion *spaP* gene was considered the primary factor mediating early attachment of *S. mutans* to tooth surface without sucrose.[20]

In the present study for adhesion in dental plaque by sucrose dependent, *gtfB* and *gbpB* gene were used to design PCR primers. The results of studies on the *gtfB* (Fig. 4.6) and *gbpB* (Fig. 4.7) from dental plaque samples of asthma patients were more express compared to the healthy group, respectively ($p < 0.05$, Mann-Whitney *U*-test).

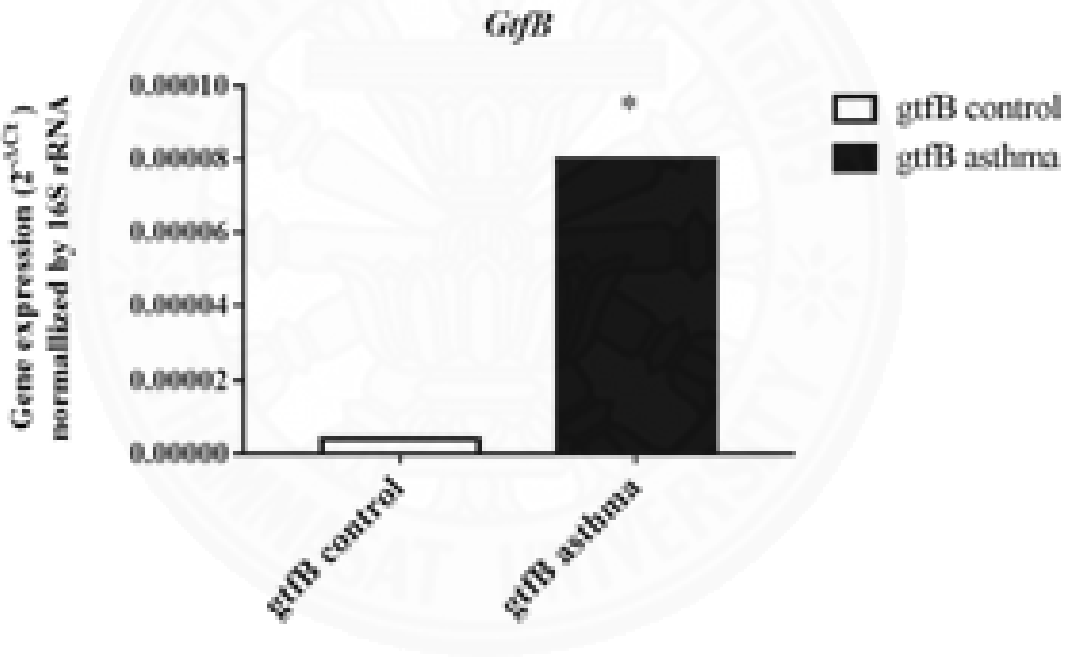


Figure 4.6 The result of Real Time PCR from selected genes based on sucrose dependent adhesion properties of *S. mutans.* The bar graph represents with * illustrating statistical difference at $p< 0.05$ (Mann-Whitney *U*-test). The bar graph showing the expression of *gtfB* gene in dental plaque samples of asthmatics was significantly higher when compared to controls ($p< 0.05$).

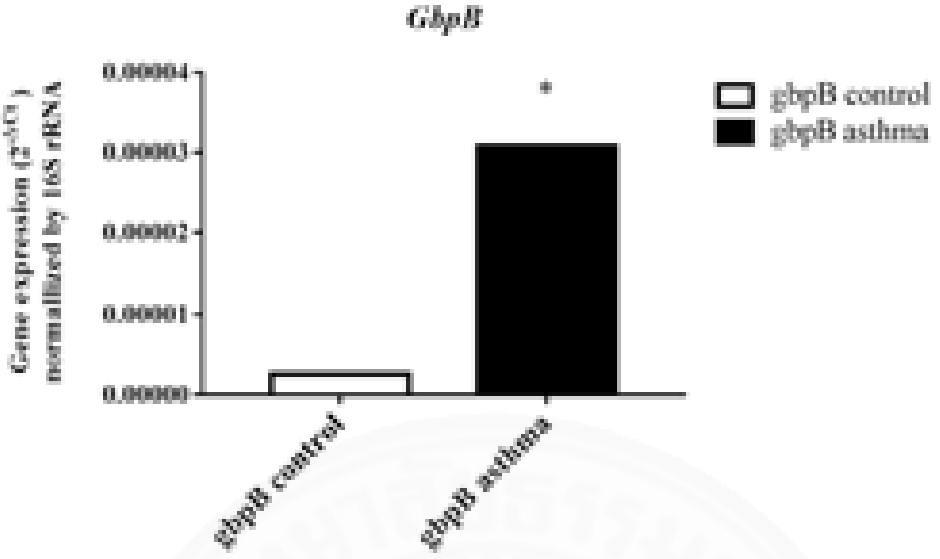


Figure 4.7 The result of Real Time PCR from selected genes based on sucrose dependent adhesion properties of *S. mutans.* The bar graph represents with * illustrating statistical difference at $p< 0.05$ (Mann-Whitney *U*-test). The bar graph showing the expression of *gbp B* gene in dental plaque samples of asthmatics was significantly higher when compared to controls ($p< 0.05$).

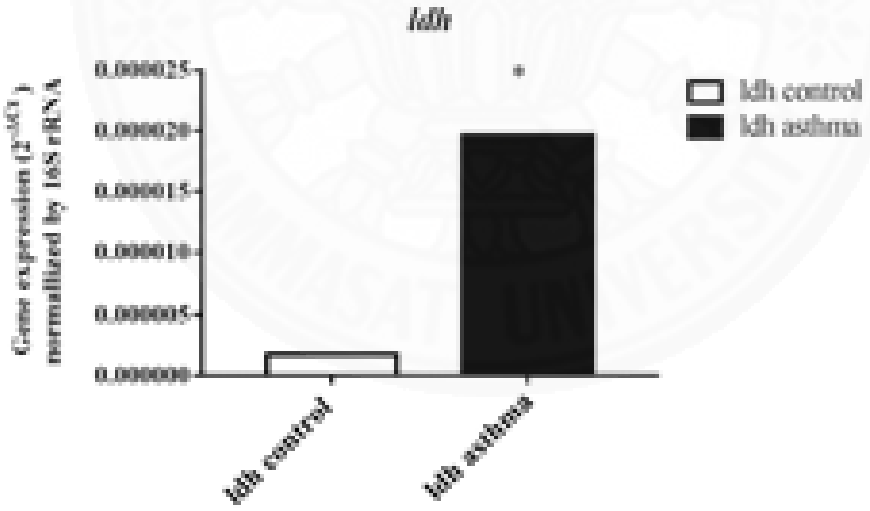


Figure 4.8 The result of Real Time PCR from selected genes based on acidogenic properties of *S. mutans.* The bar graph represents with * illustrating statistical difference at $p< 0.05$ (Mann-Whitney *U*-test). The bar graph showing the expression of *ldh* gene in dental plaque of asthmatics was significantly higher when compared to controls ($p< 0.05$)

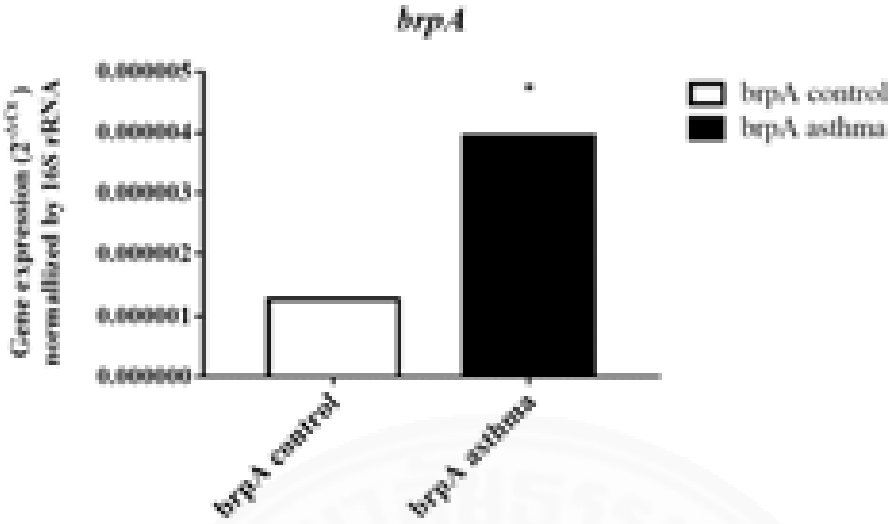


Figure 4.9 The result of Real Time PCR from selected genes based on aciduric properties of *S. mutans.* The bar graph represents with * illustrating statistical difference at $p< 0.05$ (Mann-Whitney *U*-test). The bar graph showing the expression of *brpA* gene in dental plaque of asthmatics was significantly higher when compared to healthy ($p< 0.05$).

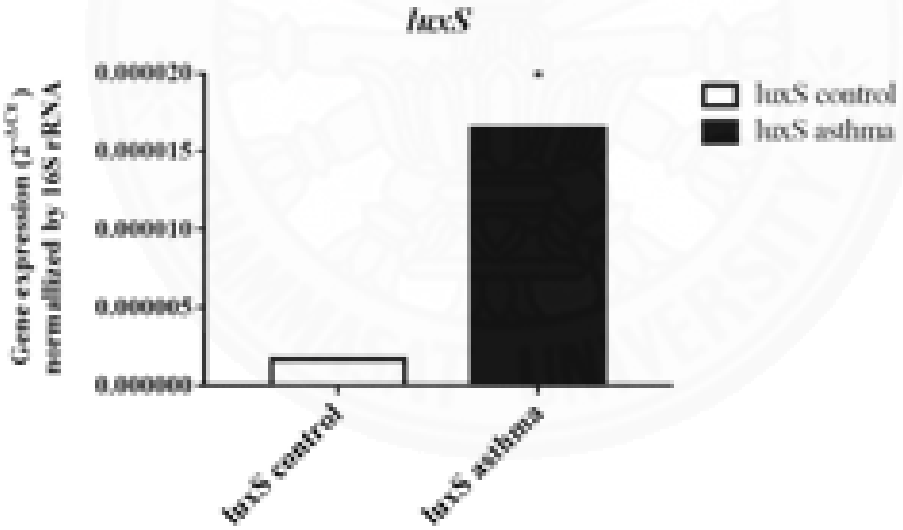


Figure 4.10 The result of Real Time PCR from selected genes based on adaptation properties of *S. mutans.* The bar graph represents with * illustrating statistical difference at $p< 0.05$ (Mann-Whitney *U*-test). The bar graph showing the expression of *luxS* gene in dental plaque of asthma patient was significantly higher when compared to healthy ($p< 0.05$).

Compared to the controls, the expression of *gtfB, gbpB*, *ldh*, *luxS*, *spaP,* and *brpA* was increased by 19.57, 11.96, 11.29, 9.73, 4.05 and 3.08 folds, respectively in asthma patients. (Fig. 4.11)

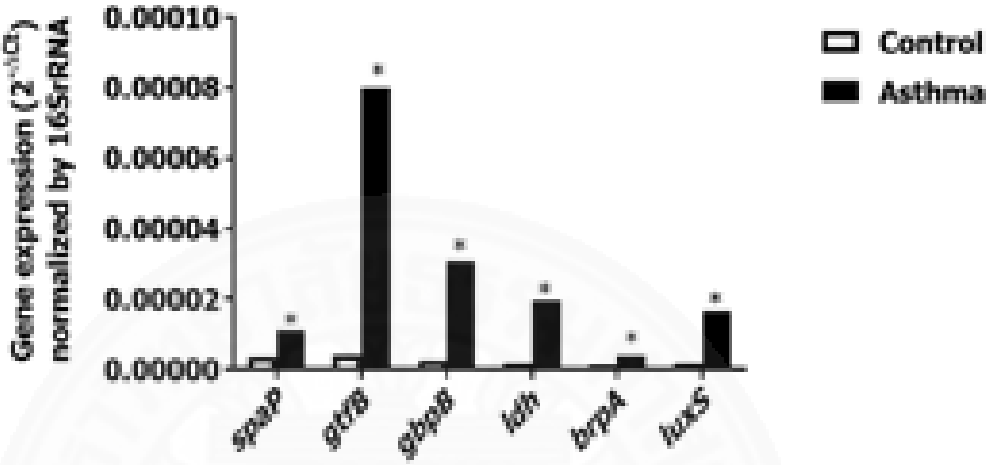


Figure 4.11 The figure represents the difference in selected genes' expression between asthmatic and control groups. Real time-PCR of *spaP, gtfB, gbpB, ldh, brpA,* and *luxS* were carried out in triplicate. Difference in expression were shown as Delta Ct values. Data presented here was generated from at least three independent sets of determinants. The bar graph represents the median expression (n = 3), with * is illustrating statistical difference in each gene at $p < 0.05$ when compared between asthmatic and control groups (Mann-Whitney *U*-test).

We found gtfB*, gbpB*, *ldh* and *luxS* were markedly upregulated in dental plaque obtained from the asthmatic patients.

Spearman's rank correlation indicated that there was the negative correlation 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 all subjects. The association between percentage of acidogenic plaque and the expression of *ldh*, ($\rho = 0.379$, $p < 0.05$), *spaP* ($\rho = 0.333$, $p < 0.05$), *luxS* ($\rho = 0.321$, $p < 0.05$), *gbpB* ($\rho = 0.310$, $p < 0.05$), and *brpA* ($\rho = 0.310$, $p < 0.05$). There was no association between

gtfB expression and percentage of acid producing plaque (ρ=0.183, p > 0.05). In contrast we could not demonstrate the association between the expression of all studied genes and percent of immature plaque (p > 0.05). Figure 4.12 demonstrated that the expression of *gtfB* expression was markedly observed in patients who used medication 1, 2, 3 or 4-6 types (p<0.05). In contrast, we could not demonstrate the difference in the numbers of drug use according to the expression of *spaP, gbpB, ldh, brpA,* and *luxS.*

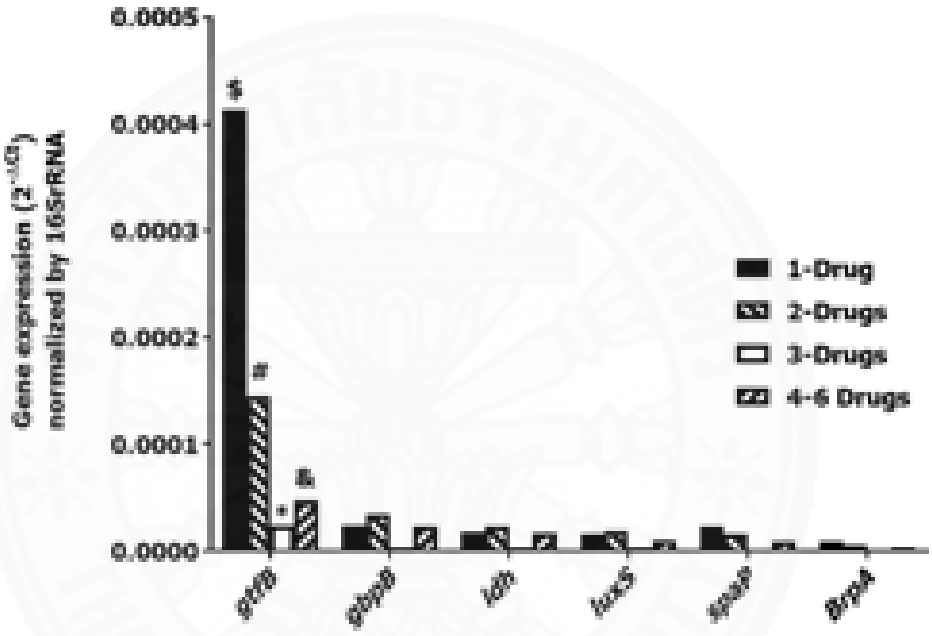


Figure 4.12 The distribution of selected gene expression according to the numbers of drug use. Real time-PCR of *gtfB, gbpB, ldh, luxS, spaP,*and ,*brpA* was carried out in triplicate. The gene expression is shown as Delta Ct values. The bar graph represents the median expression (n = 3), *symbols* illustrating statistical difference in the numbers of drug use in the respective genes at p < 0.05 (Kruskal-Wallis test follow by Dunn's multiple comparison *post- hoc* test).

Our findings showed the negative association between the expression of the studied genes and the percent of immature plaque. As expected, we found a significant moderate positive correlation between the cariogenicity of plaque and the expression of *ldh, brpA, luxS, spaP* and *gbpB* but not *gtfB* gene. This indicated the up-regulated genes might contribute to acid production and acid tolerance of certain bacteria in dental plaque [38,70]

Lactate dehydrogenase (LDH), encoded by the *ldh*, is undoubtedly the most important enzyme for the acidogenicity of caries-associated plaque[71] An increase in the expression of *brpA* involved in acid tolerance and plaque development[38,39] An increase in *luxS* might play important roles in dental plaque accumulation[72]

Surprising, we found the association between the expression of *gbpB* and *spaP* but not *gtfB* gene and an increasing of acid producing plaque. Therefore, it seems to be that an increase in virulence genes involving bacterial adherence might contribute in initial plaque formation and they may not play any important role in dental plaque metabolism. The protein spaP is known to be correlated with virulence of the organism for development of dental caries and participates in bacterial adherence to teeth via interaction with the salivary pellicle, which is termed as "sucrose-independent adhesion" [31] GBP capable of binding to glucans are hypothesized to contribute to the "sucrose-dependent adhesion" of bacteria and possibly to the cohesive architecture of the dental biofilm [34]

The association between the number of drug use and the enhancement of certain virulence gene expression for example *gtfB* implies that not only the changes in dietary habit but also the asthma medications may contribute to the expression of cariogenic related genes in asthma. To our knowledge, this is the first report concerning the expression of virulence genes in dental biofilm of asthma. Collectively, our data suggest that the medication use and/or the relatively high sugar consumption has played critical roles in upregulating caries related genes in dental plaque in asthma. These effects lead to the changes of plaque environment according to ecological plaque hypothesis and it is possible to increase caries prevalence in asthma.

CHAPTER 5
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study revealed that asthma patients have caries risk. We found the comorbidity was mostly asthma sufferers with allergic rhinitis. Asthma patients have received treatment with medication such as anticholinergics, corticosteroid, ß2 agonist and also treated with intranasal corticosteroids and antihistamines that cause salivary flow rate decrease. When salivary flow rate decreases, low salivary pH and plaque pH, low buffering and increased the composition of MS and lactobacilli would follow.

The genes that play a major role in the early stages of biofilm formation proved to appear to be more expressed in asthma patients compared to the healthy group. Genes related to bacterial adhesion properties such as *spaP, gtfB*, and *gbpB* genes are important in initial plaque formation.

Lactic acid production is catalyzed by LDH which is the strongest acid of the metabolic acid end product of oral microorganisms. The presence of LDH can affect the acid production of *S. mutans* in dental plaque samples.

The *brpA* gene plays a major role in acid and oxidative stress tolerance and biofilm formation. BrpA regulates genes that are required for stable biofilm formation. Biofilm formation is a highly regulated process and that formation of mature biofilms requires the cooperation of a wide range of gene products, including those that are involved in acid and oxidative stress tolerance. The disappearance of brpA significantly affects acid regulation and oxidative stress tolerance and biofilm formation.

The autoinducer-2 signal (AI-2) produced by the luxS protein mediates interspecies communication among Gram-positive and Gram-negative. Quorum sensing (QS) is the process of chemical communication among bacteria as a gene regulation in response to cell density and can affect many kinds of function, such as virulence, acid tolerance, and biofilm formation

Our data suggests that asthma's pathological conditions and/ or medications have affected on the properties and virulence of dental plaque. Asthma induced the expression of *spaP*, *gtfB gbpB, ldh, brpA,* and *luxS,* in dental plaque. An increase in acidogenic and mature plaque was commonly found in asthmatic patients.

Eventually, we concluded that asthmatic patients have high caries risk compared to the healthy group base on plaque staining and cariogenic genes expression

5.2 Recommendation

We recommend routine periodic dental examination. It's important to visit a dentist every six months for a regular dental checkup and professional cleaning. For patients with caries risk can visit every 4 months and for patient with free caries can visit every 1 year.

Plaque staining and gene expression assays with PCR method were useful for assessing caries risk in asthma patients. GC Tri Plaque ID Gel can be used for rapid detection of acidogenic dental plaque biofilms, and also simple and practical. The PCR method makes it possible to diagnose quickly in just one day with a very small amount of bacteria.

REFERENCES

1. Cameron AC, Widmer RPe. Dental caries In: Manton DJ,Cameron LH, eds. *Handbook of Pediatric Dentistry*. 4TH ed. Canberra, Australia: Mosby, 2014;47-62.
2. Krol DM. Dental caries, oral health, and pediatricians. *Curr Probl Pediatr Adolesc Health Care* 2003;33:253-270.
3. Mount GJ, Hume WR. Preservation and Restoration of Tooth Structure In: Ngo HC,Gaffney S, eds. *Lifestyle Impacts on Oral Health* 2 nd ed. Queensland, Australia, 2005;93.
4. Banas JA. Virulence properties of Streptococcus mutans. *Front Biosci* 2004;9:1267-1277.
5. Thomas MS, Parolia A, Kundabala M, et al. Asthma and oral health: a review. *Aust Dent J* 2010;55:128-133.
6. Network TGA. The Global Asthma Report 2018, 2018.
7. Shulman JD, Taylor SE, Nunn ME. The association between asthma and dental caries in children and adolescents: A population-based case-control study. *Caries Res* 2001;35:240-246.
8. Ersin NK, Gulen F, Eronat N, et al. Oral and dental manifestations of young asthmatics related to medication, severity and duration of condition. *Pediatr Int* 2006;48:549-554.
9. Fejerskov O, Kidd EAM, Nyvad B, et al. The Role of Saliva In: Bardow A, Lagerlöf F, Nauntofte B, et al., eds. *Dental caries: The Disease and its Clinical Management*. 2nd ed. ed. Oxford: Wiley-Blackwell, 2008;190-207.
10. Bowen WH. The Stephan Curve revisited. *Odontology* 2013;101:2-8.
11. Edgar WM, O'Mullane DM. Saliva and the Control of Plaque pH In: Higham MEaSM, ed. *Saliva and Oral Health*. London British Dental Association, 1996;81-94.

12. Heymann H, Swift EJ, Ritter AV, et al. Dental caries: Etiology, Clinical Characteristics, Risk Assesment, and Management In: Andre V. Ritter, R. Scott Eidson,Donovan TE, eds. *Sturdevant's art and science of operative dentistry*. 6th ed. ed. St. Louis, Missouri: Elsevier/Mosby, 2013;41-88.
13. Carounanidy U. Caries Risk Assessment: A Critical Look. *Journal of Operative Dentistry and Endodontics* 2018;3;1:22-27.
14. Selwitz RH, Ismail AI, Pitts NB. Dental caries. *Lancet* 2007;369:51-59.
15. Fejerskov O, Nyvad B, Kidd EAM. How big is the problem? Epidemiological features of dental caries. *Dental Caries: The Disease and Its Clinical Management*. 3 rd ed. Oxford: John Wiley& Sons, Ltd, 2015;37-40.
16. Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. *J Dent Res* 2011;90:294-303.
17. Takahashi N, Yamada T. Acid-induced acid tolerance and acidogenicity of non-mutans streptococci. *Oral Microbiol Immunol* 1999;14:43-48.
18. Marsh PD, Martin MV. Oral Microbiology. *Dental Plaque and Plaque-mediated diseases – dental caries and periodontal diseases* Fifth ed: Elsevier, 2009;74-102 103-116.
19. Nishikawara F, Nomura Y, Imai S, et al. Evaluation of cariogenic bacteria. *Eur J Dent* 2007;1:31-39.
20. Wen ZT, Yates D, Ahn SJ, et al. Biofilm formation and virulence expression by Streptococcus mutans are altered when grown in dual-species model. *BMC Microbiol* 2010;10:111.
21. Ajdic D, McShan WM, McLaughlin RE, et al. Genome sequence of Streptococcus mutans UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A* 2002;99:14434-14439.
22. Samaranayake LP. Lactobacilli, Corynebacteria, and propionibacteria. *Essential microbiology for dentistry*. 4th ed. Edinburgh ; New York: Churchill Livingstone Elsevier, 2012;129-131.

23. Demirci M, Tuncer S, Yuceokur AA. Prevalence of caries on individual tooth surfaces and its distribution by age and gender in university clinic patients. *Eur J Dent* 2010;4:270-279.
24. Kolenbrander PE, Andersen RN, Blehert DS, et al. Communication among oral bacteria. *Microbiol Mol Biol Rev* 2002;66:486-505, table of contents.
25. Wan AK, Seow WK, Purdie DM, et al. A longitudinal study of Streptococcus mutans colonization in infants after tooth eruption. *J Dent Res* 2003;82:504-508.
26. Jenkinson HF, Demuth DR. Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. *Mol Microbiol* 1997;23:183-190.
27. Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. *J Dent Res* 2013;92:1065-1073.
28. Schilling KM, Bowen WH. Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for Streptococcus mutans. *Infect Immun* 1992;60:284-295.
29. Struzycka I. The oral microbiome in dental caries. *Pol J Microbiol* 2014;63:127-135.
30. Kawada-Matsuo M, Oogai Y, Komatsuzawa H. Sugar Allocation to Metabolic Pathways is Tightly Regulated and Affects the Virulence of Streptococcus mutans. *Genes (Basel)* 2016;8.
31. Matsumoto-Nakano M. Role of Streptococcus mutans surface proteins for biofilm formation. *Jpn Dent Sci Rev* 2018;54:22-29.
32. Koo H, Xiao J, Klein MI, et al. Exopolysaccharides produced by Streptococcus mutans glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *J Bacteriol* 2010;192:3024-3032.
33. Costa Oliveira BE, Cury JA, Ricomini Filho AP. Biofilm extracellular polysaccharides degradation during starvation and enamel demineralization. *PLoS One* 2017;12:e0181168.

34. Banas JA, Vickerman MM. Glucan-binding proteins of the oral streptococci. *Crit Rev Oral Biol Med* 2003;14:89-99.
35. Fujita K, Takashima Y, Inagaki S, et al. Correlation of biological properties with glucan-binding protein B expression profile in Streptococcus mutans clinical isolates. *Arch Oral Biol* 2011;56:258-263.
36. Matsumoto-Nakano M, Fujita K, Ooshima T. Comparison of glucan-binding proteins in cariogenicity of Streptococcus mutans. *Oral Microbiol Immunol* 2007;22:30-35.
37. Nishimura. Advances in Microbiology. Biofilm Formation by S.mutans and Related bacteria: Scientific Research., 2012.
38. Bitoun JP, Liao S, Yao X, et al. BrpA is involved in regulation of cell envelope stress responses in Streptococcus mutans. *Appl Environ Microbiol* 2012;78:2914-2922.
39. Wen ZT, Baker HV, Burne RA. Influence of BrpA on critical virulence attributes of Streptococcus mutans. *J Bacteriol* 2006;188:2983-2992.
40. Takahashi N. International congress series. Microbial ecosystem in the oral cavity: Metabolic diversity in an ecological niche and its relationship with oral diseases. Amsterdam ; New York: Elsevier Science., 2005.
41. Huang Z, Meric G, Liu Z, et al. luxS-based quorum-sensing signaling affects Biofilm formation in Streptococcus mutans. *J Mol Microbiol Biotechnol* 2009;17:12-19.
42. He Z, Liang J, Tang Z, et al. Role of the luxS gene in initial biofilm formation by Streptococcus mutans. *J Mol Microbiol Biotechnol* 2015;25:60-68.
43. GINA. Global strategy for asthma management and prevention. Global Initiative for asthma(GINA). 2012.
44. Trakultivakorn M. Economic burden of asthma in Thailand. *Asian Pac J Allergy Immunol* 2012;30:1-2.
45. Kaur S, Singh V. Asthma and Medicines - Long-Term Side-Effects, Monitoring and Dose Titration. *Indian J Pediatr* 2018;85:748-756.

46. Nakagawa S, Cuthill IC. Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol Rev Camb Philos Soc* 2007;82:591-605.
47. Association ACoTT. Asthma Diagnosis and Treatment Guidelines in Thailand. 2012.
48. Alaki SM, Ashiry EA, Bakry NS, et al. The effects of asthma and asthma medication on dental caries and salivary characteristics in children. *Oral Health Prev Dent* 2013;11:113-120.
49. Ryberg M, Moller C, Ericson T. Effect of beta 2-adrenoceptor agonists on saliva proteins and dental caries in asthmatic children. *J Dent Res* 1987;66:1404-1406.
50. Sowmya K. R. RCVK, Veeresh D.J. Oral health status and treatment needs of asthmatics children. *Journal of Indian Asscociation of Public Health Dentistry* 2007;2007:13-17.
51. Rugg-Gunn AJ, Hackett AF, Appleton DR, et al. Relationship between dietary habits and caries increment assessed over two years in 405 English adolescent school children. *Arch Oral Biol* 1984;29:983-992.
52. Wongkamhaeng K, Poachanukoon O, Koontongkaew S. Dental caries, cariogenic microorganisms and salivary properties of allergic rhinitis children. *Int J Pediatr Otorhinolaryngol* 2014;78:860-865.
53. Mazzoleni S, Stellini E, Cavaleri E, et al. Dental caries in children with asthma undergoing treatment with short-acting beta2-agonists. *Eur J Paediatr Dent* 2008;9:132-138.
54. Paganini M, Dezan CC, Bichaco TR, et al. Dental caries status and salivary properties of asthmatic children and adolescents. *Int J Paediatr Dent* 2011;21:185-191.
55. Dental GA. A new perspective on biofilms, GC Tri Plaque ID Gel., 2011.

56. Utispan K, Chitkul B, Monthanapisut P, et al. Propolis Extracted from the Stingless Bee Trigona sirindhornae Inhibited S. mutans Activity In Vitro. *Oral Health Prev Dent* 2017;15:279-284.
57. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-408.
58. Tootla R, Toumba KJ, Duggal MS. An evaluation of the acidogenic potential of asthma inhalers. *Arch Oral Biol* 2004;49:275-283.
59. Stensson M, Wendt LK, Koch G, et al. Caries prevalence, caries-related factors and plaque pH in adolescents with long-term asthma. *Caries Res* 2010;44:540-546.
60. Meldrum AM, Thomson WM, Drummond BK, et al. Is asthma a risk factor for dental caries? Finding from a cohort study. *Caries Res* 2001;35:235-239.
61. Eloot AK, Vanobbergen JN, De Baets F, et al. Oral health and habits in children with asthma related to severity and duration of condition. *Eur J Paediatr Dent* 2004;5:210-215.
62. Vichyanond P, Suratannon C, Lertbunnaphong P, et al. Clinical characteristics of children with non-allergic rhinitis vs with allergic rhinitis. *Asian Pac J Allergy Immunol* 2010;28:270-274.
63. Wu F-y, Liu J-f. Asthma medication increases dental caries among children in Taiwan: An analysis using the National Health Insurance Research Database. *Journal of Dental Sciences* 2019.
64. Konde S, Agarwal M, Chaurasia R. Effects of inhalational anti-asthmatic medications on oral health between 7 and 14 years of age. *Indian Journal of Allergy, Asthma and Immunology* 2018;32:70.
65. Chala S, Rouiffi S, Soualhi M, et al. Association between untreated carious lesions and asthma in adults at Rabat University Hospital, Morocco: a cross sectional study. *BMC Res Notes* 2017;10:221.

66. Al-Zalabani AH, Noor Elahi I, Katib A, et al. Association between soft drinks consumption and asthma: a systematic review and meta-analysis. *BMJ Open* 2019;9:e029046.
67. Walsh LJ. Dental plaque fermentation and its role in caries risk. *INTERNATIONAL DENTISTRY* 2006;8 . no.5:34-40.
68. Walsh LJ. New paradigms for assessing caries risk and lesion activity. *Auxillary* 2011;5:28-33.
69. Kargul B, Tanboga I, Ergeneli S, et al. Inhaler medicament effects on saliva and plaque pH in asthmatic children *J Clin Pediatr Dent* 1998;22:137-140.
70. He Z, Huang Z, Jiang W, et al. Antimicrobial Activity of Cinnamaldehyde on Streptococcus mutans Biofilms. *Front Microbiol* 2019;10:2241.
71. Tanaka H, Tamura M, Kikuchi K, et al. An enzymological profile of the production of lactic acid in caries-associated plaque and in plaque formed on sound surfaces of deciduous teeth. *Caries Res* 1993;27:130-134.
72. Yoshida A, Ansai T, Takehara T, et al. LuxS-based signaling affects Streptococcus mutans biofilm formation. *Appl Environ Microbiol* 2005;71:2372-2380.

APPENDICES

APPENDIX A
CONSENT FORM

Code

หนังสือแสดงเจตนายินยอมเข้าร่วมการวิจัย

(Consent Form)

โครงการวิจัยเรื่อง Evaluation caries status in asthmatic patient by using Tri-plaque disclosing agent and gtfB gene expression

การประเมินสภาวะฟันผุในผู้ป่วยโรคหืดด้วยผลิตภัณฑ์ย้อมคราบจุลินทรีย์แบบ 3 สี และการแสดงออกของยีน gtfB

วันที่ให้คำยินยอม วันที่ ____________ เดือน ________________ พ.ศ. ________________

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

1) [illegible]

คณะทันตแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

[illegible]

Code

2) รศ.ทพ.[illegible]
คณะทันตแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์
เบอร์ติดต่อ 087-5081717

3) รศ.ทพ.[illegible]
คณะทันตแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์
เบอร์ติดต่อ 090-968-7678

4) รศ.ทพ.[illegible]
คณะทันตแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์
เบอร์ติดต่อ 086-388-4704

ลงนาม..ผู้ยินยอม
(..)
ลงนาม..พยาน
(..)
ลงนาม..พยาน
(..)

ในกรณีที่การศึกษาวิจัยครั้งนี้ มีความจำเป็นอย่างยิ่งที่จะต้องให้ผู้เยาว์และหรือบุคคลไร้ความสามารถมีส่วนร่วมในการวิจัย ข้าพเจ้าได้อ่านข้อความข้างต้นแล้ว มีความเข้าใจในสิทธิและหน้าที่ของผู้เข้าร่วมโครงการวิจัยทุกประการ และยินยอมให้ผู้เยาว์ และหรือ บุคคลไร้ความสามารถเข้าร่วมการวิจัยได้ จึงลงลายมือชื่อไว้เป็นหลักฐาน

ลงนาม..
(..)
ผู้ปกครอง ผู้แทนโดยชอบธรรม หรือ ผู้มีอำนาจกระทำการแทน

ลงนาม..พยาน
(..)
ลงนาม..พยาน
(..)

Code

Consent Form

Research Project's Topic Evaluation caries status in asthmatic patient by using Tri-plaque disclosing agent and *gtfB,* gene expression

Date of Consent:

Date……Month……Year…….

Before signs consent form for this research project, I, consenter, have been informed by researcher on objectives of this research project, method of this research project, hazard or possible symptoms those could occur from this research and drugs using in research, as well as benefit from this research in detail and understand thoroughly.

I, consenter, have right to cancel participation to this research experiment as I wish without losing right in medical treatment that following the experiment.

Researchers guarantee that the data they collect related to me, consenter, is secret and will be disclosed only in form of research summary.

Disclosure of data related to me, consenter, to related organizations and departments could only be done for academic reasons and only after receive written consent form from me, participant.

Researchers guarantee if there is any complication that caused by this research, I, consenter, will receive medical treatment without cost and/or compensation fee as well as suitable compensation for disability that occur.

I, consenter, agree for research conductor, inspectors of human-research ethic committee and drug-control committee to able to check record of my medical data to confirm procedure of this clinical research project without considered this as violation of right to conceal personal data of participants according to framework of law and regulation allowing to.

I, participant, had already read all of above paragraphs and had completely understood all of their content and have signed this consent form voluntarily.

If I, consenter, am illiterate, researcher had already read all content in this consent form for me to listen and had already completely understood the detail. I have signed this consent form voluntarily.

I, consenter, could contact researchers at faculty of Dentistry at Thammasat University, Klongluang district, Patum Thani province, Thailand.

Persons who responsible for this research project are.

1) Professor Dentist Doctor Sittichai Kuntongkaew
   Faculty of Dentistry Thammasat University
   Phone: 02-9869213 to 7126
2) Miss Sirirat Raeongsuwat
   Faculty of Dentistry Thammasat University
   Phone: 087-5091717
3) Miss Vacharee Klaengkaew
   Faculty of Dentistry Thammasat University
   Phone: 090-9697678
4) Miss Pornvacharinth Limwatanachai
   Faculty of Dentistry Thammasat University
   Phone: 086-388-4704

Code

Sign…….Consenter
(…….)
Sign…….Witness
(…….)
Sign…….Witness
(…….)

In case of this research study utmost need for minor or disability person participate in this research, I, consenter, had already read content above and understand all about rights and obligations of research project's participants and allow minor or disability person to participate in research. So l left written sign as proof.

Sign…….
(…….)
Parent, Legal Representative, Authorized Representative
Sign…….Witness
(…….)
Sign…….Witness
(…….)

APPENDIX B
QUESTIONNAIRE

Code

รูปแบบของแบบสอบถามในการเก็บรวบรวมข้อมูลทั่วไป ข้อมูลของการเป็นโรคหืด การใช้ยารักษา ระยะเวลาในการใช้ยา ระดับการควบคุมโรค ระยะเวลาในการเป็นโรคหอบหืด ระดับการควบคุมโรคหืด การดูแลสุขภาพช่องปาก และการบันทึกการบริโภคอาหาร เพื่อนำมาใช้ในการศึกษาครั้งนี้

แบบสอบถามงานวิจัย

<u>ขั้นตอน</u> กรุณาตอบคำถาม และทำเครื่องหมาย ✓ หน้าคำตอบที่ท่านเลือก

<u>ส่วนที่ 1</u> ข้อมูลทั่วไป

1) อายุ ..... ปี

2) เพศ ❒ ชาย ❒ หญิง

3) รายได้เฉลี่ยต่อเดือน

❒ ไม่มีรายได้ ❒ รายได้ 15,001-30,000 บาท

❒ รายได้ 1-5,000 บาท ❒ รายได้ 30,001-50,000 บาท

❒ รายได้ 5,001-15,000 บาท ❒ รายได้ตั้งแต่ 50,001 บาทขึ้นไ

4) ระดับการศึกษาของตนเอง หรือระดับการศึกษาของผู้ปกครอง (กรณียังไม่บรรลุนิติภาวะ)

❒ ไม่เคยเรียน ❒ ปวส./อนุปริญญา

❒ ประถมศึกษา ❒ ปริญญาตรี

❒ มัธยมศึกษาตอนต้น ❒ สูงกว่าปริญญาตรี

❒ มัธยมศึกษาตอนปลาย/ปวช.

5) มีโรคประจำตัว หรือโรคทางระบบที่แพทย์ระบุหรือไม่

❒ ไม่มี ❒ มี (ระบุ .....)

Code

<u>ส่วนที่ 2</u> ข้อมูลผู้ป่วยโรคหืด

1) ระยะเวลาดำเนินโรค …….

2) ระดับการควบคุมโรค ❒ Controlled ❒ Partly controlled ❒ Uncontrolled

3) การวินิจฉัย ❒ Asthma ❒ Asthma & allergic rhinitis ❒ Asthma & sinusitis ❒ Other

4) ยาที่ใช้รักษาโรคหืดในปัจจุบัน

>> ยาที่ใช้ในการควบคุมโรค (Controller medication)

✓	ยาที่ใช้	ปริมาณยา	วิธีการใช้	ความถี่ (ครั้ง/วัน)	ระยะเวลาที่ใช้
	Corticosteroids				
	Long-acting β2 agonist (LABA)				
	ICS + LABA				
	Leukotriene modifier				

>> ยาบรรเทาอาการ (Relieve medications)

✓	ยาที่ใช้	ปริมาณยา	วิธีการใช้	ความถี่ (ครั้ง/วัน)	ระยะเวลาที่ใช้
	Short-acting β2 agonist				

>> ยาอื่นๆ

✓	ยาที่ใช้	ปริมาณยา	วิธีการใช้	ความถี่ (ครั้ง/วัน)	ระยะเวลาที่ใช้
	Antihistamine				
	Antibiotics				
	อื่นๆ ระบุ **1. ……** **2. ……** **3. ……**				

Code

5) กรณียาพ่นได้ใช้ Spacer หรือไม่

❒ ไม่ใช้

❒ ใช้

6) การใช้ยา

❒ ใช้ยามากกว่า 80%

❒ ใช้ยาน้อยกว่า 80%

Code

<u>ส่วนที่ 3</u> พฤติกรรมทันตสุขภาพ

1) ทำความสะอาดช่องปากด้วยวิธีใดเป็นประจำ (เลือกตอบวิธีที่ทำเป็นประจำเพียง 1 ข้อ) และทำเวลาใดบ้าง (ตอบได้มากกว่า 1 ข้อ)

❒ ไม่ได้ทำอะไรเลย

❒ บ้วนปาก [ ] หลังตื่นนอนเช้า [ ] หลังมื้อเช้าเที่ยง [ ] หลังมื้อเที่ยง [ ] หลังมือเย็น [ ] ก่อนนอน

❒ ใช้มือถู [ ] หลังตื่นนอนเช้า [ ] หลังมื้อเช้าเที่ยง [ ] หลังมื้อเที่ยง [ ] หลังมือเย็น [ ] ก่อนนอน

❒ ใช้แปรงสีฟันถู [ ] หลังตื่นนอนเช้า [ ] หลังมื้อเช้าเที่ยง [ ] หลังมื้อเที่ยง [ ] หลังมือเย็น [ ] ก่อนนอน

❒ อื่นๆ [ ] หลังตื่นนอนเช้า [ ] หลังมื้อเช้าเที่ยง [ ] หลังมื้อเที่ยง [ ] หลังมือเย็น [ ] ก่อนนอน

ระบุ.....

2) ปัจจุบันใช้ยาสีฟันมีฟลูออไรด์หรือไม่

❒ ไม่มีฟลูออไรด์ ระบุ.....

❒ มีฟลูออไรด์ ระบุ.....

3) ในรอบปีที่ผ่านมา เคยไปหา หมอฟัน (ทันตแพทย์/เจ้าพนักงานทันตสาธารณสุข) บ้างหรือไม่

❒ ไม่เคยไป

❒ เคยไป ระบุจำนวนครั้ง.....ครั้ง

❒ จำไม่ได้

Code

4) การทำความสะอาดช่องปากภายหลังใช้ยารักษาโรคหืดชนิดสูดพ่น

❒ ไม่ได้ทำอะไรเลย

❒ ทำความสะอาด (ระบุ .....)

ดัดแปลงจาก
:รายงานผลการสำรวจสภาวะสุขภาพช่องปากระดับประเทศ ครั้งที่ 7 ประเทศไทย พ.ศ. 2555 & Oral health survey basic method, 1994.

Code

<u>ส่วนที่ 4</u> พฤติกรรมการบริโภค

ข้อแนะนำการบันทึกแบบบันทึกการบริโภคอาหาร

1. บันทึกแบบบันทึกการบริโภคอาหารเป็นระยะเวลา 3 วันติดต่อกัน โดยวันที่บันทึกจะต้องมีทั้ง วันธรรมดาและวันหยุด เช่น วันพฤหัสบดี ศุกร์ และเสาร์ หรือ วันอาทิตย์ วันจันทร์ และวันอังคาร
2. บันทึกอาหารและเครื่องดื่มทุกประเภทที่บริโภคทั้งในมื้อและระหว่างมื้อ
3. บันทึกเวลาที่บริโภคอาหารและเครื่องอย่างชัดเจนทั้งในมื้อและระหว่างมื้อ
4. บันทึกปริมาณต่อหน่วยบริโภคอย่างชัดเจน
5. บันทึกเวลาที่เข้านอน

ตัวอย่างการบันทึกการบริโภคอาหาร

ผู้บันทึก นายไก่ ใจดี

วันที่บันทึก : วันที่1 วันศุกร์ 13 มกราคม 2554

เวลา	อาหารและเครื่องดื่ม	ปริมาณการรับประทาน
6.00-6.30	หมูปิ้ง	3 ไม้
	ข้าวเหนียว	1 ห่อ
	นมรสหวาน	1 กล่อง
10.00-10.15	น้ำชานมไข่มุก	1 แก้ว
11.30-12.30	ก๋วยเตี๋ยวหมูถั่วงอก	1 ถ้วย
	ขนมปัง 10 บาท	2 ชิ้น
15.30-16.00	ลูกอมซูกัส	5 เม็ด
	ลูกชิ้น ผักแตงกวา	2 ไม้
18.00-19.00	ไอศกรีมช็อคโกแลต	1 ถ้วยเล็ก
	น้ำโค้ก	1 ขวดเล็ก
20.30-21.30	ข้าวเปล่า	1 จาน
	ไข่เจียว	ครึ่งฟอง
	ต้มแซ่บเนื้อ	2 ทัพพี
	น้ำพริกกะปิ	1 ถ้วยเล็ก
	ผัดฟักทอง	1 ถ้วยเล็ก
23.00	เข้านอน	

แบบบันทึกการบริโภคอาหาร

ผู้บันทึก _____

Code

วันที่บันทึก : วันที่1 _____

เวลา	อาหารและเครื่องดื่ม	ปริมาณการรับประทาน

ส่วนเฉพาะผู้ทำวิจัย

ความถี่ของการบริโภคอาหารทั้งหมด (Food taken each per day)……..……,,,,,ครั้ง/วัน

- ความถี่ของการบริโภคอาหารระหว่างมื้อ (Between meal snacks per day)…… ครั้ง/วัน

- ความถี่ของการบริโภคน้ำตาล (Intakes of sugars/day)…...…… ครั้ง/วัน

- ประเภทของน้ำตาลที่บริโภค……

Code

แบบบันทึกการบริโภคอาหาร

ผู้บันทึก _____

วันที่บันทึก : วันที่2 _____

เวลา	อาหารและเครื่องดื่ม	ปริมาณการรับประทาน

ส่วนเฉพาะผู้ทำวิจัย

- ความถี่ของการบริโภคอาหารทั้งหมด (Food taken each day) …….……ครั้ง/วัน

- ความถี่ของการบริโภคอาหารระหว่างมื้อ (Between meal snacks per day) ..,…..…. ครั้ง/วัน

- ความถี่ของการบริโภคน้ำตาล (Intakes of sugars/day).…,,,…….. ครั้ง/วัน

- ประเภทของน้ำตาลที่บริโภค……

Code

แบบบันทึกการบริโภคอาหาร

ผู้บันทึก _____

วันที่บันทึก : วันที่3 _____

เวลา	อาหารและเครื่องดื่ม	ปริมาณการรับประทาน

- ความถี่ของการบริโภคอาหารทั้งหมด (Food taken each day)……..……ครั้ง/วัน

- ความถี่ของการบริโภคอาหารระหว่างมื้อ (Between meal snacks per day)……..… ครั้ง/วัน

- ความถี่ของการบริโภคน้ำตาล (Intakes of sugars/day)..…… ครั้ง/วัน

- ประเภทของน้ำตาลที่บริโภค……

ดัดแปลงจาก : Wongkamhaeng,K et al., Dental caries, cariogenic microorganisms and salivary properties of allergic rhinitis children, 2014.

Code

สรุปข้อมูล

ลักษณะการรับประทานอาหาร	Day 1	Day 2	Day 3	Average
ความถี่ของการบริโภคอาหารทั้งหมด (Food taken each day)				
ความถี่ของการบริโภคอาหารระหว่างมื้อ (Between meal snacks per day)				
ความถี่ของการบริโภคน้ำตาล (Intakes of sugars/day)				
ประเภทของน้ำตาลที่บริโภค				

Code

แบบบันทึกการตรวจช่องปาก

(Oral examination record)

Dentition: ❒ Primary dentition ❒ Mixed dentition ❒ Permanent dentition

Score	Tri-plaque disclosing score
0	No plaque
1	Plaque stained in red/pink (new plaque)
2	Plaque stained in blue/purple (old plaque)
3	Plaque stained in light blue (cariogenic bacteria)

Analysis

1. Tri-plaque disclosing score

Score	0	1	2	3
No. of teeth				

ดัดแปลงจาก :รายงานผลการสำรวจสภาวะสุขภาพช่องปากระดับประเทศ ครั้งที่ 7 ประเทศไทย พ.ศ. 2555 & Reliability and discriminatory power of methods for dental plaque quantification, 2010 & Preventive community dentistry,2006

Code

Research Questionannaire

To assess caries in patients with asthma agent, 3-color and expression of genes gtfB, spaP, gbpB, ldh,brpA, and luxS
Please answer the questions ✓page and mark the answer you choose.
Part 1:

1) Age ……
2) Sex ❒ male ❒ female
3) Income of the parents per month.
   - ❒ No income
   - ❒ Income 1-5000 baht
   - ❒ Income 5001-15000 baht
   - ❒ Income 15,001-30,000 baht.
   - ❒ Income 30,001-50,000 baht.
   - ❒ Income more than 50,001 baht.

4) **E**ducation (in case of patient who are younger than 18 years old, identify education of parent)
   - ❒ Never studied
   - ❒ Elementary Education
   - ❒ Junior high school education
   - ❒ Vocational course
   - ❒ Bacchelor
   - ❒ Diploma/Master degree
   - ❒ Postgraduate

5) Have congenital disease or systemic disease that the doctor specifies?
   - ❒ without ❒ indicate

Code

ส่วนที่ 2 ข้อมูลผู้ป่วยโรคหืด / Part 2 of the asthmatic patients.

1) ระยะเวลาการดาเนินโรค / The duration of the operation.……

2) ระดับการควบคุมโรค / the degree of control □□Controlled □□Partially □□Uncontrolled

3) โรคประจาตัวและโรคร่วมของผู้ป่วย / chronic diseases and comorbidities of the patient

□□โรคหืด / Asthma □□โรคหืด และโรคภูมิแพ้อากาศ / asthma and allergies

□□โรคหืด และโรคไซนัสอักเสบ / asthma and sinusitis □□อื่นๆ / Other.....

4) Drugs used to treat asthma today

Drugs used to control diseases (Controller medication)

✓	**Drug use**	**Dosage**	**How to use**	**frequency/times/day**	**terms of use**
	Corticosteroids				
	Long-acting β2 agonist (LABA)				
	ICS + LABA				
	Leukotriene modifier				

Relieve medications

✓	**Drug use**	**Dosage**	**How to use**	**frequency/times/day**	**terms of use**
	Short-acting β2 agonist				

Code

Other drugs

✓	Drug use	Dosage	How to use	frequency/times/ day	terms of use
	Antihistamine				
	Antibiotics				
	Others **1. ……** **2. ……** **3. ……**				

5) In the case of sprayers, use Spacer or not?

□ **Do not use**

□ **use**

6) Drug use

□ **Use medication more than 80%**

□ **Use medication less than 80%**

Code

Part 3 Dental health behavior

Regularly clean the mouth with any methods (Choose to answer only 1 routine) and when? (Can answer more than 1 question)

- ❒ Didn't do anything
- ❒ Rinse mouth [] after wake up in the morning [] after breakfast, lunch [] after lunch [] after dinner [] at bedtime.

- ❒ Hand rub [] Iwake up early [] after breakfast, lunch [] after lunch [] after dinner [] at bedtime.
- ❒ Use a toothbrush to scrub [] I wake up early [] after breakfast, lunch [] after lunch [] after dinner [] at bedtime.
- ❒ Other [] Iwake up early [] after breakfast, lunch [] after lunch [] after dinner [] at bedtime.

Identified .....

2) **Currently.** Did you use toothpaste with fluoride or not?
   - ❒ No fluoride identified. .....
   - ❒ With fluoride identified. .....

3) In the past year, have you ever been to a dentist (dentist / public health official)?
   - ❒ never
   - ❒ number of times……
   - ❒ I do not remember

4) Cleaning the mouth after using asthma medication inhaler type.
   - ❒ Do not do anything
   - ❒ Cleaning (specified ……)

Adapted from: Report of a national survey of oral health status of the 7th Thailand, 2012 & Oral health survey basic method, 1994.

Code

Part 4: Consumption Behavior

Suggestion on writing report on food consumption.

1) Record note on food consumption for 3 days continuously and days in report need to including both weekdays and weekend. Example Thursday-Friday-Saturday or Sunday-Monday-Tuesday.
2) Record every foods and beverages consumed both in and between meals.
3) Record time of consumption of foods and beverages clearly of both in and between meals.
4) Record amount per consumption unit clearly.
5) Record bed time as well.

Example of record on food consumption.

Recorder_____
Date of Recording: Day 1_____

Time	Foods and Beverages	Amount of Consumption

Code

Record Form of Food Consumption

Recorder_____

Date of Recording: Day 2_____

Time	Foods and Beverages	Amount of Consumption

This part is for Researcher only

- Frequency of food intake (Food taken each day)…….. times/day
- Frequency of food intake between meals (Between meal snack per day)……..times/day
- Frequency of sugar intake (Intake of sugar/day)…….times/day
- Type of sugar intake…….

Code

Record Form of Food Consumption

Recorder_____

Date of Recording: Day 3_____

Time	Foods and Beverages	Amount of Consumption

This part is for Researcher only

- Frequency of food intake (Food taken each day)…….. times/day
- Frequency of food intake between meals (Between meal snack per day)……..times/day
- Frequency of sugar intake (Intake of sugar/day)…….times/day
- Type of sugar intake…….

Code

Data summary

Eating characteristics	Day 1	Day 2	Day 3	Average
(Food taken each day)				
(Between meal snacks per day)				
(Intakes of sugars/day)				
The type of sugar consumed				

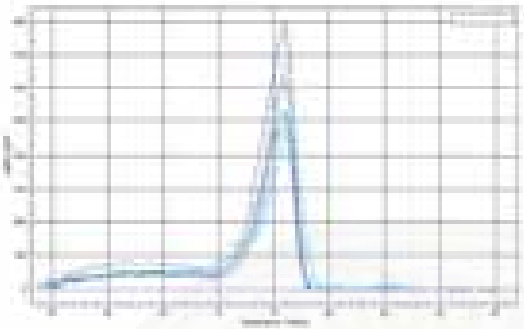
APPENDIX C
MELTING CURVE ANALISYS OF REAL TIME PCR

APPENDIX C
MELTING CURVE ANALISYS OF REAL TIME PCR

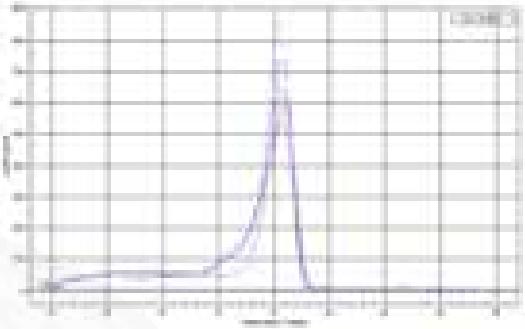
C.1. *spaP* gene

Melting curve *spaP* gene

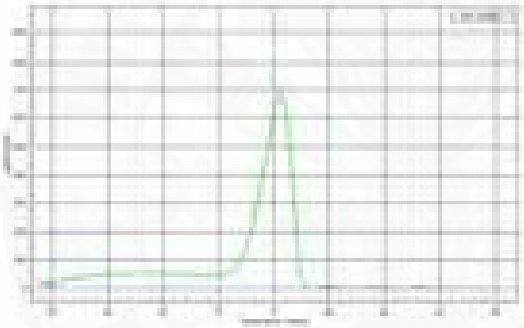
Asthma group



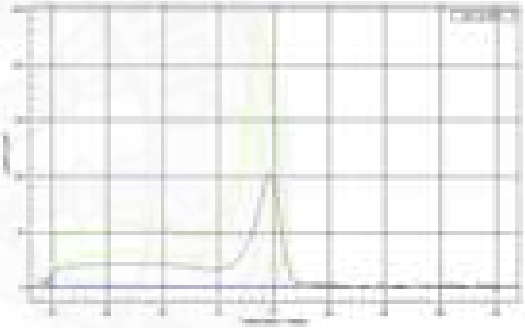
Healthy group



Control positive



Control negative

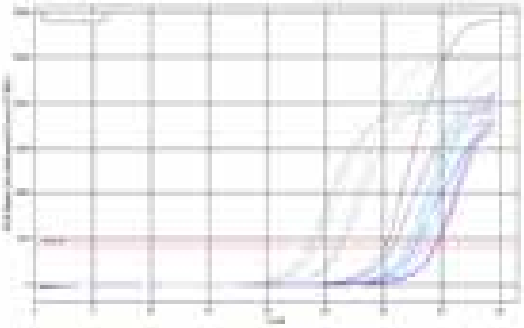


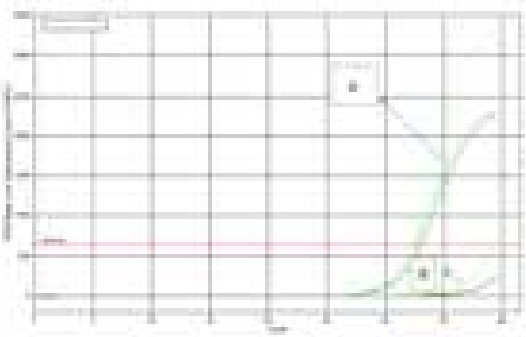
Treshold cycle *spaP* gene

A: Positive control

B: Negative control

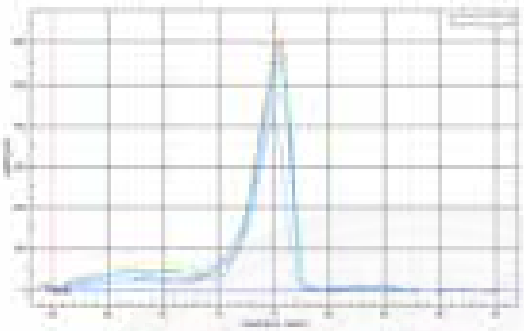
Tresholdcycle asthma group



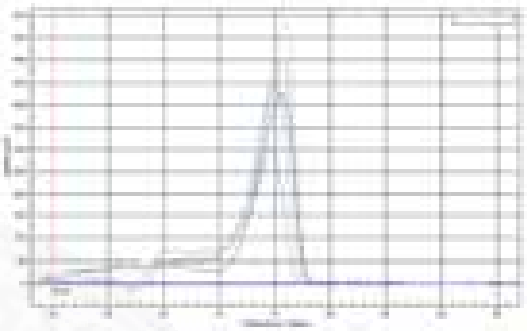


Melting curve *spaP* gene

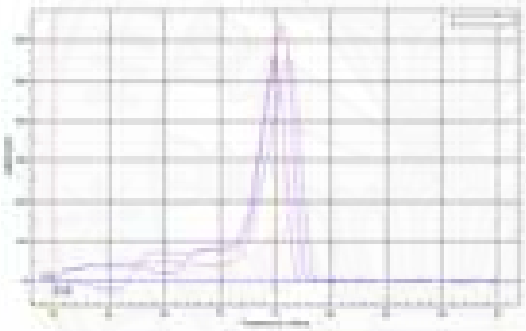
Asthma group



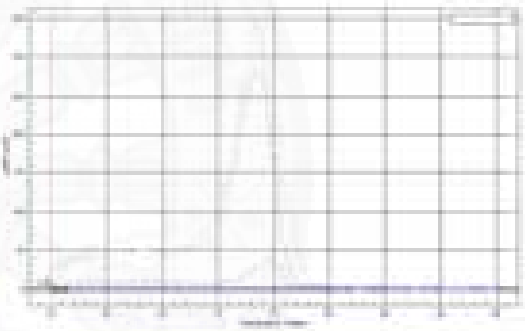
Healthy group



Control positive (*S. mutans*)

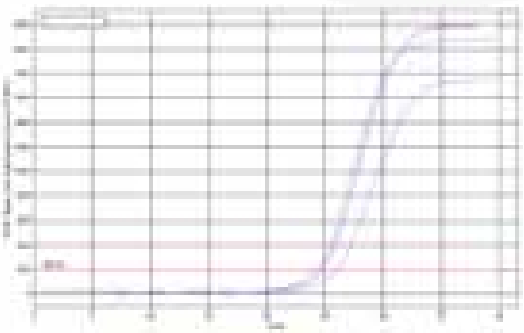


Control negative

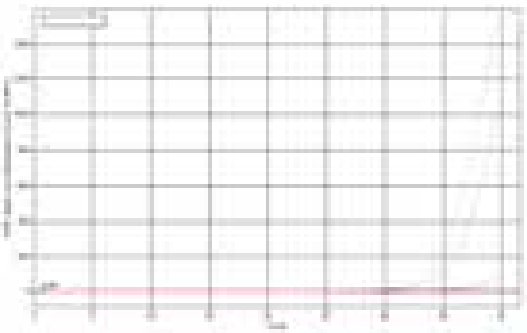


Treshold cycle *spaP* gene

Control positive



Control negative

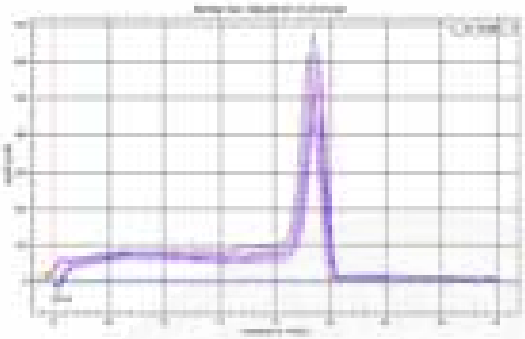


APPENDIX C
MELTING CURVE ANALISYS OF REAL TIME PCR

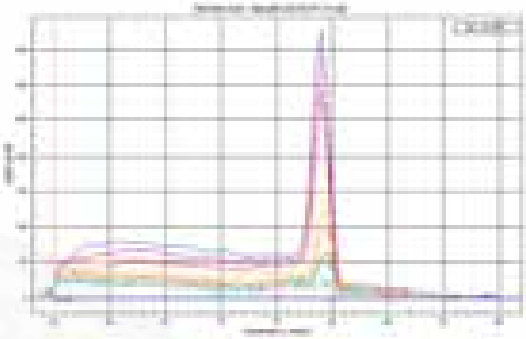
C.2. *gtf B* gene

Melting curve *gtfB* gene

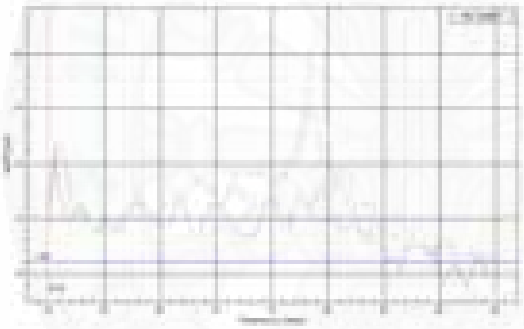
Asthma group



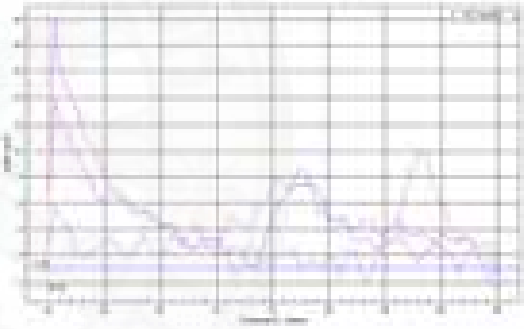
Healthy group



Negative control asthma

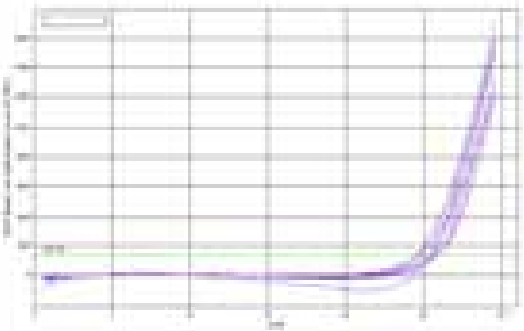


Negative control healthy

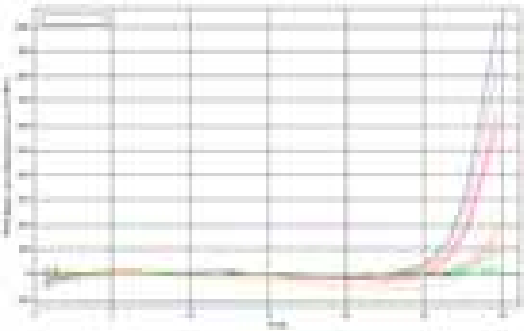


Treshold cycle *gtfB* gene

Asthma group



Healthy group

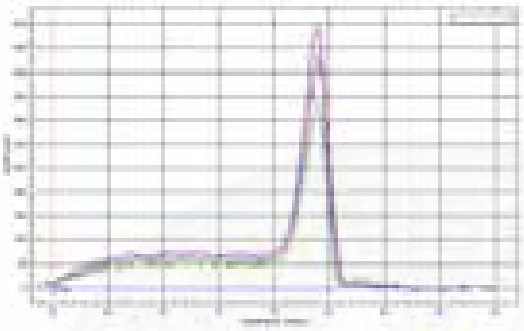


APPENDIX C
MELTING CURVE ANALISYS OF REAL TIME PCR

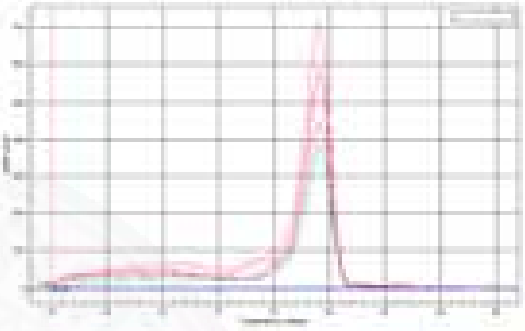
C.3. *gbp B gene*

Melting curve *gbpB* gene

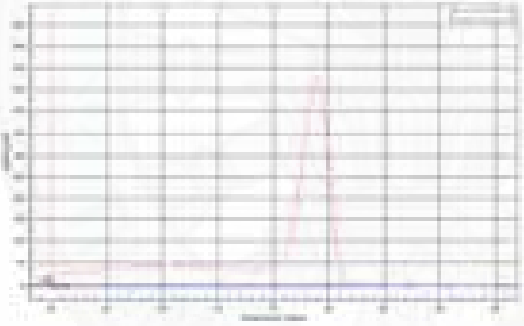
Asthma group



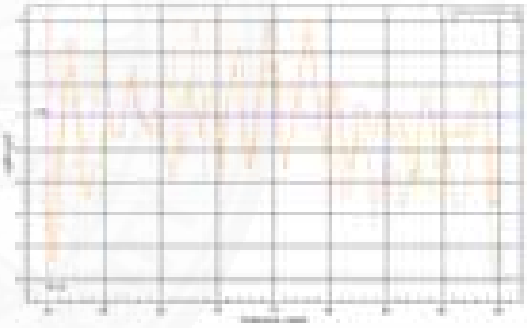
Healthy group



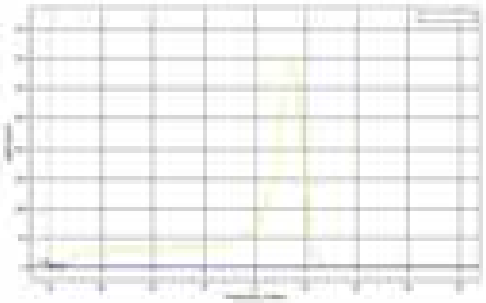
Control positive (*S. mutans*)



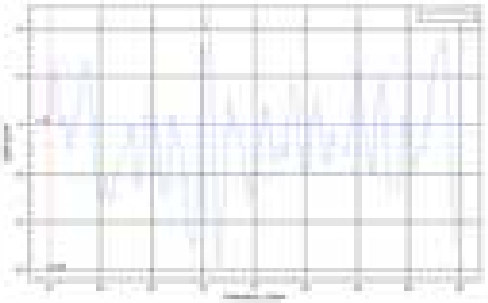
Control negative



Positive control

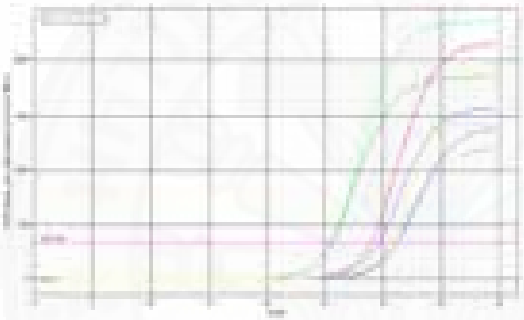


Negative control

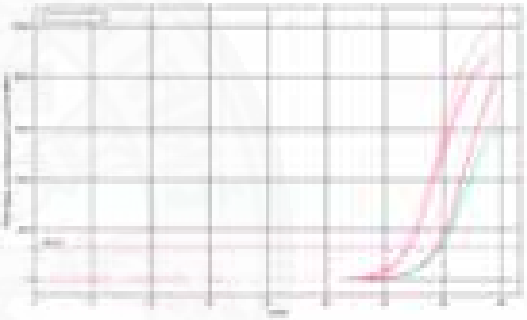


Treshold cycle *ghpB* gene

Asthma group



Healthy group

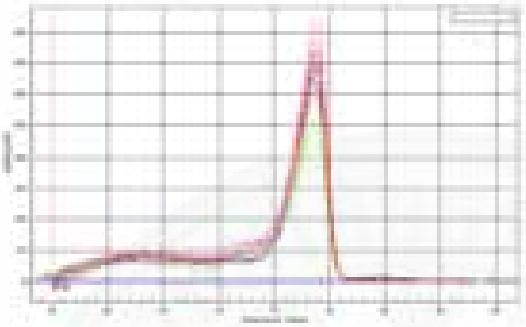


APPENDIX C
MELTING CURVE ANALISYS OF REAL TIME PCR

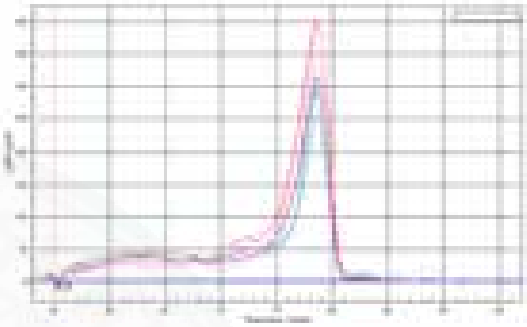
C.4. *ldh gene*

Melting curve *ldh* gene

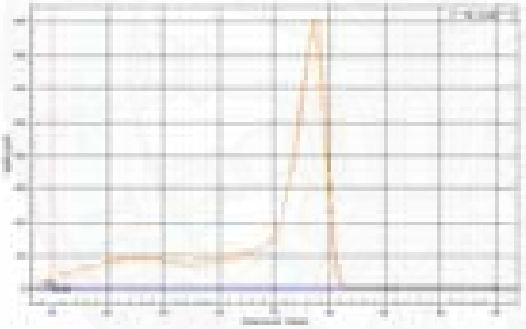
Asthma group



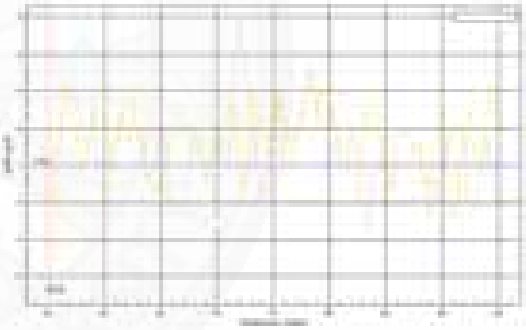
Healthy group



Positive control

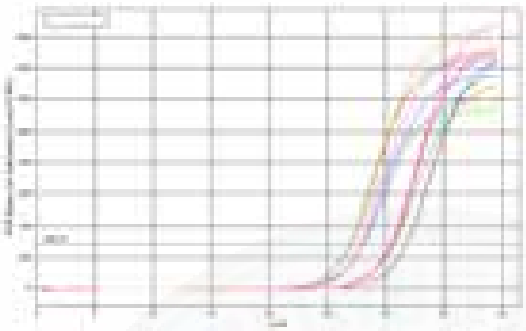


Negative control

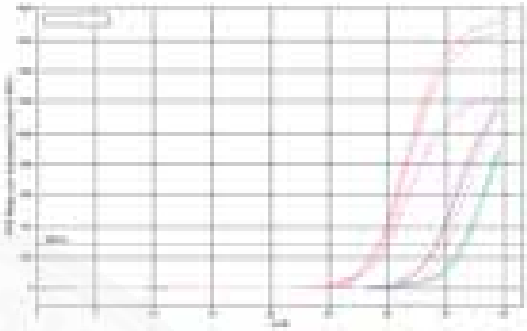


Tresholdcycle of *ldh* gene

asthma



control

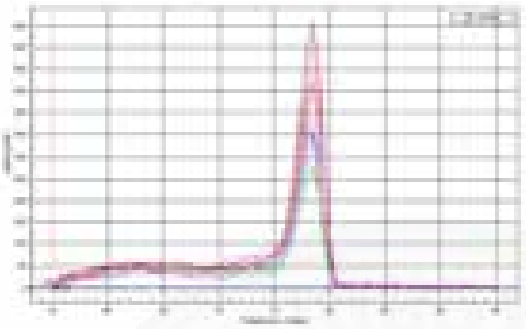


APPENDIX C
MELTING CURVE ANALISYS OF REAL TIME PCR

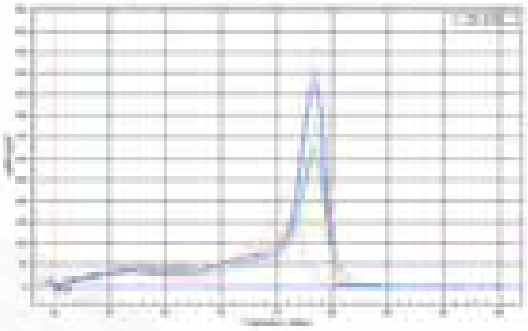
C.5. *brpA* gene

Melting curve *brpA* gene

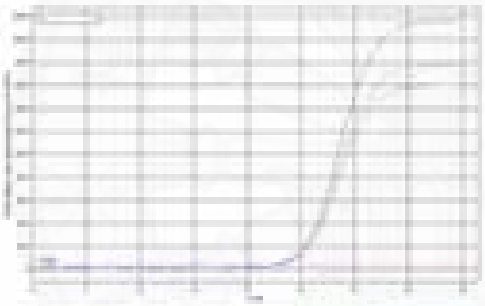
Asthma group



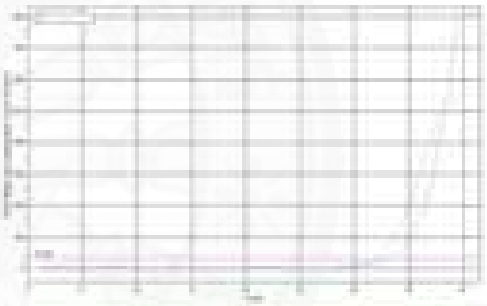
Healthy group



Positive control

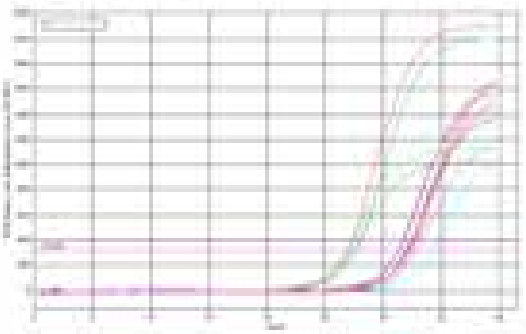


Negative control

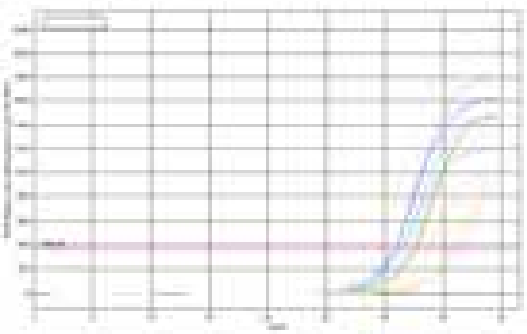


Tresholdcycle of *brpA* gene

Asthma group



Healthy group

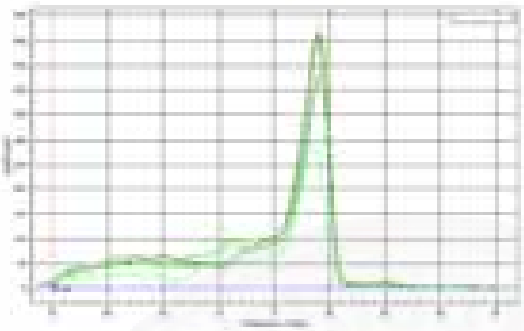


APPENDIX C
MELTING CURVE ANALISYS OF REAL TIME PCR

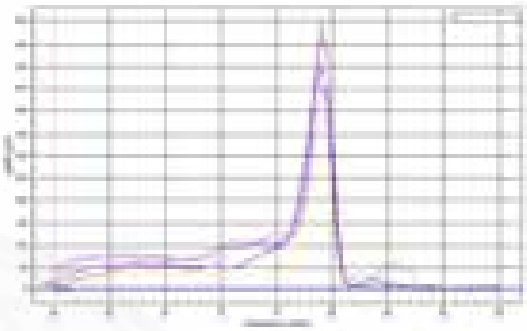
C.6. *luxS* gene

Melting curve *luxS* gene

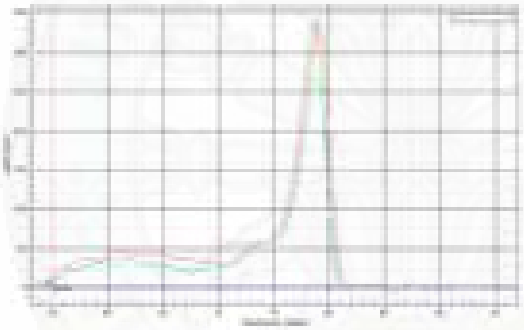
Asthma group



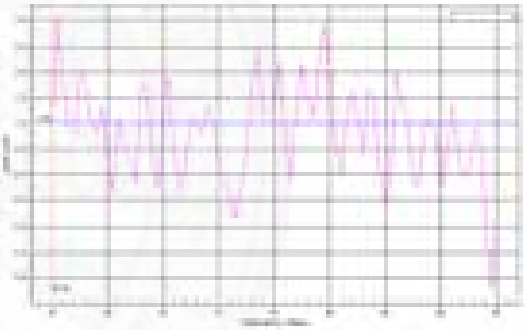
Healthy group



Control positive

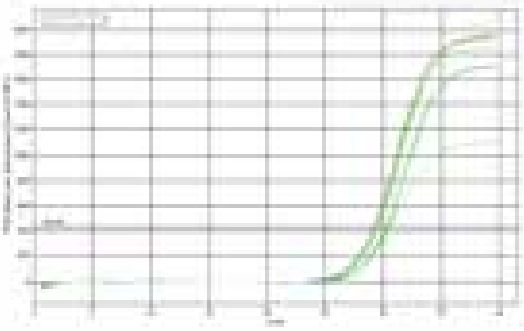


Control negative

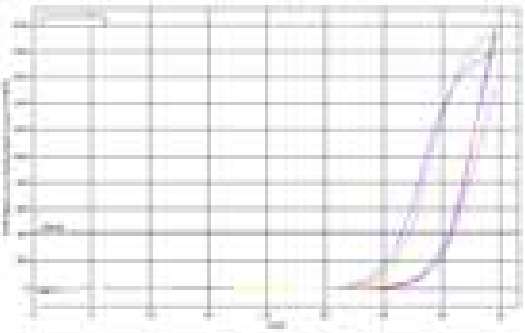


Treshold cycle *luxS* gene

Asthma group



Healthy group



BIOGRAPHY

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