

**FACTORS INVOLVED IN TRANSMISSION OF  
*STREPTOCOCCUS MUTANS***

**Ph.D. THESIS**

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**DEPARTMENT OF PEDIATRIC DENTISTRY  
GRADUATE SCHOOL OF BIOMEDICAL SCIENCES  
HIROSHIMA UNIVERSITY  
JAPAN  
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# **THESIS**

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## Preface

Caries is one of the problems which occur almost in every country included Indonesia and this disease mostly occur in children, with the prevalence of the caries in Indonesia for general population study is about 85 - 95 % and about 89% is in children. Caries could cause discomfort and also could impact in health development by interfering eating patterns, appetite and sleep. Mutans streptococci (MS), especially *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) has become the bacteria which are closely related to the incidence of caries. Some study stated that at first when children born are MS free, then being colonized by MS as they start to grow. Colonization related to transmission of MS is important event in the pathogenesis of these bacteria, in which mode of transmission could be intra familial or extra familial. Parents, particularly mother, is the major source of the transmission of MS alongside the fact that mother acted as the first contact and as the primary care giver for the children. Mother who share food, drinks, utensils and other items with their children have the highest risk of transmitting MS to their children. In addition, father may also transmit MS to his children. Furthermore, the day nursery is also a favorable environment for the transmission of MS, since the long duration time children stay and do activities together may also increased the risk of bacteria transfer other than within the family. Colonization of MS is associated to its factor in transmission, such as ability to produce bacteriocins which inhibit the growth of the other bacterial colony

genetically related or closely related species. Bacteriocin could support the colonization of the *S. mutans* producers which promote dental biofilm formation. Biofilm formation by *S. mutans* is essential for its ability to survive and thrive in competitive environment inside oral cavity. Biofilm can be formed through two ways of formation: sucrose independent adherence and sucrose dependent adherence. Sucrose independent adherence is interaction between adhesive particles in *S. mutans*, with the acquired enamel pellicle on the tooth surface that promote bacterial adherence. The sucrose dependent adherence of *S. mutans* is related to synthesis of glucans in enamel that provide avid site for colonization of these microorganisms. Glucans is synthesized by glucosyltransferase and *S. mutans* expresses three glucosyltransferase which are GTFB, GTFC and GTFD. The GTFB synthesize water insoluble glucans is encoded by *gtfB*, GTFC produces water soluble and water insoluble glucans is encoded by *gtfC*, and GTFD synthesizes water soluble is encoded by *gtfD*. The aims of this study are; to identify *S. mutans* from the current study place, to observe genotypes that might be related to the transmission of *S. mutans* and to examine factors that involved in the transmission of *S. mutans*.



## Introduction

Caries is one of the problems which occur almost in every country. Indonesia is one of the countries which also dealing with oral health problem related to the caries. Dental caries could cause discomfort and also could impact in health development by interfering eating patterns, appetite and sleep <sup>(1)</sup>. According to previous study, the prevalence of the caries in Indonesia for general population study is about 85 - 95 %. More over about 89% is in children <sup>(2)</sup>. The bacteria which mostly related to the incidence of caries are mutans streptococci (MS), in which predominantly are *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) <sup>(3)</sup>. Previous study suggested that the average age about 26 month, were the time children initially colonized by MS. Colonization related to transmission of MS is important event in the pathogenesis of this bacteria <sup>(4)(5)</sup>.

The possible modes of transmission of MS are intra-familial transmission between mother, father and child or extra-familial transmission <sup>(6) (7)</sup>. Previous studies suggested that parents, particularly mother, was the major source of the transmission of MS alongside the fact that mother acted as the first contact and as the primary care giver for the children <sup>(8)(1)</sup>. Mother who share food, drinks, utensils and other items with their children have the highest risk of transmitting MS to their children <sup>(4)</sup>. The day nursery is also a favorable environment for the transmission of MS <sup>(7)</sup>. In addition, another studies showed that father may also transmit MS to his children <sup>(9)(10)</sup>. The study found that, there are similar transmitted genotypes found

in children and their parents, nevertheless non-similar (not transmitted) genotypes are also found between children and their parents <sup>(9)</sup>.

Colonization of MS is associated to its factor in transmission, such as ability to produce bacteriocin which variant in *S. mutans* is mutacin. Mutacins are antimicrobial peptides that inhibit the growth of the other bacterial colony which are genetically related or closely related species. Mutacin has selective antagonism mechanism to other bacteria. The ability to produce broad spectrum of mutacins could promote the colonization of the *S. mutans* producers which in turn could promote dental biofilm, a great factor for dental caries. Theoretically, mutacin activity of *S. mutans* may facilitate the transmission between parents and child due to its selective antagonism mechanism <sup>(11)(12)</sup>.

Successful colonization of MS is also depending on the favorable environment to make biofilm formation. Biofilm formation by MS is essential for its ability to survive and thrive in competitive environment. There are two ways of biofilm formation: sucrose independent adherence and sucrose dependent adherence <sup>(13)</sup>. Sucrose independent adherence is interaction between adhesive particles in *S. mutans*, which is antigen I/II (also known as SpaP) with the acquired enamel pellicle on the tooth surface that promote bacterial adherence <sup>(14)</sup>. The sucrose dependent adherence of *S. mutans* is related to synthesize of glucans in enamel which would provide site for avid colonization by these microorganisms <sup>(15)</sup>. Glucan formation is catalyzed by an enzyme group glucosyltransferase (GTF). *S. mutans* expresses three glucosyltransferase, which are GTFB, GTFC and GTFD. GTFC will be absorbed to enamel within pellicle, along with GTFB which binds avidly to

bacteria, to promote tight cell clustering and enhancement of cohesion plaque, and GTFD will form a soluble polysaccharide and become primer for GTFB<sup>(15)</sup>. GTFB synthesizes water insoluble glucans and encoded by gene *gtfB*. GTFC produces water soluble and water insoluble glucans which is encoded by *gtfC*. GTFD which synthesizes water soluble glucans is encoded by *gtfD*<sup>(16)(17)</sup>.

The aims of this study are to identify *S. mutans* from current study place, to observe genotype that might be related to the transmission of *S. mutans* and to examine factors that involved in the transmission of *S. mutans*. In this study, factors involved in transmission of *S. mutans* genotypes examined by using bacteriocin assay, biofilm formation assay and mRNA expression from *gtf* genes. Hypothesis in this study is that there is transmission in genotypes of *S. mutans*, and there are differences in factors involved in transmission, which are bacteriocin activity, biofilm formation and also the adherence ability.

## **Chapter 1**

### **Detection of mutans streptococci and genotypes of *S. mutans***

Children who harbor MS may have risk to suffer from dental caries, therefore it is importance to detect the presence of *S. mutans* and *S. sobrinus*. PCR methods provide a more sensitive detection for MS compared with conventional cultural techniques. The acquisition of MS is closely related to the transmission of these bacteria. The route of transmission from *S. mutans* is able to reveal by using restriction endonuclease analysis.

#### **1.1 Experiment 1-1. Detection of *S.mutans* and *S. sobrinus* (PCR)**

##### **1.1.1 Materials and methods**

###### **1.1.1.1 Subjects**

Thirty seven children (18 boys and 19 girls) aged from 1 to 6 years old whose at the day nursery in Hiroshima University were examined. Written consent of participation in this study was collected from their parents.

###### **1.1.1.2 Specimen collection**

Dental plaque was collected by brushing all of the erupted teeth with sterile toothbrush. Plaques which were adhered to the toothbrush were removed by washing two times in sterile tube contains sterile distilled water. The plaque samples were immediately transported to the laboratory for DNA extraction.

### **1.1.1.3 Preparation of chromosomal DNA**

The chromosomal DNA was prepared according to standard mini prep method <sup>(18)</sup> with some modification as followed. Plaque sample in distilled water were centrifuged 7.000 rpm for 5 min. until a pellet formed and the supernatant was discarded. Pellet was then re-suspended in 567 µl of TE buffer and all transferred to the new micro centrifuge tube added with glass beads 0.150 g. After addition of 30 µl of 10% SDS (Nacalai Tesque Inc., Kyoto, Japan), 3 µl of 20 mg/ml proteinase K (Nacalai Tesque Inc., Kyoto, Japan) was added and specimens were put in vortex for 3 min. followed with incubation at 37°C (block incubator B1-516 S, ASTEC Co., Fukuoka, Japan) for 1 hrs. Then, 100 µl 5 M NaCl was added followed by addition of 80 µl hexadecyltrimethyl-amonium bromide (CTAB) (Nacalai Tesque Inc., Kyoto, Japan) and incubated at 65°C for 10 min. Next, 0.7 ml of Chloroform/ isoamyl alcohol (24:1) was added and the solution was mixed thoroughly. After 13.000 rpm of centrifugation for 5 min., supernatant was collected and equal volume of phenol/ chloroform/ isoamyl alcohol 25:24:1 (Nacalai Tesque Inc., Kyoto, Japan) was added. After that, a centrifugation at 13.000 rpm for 5 min. was performed again. The supernatant was then transferred to the new micro centrifuge tube, with 0.6 ml isopropanol (Nacalai Tesque Inc., Kyoto, Japan) was added, and stored in at -30°C overnight. The following day, the suspension was centrifuged at 13.000 rpm for 5 min. The precipitate was added 0.7 ml 70% ethanol (Nacalai Tesque Inc., Kyoto, Japan) followed with centrifugation at 13.000 rpm for 5 min. The supernatant was discarded and pellet was dried for 1 hrs. 30 min. at room temperature. The precipitate containing DNA was suspended in 30 µl TE buffer and stored in -30°C.

#### 1.1.1.4 Detection of *S. mutans* and *S. sobrinus* by PCR

*S. mutans* and *S. sobrinus* were used as controls. PCR was performed using primers as described by Igarashii *et al.* <sup>(19)</sup> <sup>(20)</sup>. Primer for *S. mutans* strain Ingbritt (SD1 and SD2) specifically amplified a 1272-bp fragment sequences were F: 5' - TAT GCT GCT ATT GGA GGT TC - 3' (position 973 to 992) and R: 5' - AAG GTT GAG CAA TTG AAT CG - 3' (position 2225 to 2244) respectively. Primer for *S. sobrinus* UAB66 (SOF14 and SOR1623) amplified a 1610-bp fragment sequences were F: 5' - TGC TAT CTT TCC CTA GCA TG - 3' (position 134-153) and R: 5' - GGT ATT CGG TTT GAC TGC - 3' respectively. Amplification of PCR was performed in 25 µl reaction mixtures (Biotaq DNA polymerase, Bioline, Tauton, Massachusetts, USA) the reaction mixture contains 2.5 µl 10x NH<sub>4</sub> Buffer, 1 µl 50 mM MgCl<sub>2</sub>, 2.5 µl DNTP, 2.5 µl Taq polymerase, 0.5 µl of appropriate primers, 16.75 µl dH<sub>2</sub>O, 1 µl DNA sample in iCycler Thermal cycler (Bio-rad iCycler, Berkeley, California, USA). Positive and negative controls were used to minimize the impact of false positives. The thermal cycle parameters were performed at 94°C for 2 min., 30 cycles of 94°C for 20 sec., 50°C for 30 sec., 72°C for 90 sec. PCR reactions ended with final elongation step at 72°C for 5 min. and 4°C until usage. PCR products were separated on 1 % agarose gel (Type III, Sigma, Sigma Chemical Co, St. Louis, Missouri, USA) electrophoresis in Tris-borate-EDTA (TBE), stained with ethidium bromide (EtBr).

#### 1.1.2 Result

From the detection of *S. mutans* and *S. sobrinus* in this research it was found that there were children who harbor *S. mutans*, *S. sobrinus* and both *S. mutans*

and *S. sobrinus*. Prevalence of these bacteria in age of groups was shown in table.1. From the result it was suggested that most of the children in this population of study harbor for *S. sobrinus* only (70.3%), then followed by harboring both, *S. mutans* and *S. sobrinus* about 27% of the population study. There were 2.7% children from this population of study who harbor *S. mutans* only. Moreover, none children of this population study free from *S. mutans* and *S. sobrinus*. The children aged below 3 until 4 years old harbored whether *S. sobrinus* only or both *S. mutans* and *S. sobrinus*. None of the children harbor *S. mutans* only. On the other hand, children from 5 years old above showed that there were children who harbor *S. mutans* only, *S. sobrinus* only and also both *S. mutans* and *S. sobrinus*.

### **1.1.3 Summary**

The children were positively detected *S. mutans* only in 1 (2.7%) child, *S. mutans* and *S. sobrinus* in 10 (27%) children, and *S. sobrinus* only in 26 (70.3%) children.

## **1.2 Experiment 1-2. *S. mutans* isolation and DNA fingerprinting from children and parents (Restriction endonuclease analysis)**

### **1.2.1 Materials and methods**

#### **1.2.1.1 Specimen collection**

The plaque was collected from children and parents by swabbing all surfaces of the erupted teeth with sterile cotton swab stick. The swab stick then

washed in sterile tube containing 1 ml of phosphate buffered saline (PBS) <sup>(21)</sup>. Furthermore, the plaque samples were immediately transported to the laboratory for isolation of strains.

#### **1.2.1.2 Isolation of strains**

The plaque samples were serial diluted in PBS, 100 µl of suspension was inoculated in to the Modified mitis-salivarius-bacitracin (MSB) agar <sup>(22)</sup> and incubated anaerobic ally at 37°C for 48 hrs. in a CO<sub>2</sub> Gas Pack system (AnaeroPack. Anaero, Mitsubishi Gas Chemical Co., Inc, Tokyo, Japan). Presumptive colonies of *S. mutans* were picked from modified MSB. Each colony was inoculated to 5 ml of BHI broth (Difco, Sparks, Maryland, USA) and incubated 37°C for 24 hrs. This process was repeated to obtain isolate colony and eventually identified as *S. mutans* by PCR detection.

#### **1.2.1.3 Preparation of chromosomal DNA for digestion**

The chromosomal DNA for digestion was prepared according to Kozai *et al.* <sup>(9)</sup>. A 0.5 ml overnight culture of each strain of *S. mutans* was inoculated into 4.5 ml BHI broth (Difco, Sparks, Maryland, USA) and incubated 37°C. As growth reached the optimal density 0.3 to 0.5 at 550 nm measured by a spectrophotometer (Spectronic 21, Milton Roy Co., Rochester, Minnesota, USA) 0.25 g of glycine (Sigma Chemical Co., St. Louis, Missouri, USA) added to the culture and incubated at 37°C for 45 min. The *S. mutans* cells were centrifuged at 7.000 rpm for 5 min. The cell pellets were washed with 0.75 ml of 0.1M Tris-HCl (pH 8.0) (Trizma base, Sigma Chemical Co., St. Louis, Missouri, USA) in eppendorf tube, then centrifuged at 15.000 rpm for 10 min. Then, continued with preparation of chromosomal DNA.



The chromosomal DNA was prepared according to standard mini preparation method <sup>(18)</sup> with some modification as mentioned before.

#### **1.2.1.4 Digestion and electrophoresis for fingerprinting**

Digestion was performed according to Kozai *et al.* <sup>(9)</sup> by using *EcoRI* restriction enzyme with some modification as follow: 12 µl of the DNA solution and 8 µl of incubation buffer (3 µl dH<sub>2</sub>O, 2 µl 10x buffer Toyobo Co., Osaka, Japan, and 3 µl of *EcoRI* (Toyobo Co., Osaka, Japan)) were mixed and incubated at 37°C in Dry Thermo Unit (DTU-28, TAITEC Co., Tokyo, Japan) for 6 hrs. In order to verify the fingerprint precisely, *HindIII* (Toyobo Co., Osaka, Japan) was also used. Four microliters of 6x loading dye (BPB, OrG Toyobo Co., Osaka, Japan) was added to the sample. The tube was then incubated at 55°C (*EcoRI*) or 70°C (*HindIII*) for 5 min. for enzyme denaturation.

Electrophoresis was carried out on 0.7% agarose gel (Type III, Sigma, Sigma Chemical Co, St. Louis, Missouri, USA) in Tris-borate-EDTA (TBE) buffer at 40 volt for 15 hrs. at room temperature with a submarine apparatus (gel size 15x18 cm, 16 wells, Nihon Eido Co., Tokyo, Japan). The gel was stained with EtBr (0.5 µg/ml) for 30 min. and de-stained for 15 min. in ultra pure water (Millipore Direct-Q, Merck Millipore., Billerica, Massachusetts, USA). Detection of band was taken under UV transilluminator (Bio-rad Molecular Imager Gel Doc., Bio-Rad Laboratories., Berkeley, California, USA). Fingerprints were visually compared and were considered as unmatched if there were no pairs of identical patterns. Six micro liters of 6x loading dye (BPB, OrG Toyobo Co., Osaka, Japan) and  $\lambda$  /Hind III (Toyobo Co., Osaka, Japan) were used as a marker.

### **1.2.2 Result**

From the study there were 26 genotypes from 27 subjects of population study. Furthermore, genotypes that harbored from individual and families shown in table 2.

There were up to 3 number of genotypes harbor in individual, in which 21 subjects has one genotype (77.8%). Number of genotypes that harbor by family showed that in one family may harbor 2 to 5 genotypes, whereas most of the families in this population of study harbor 4 genotypes (37.5%). All of children in this population study harbour only one genotype. From the fingerprinting pattern analysis (Fig.11) it was found that there were 3 families which showed similar fingerprint patterns between children and parents, which were in family No.1, 4 and 6. Furthermore, the similar fingerprint patterns between children from the same family were found in children family No. 4 and also in other different families, which were shown in child from family No. 2 with child from family No. 7. Similar fingerprint patterns between parents from the same family were found in 2 families, which were in family No. 1 and No. 8.

### **1.2.3 Summary**

Fingerprint analysis showed that there were transmitted genotypes between parents and children, between children them self and also between parents them self from the same family. In this study it was found 8 transmitted and 18 non-transmitted *S. mutans* genotypes.

## Tables Chapter 1

Table.1 Prevalence of *S. mutans* and *S. sobrinus* in age of groups.

<i>S. m</i>	<i>S. s</i>	Age				Total
		< 3	3 ≤ age < 5	≥ 5		
+	+	1	5	4	10	27.0%
+	-	0	0	1	1	2.7%
-	+	1	14	11	26	70.3%
-	-	0	0	0	0	0.0%
Total					37	100.0%

Table.2 Number of genotypes *S. mutans* harbored by individual and families

	Number of genotypes	Number of individual (%)	Number of genotypes	Number of families (%)
	1	21 (77.8)	2	2 (25.0)
	2	5 (18.5)	3	2 (25.0)
	3	1 (3.7)	4	3 (37.5)
			5	1 (12.5)
<b>Total</b>		27 (100.0)		8 (100.0)

## Figures Chapter 1

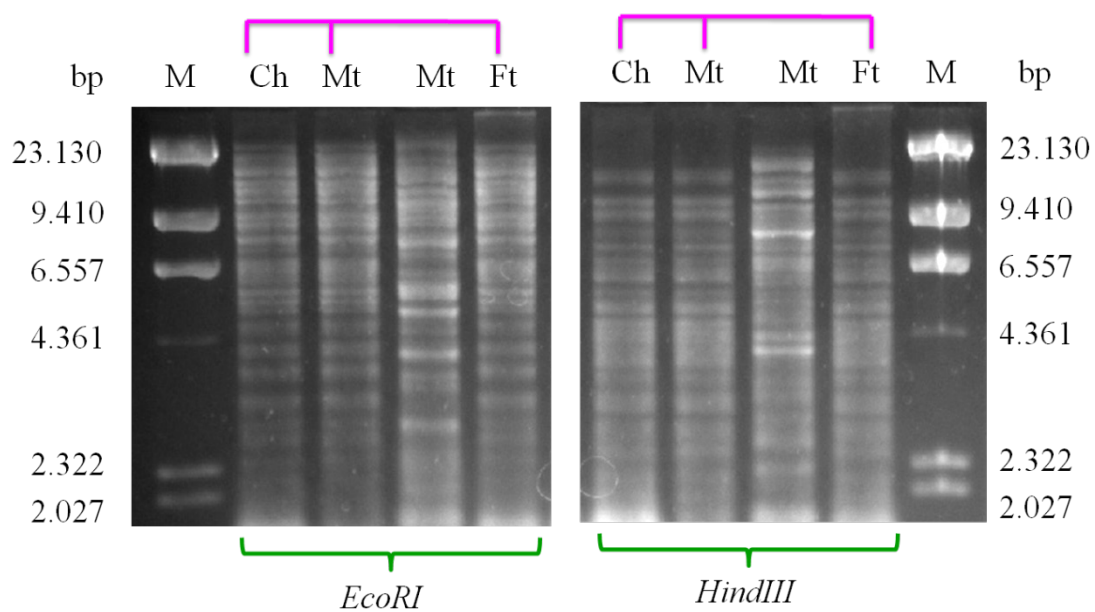


Fig.1. DNA fingerprint analysis by electrophoresis from family No.1.

Abbreviation : bp = Basepair, M = Marker, Ch = Child, Mt = Mother, Ft = Father.

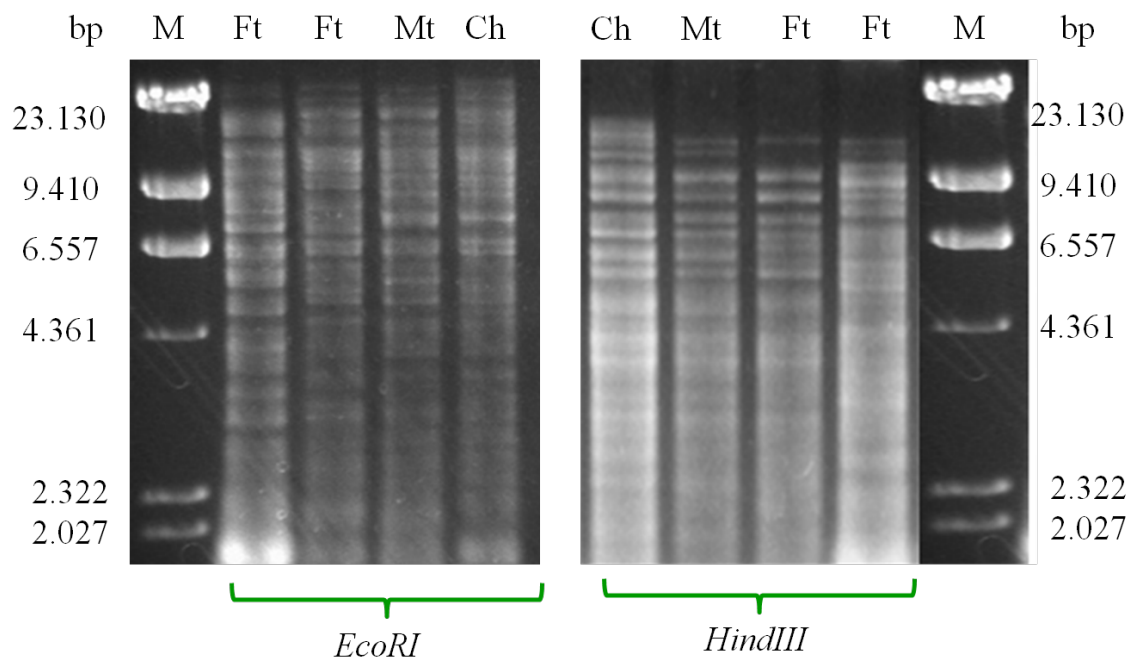


Fig.2. DNA fingerprint analysis by electrophoresis from family No.2.

Abbreviation : bp = Basepair, M = Marker, Ch = Child, Mt = Mother,

Ft = Father.

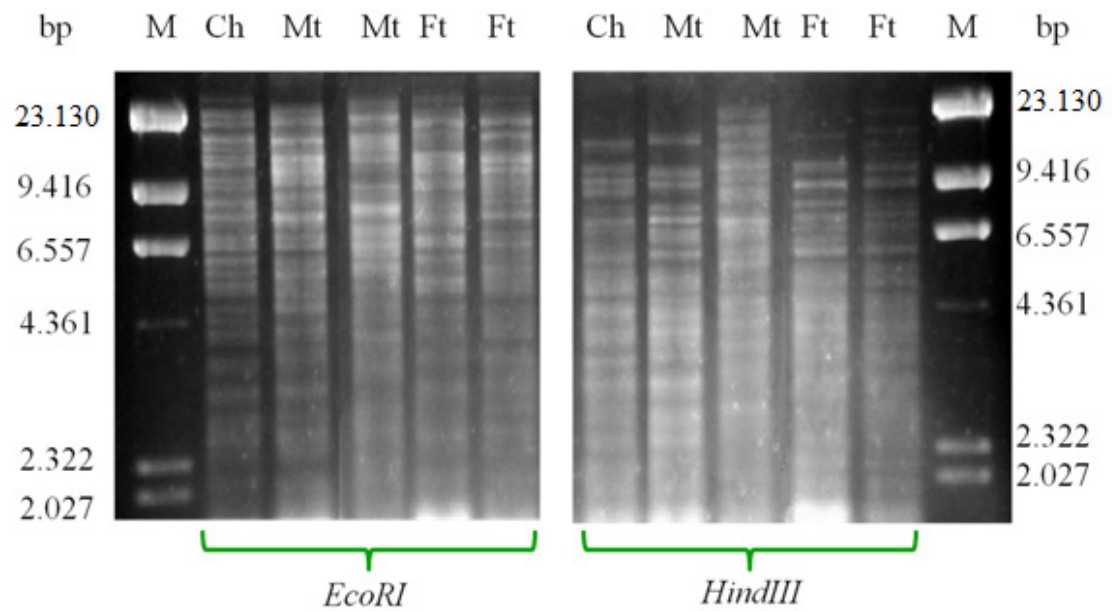


Fig.3. DNA fingerprint analysis by electrophoresis from family No.3.

Abbreviation : bp = Basepair, M = Marker, Ch = Child, Mt = Mother, Ft = Father.

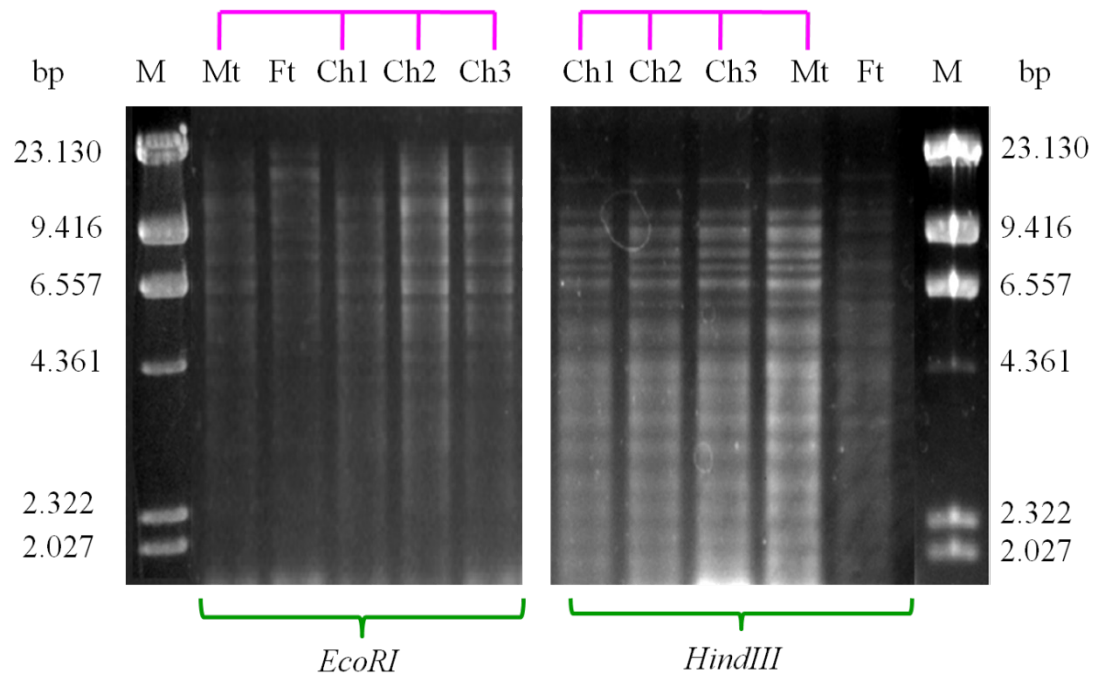


Fig.4. DNA fingerprint analysis by electrophoresis from family No.4.

Abbreviation : bp = Basepair, M = Marker, Ch = Child, Mt = Mother,

Ft = Father.

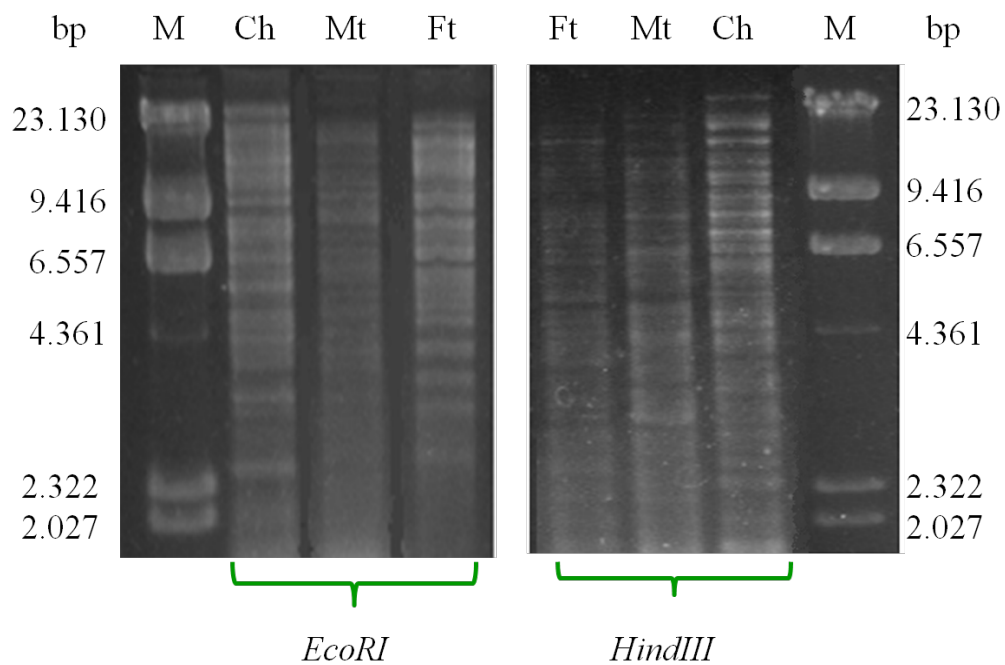


Fig.5. DNA fingerprint analysis by electrophoresis from family No.5.

Abbreviation : bp = Basepair, M = Marker, Ch = Child, Mt = Mother, Ft = Father.



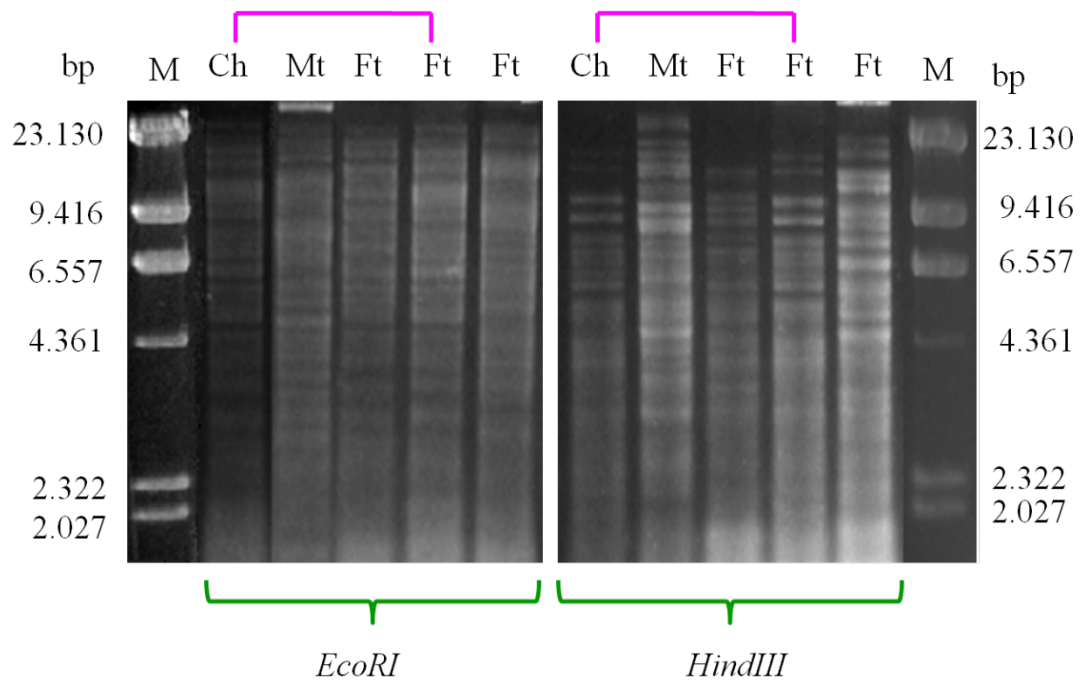


Fig.6. DNA fingerprint analysis by electrophoresis from family No.6.

Abbreviation : bp = Basepair, M = Marker, Ch = Child, Mt = Mother,

Ft = Father.

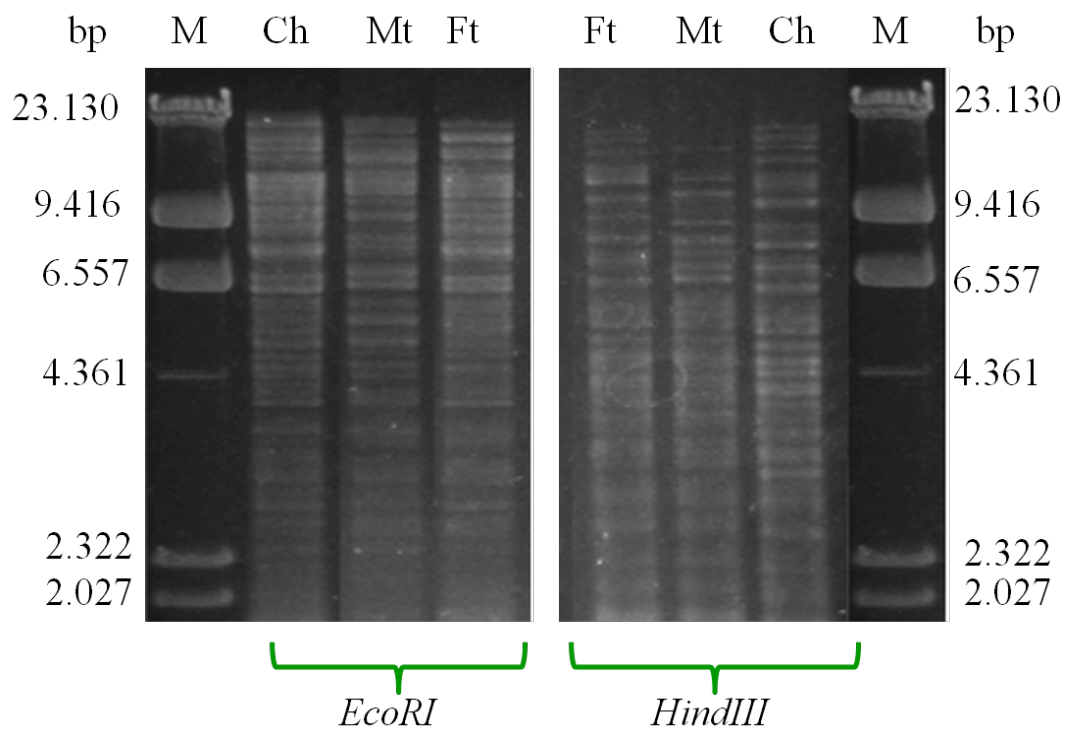


Fig.7. DNA fingerprint analysis by electrophoresis from family No.7.

Abbreviation : bp = Basepair, M = Marker, Ch = Child, Mt = Mother,  
Ft = Father.

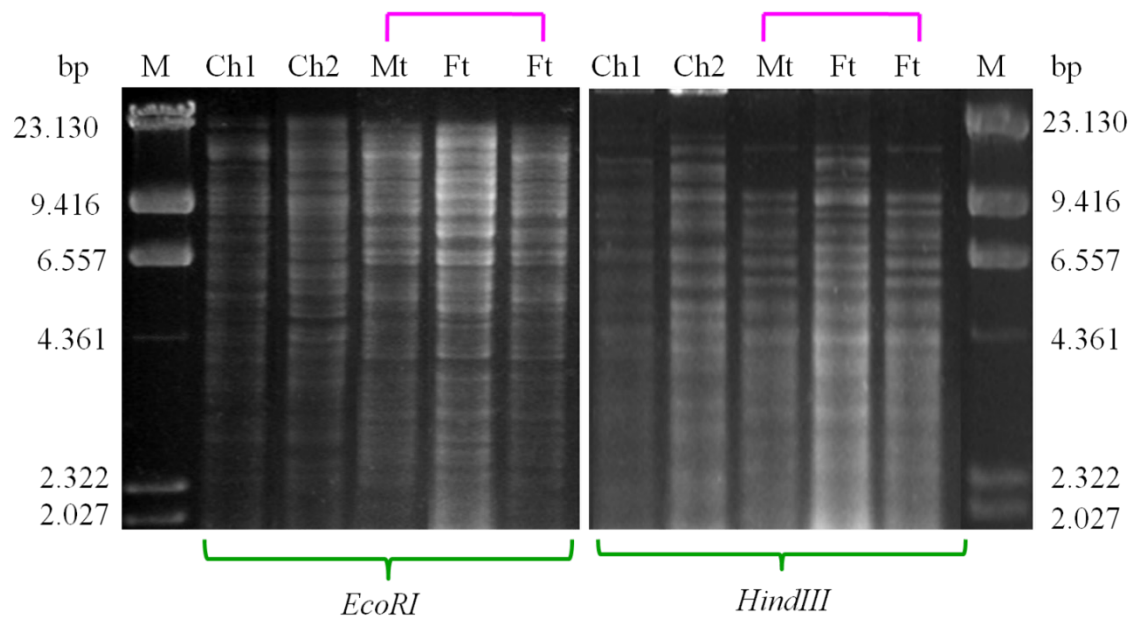


Fig.8. DNA fingerprint analysis by electrophoresis from family No.8.

Abbreviation : bp = Basepair, M = Marker, Ch = Child, Mt = Mother, Ft = Father.

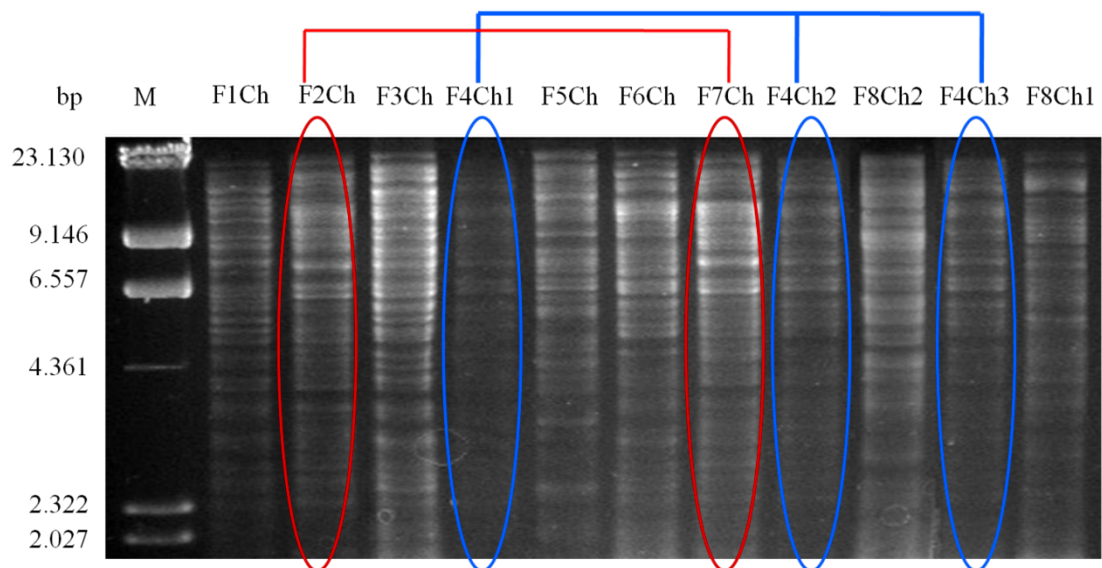


Fig.9. Fingerprint patterns between children using *EcoRI*.

Abbreviation : bp = Basepair, M = Marker, F = Family, Ch = Child

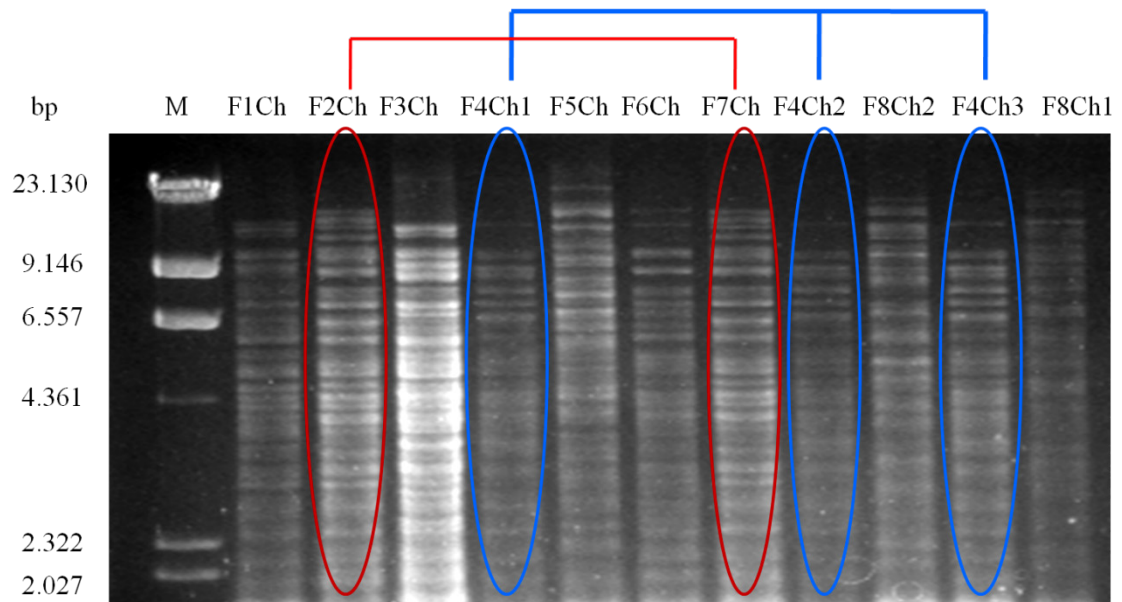


Fig.10. Fingerprint patterns between children using *HindIII*.

Abbreviation : bp = Basepair, M = Marker, F = Family, Ch = Child

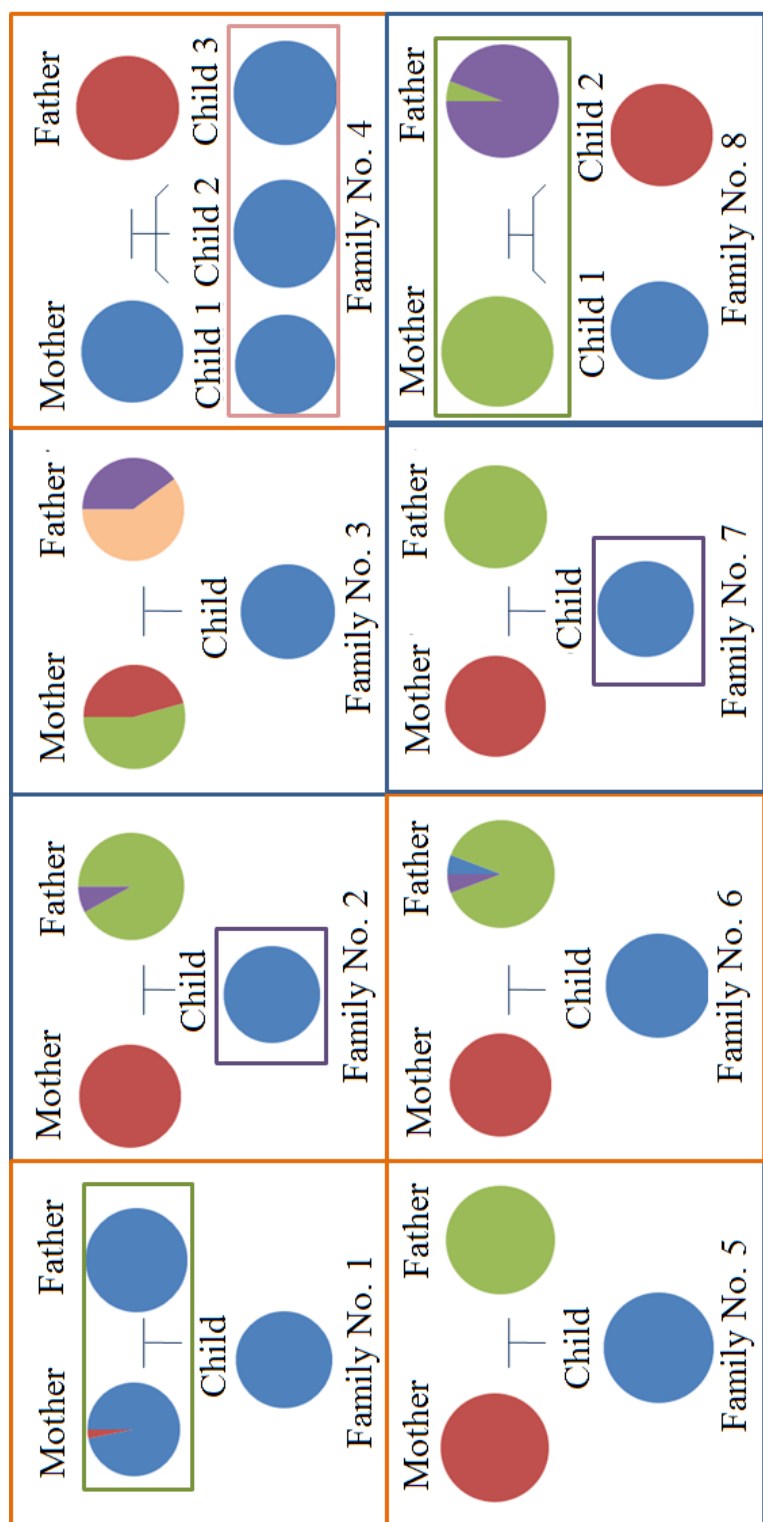


Fig. 11 Genotypes from all of the subjects in the families

## Chapter 2

### Factors involved in transmission of *S. mutans*

Transmission of cariogenic bacteria *S. mutans* may cause the risk in dental caries. These bacteria were transmitted via saliva and promote their colonization to persist in oral cavity. The existence of these bacteria in oral cavity might relate to the factors that involved in transmission such as : bacteriocin activity, biofilm formation and adherence ability.

#### 2.1 Experiment 2-1. Bacteriocin activity assay (Agar diffusion)

##### 2.1.1 Materials and methods

Bacteriocin activity profiles were carried out according to Khalid *et al.* <sup>(23)</sup> and van Lovere *et al.* <sup>(24)</sup> by agar well diffusion assay with some modifications. From each of transmitted and non-transmitted genotypes, 6 genotypes were randomly selected and inoculated to 5 ml TS (Tryptic soy, BD, Sparks, Maryland, USA) then incubated for overnight at 37°C after pre cultured with BHI. Prepoured 2% TSA (Trypticase soy agar, Difco, Detroit, Michigan, USA) plates were overlaid with 4.5 ml of 0.8% TSA (Difco, Detroit, Michigan, USA) containing 0.5 ml of *S. mutans* UA 159 as indicator cultures. Wells was performed by using multipoint inoculators placed in pre poured TSA (Difco, Detroit, Michigan, USA) plates and 100 µl of the specimen strains were placed into each well. Additional incubation for overnight at 37°C was performed, followed by measurement of the inhibition zone.

### **2.1.2 Statistic analysis**

Data were statistic analysis using Student's *t*-test to evaluate the difference bacteriocin inhibition zone between the transmitted genotypes with non-transmitted genotypes. Data was performed as mean  $\pm$  Standard Deviation.

### **2.1.3 Result**

The bacteriocin activity assay from the transmitted genotypes and the non-transmitted genotypes is shown in fig.13. The figure showed inhibition zone produced against the indicator strains. The inhibition zones from the transmitted genotypes were range 14.5 – 16.5 mm ( $15.2 \pm 0.7$ ), while for the non-transmitted genotypes were range 15 – 18.5 mm ( $15.8 \pm 1.3$ ).

### **2.1.4 Summary**

The inhibition zone produce showed no significant differences between transmitted and non-transmitted genotypes. However, mother of family No.1 from the non-transmitted genotypes in this study showed the biggest inhibition zone (Fig.13).

## **2.2 Experiment 2-2. Biofilm formation assay**

### **2.2.1 Materials and methods**

Biofilm formation assay was performed according to Bedran *et al* <sup>(25)</sup> with modification as followed : an 18 hours. BHI (Difco, Sparks, Maryland, USA) pre cultured *S. mutans* was diluted in to the new BHI (Difco, Sparks, Maryland, USA)



which contained 2% sucrose (Nacalai Tesque Inc., Kyoto, Japan). Samples (200  $\mu$ l) were added to the wells of a 96-well polystyrene tissue culture plate. After 2 days incubation at 37°C, culture medium and the free floating bacteria were then removed by aspiration, the wells were washed twice with distilled water, and the biofilms were stained with 50  $\mu$ l 0.1% crystal violet dye for 15 min. at room temperature. The wells were washed three times with distilled water to remove the unbound crystal violet dye and then they were dried for a few minutes at room temperature. After that, 200  $\mu$ l 99% ethanol (Nacalai Tesque Inc., Kyoto, Japan) added to each well and after 2 hrs. liquid was transferred to the new plate. Then, an absorbance at 590 nm was determined to quantify biofilm formation.

### **2.2.2 Statistic analysis**

Data were statistic analysis using *Student t-test* to evaluate the difference biofilm formation between the transmitted genotypes with non-transmitted genotypes. Data was performed as mean  $\pm$  Standard Deviation.

### **2.2.3 Result**

The biofilm formation analysis is shown in fig.14. Figure 14 showed that there were biofilm formation differences between transmitted and non-transmitted genotypes of *S. mutans*. The biofilm formation from the transmitted genotypes were range 0.82 – 1.89 O.D. ( $1.58 \pm 0.4$ ) at 590 nm, while the non-transmitted genotypes were range 0.02 – 1.80 O.D. ( $1.06 \pm 0.8$ ) at 590 nm.

#### **2.2.4 Summary**

Biofilm formation analysis from the transmitted and non-transmitted genotypes showed differences, however statistically not significant.

### **2.3 Experiment 2-3. mRNA expression of glucosyltransferase (*gtf*) genes (reverse transcriptase and quantitative PCR)**

#### **2.3.1 Materials and methods**

##### **2.3.1.1 Extraction of RNA**

RNA samples were extracted according to Chomczynski *et al.* <sup>(26)</sup> and Open wet Ware. <sup>(27)</sup>, with some modifications : cultured cells well processed immediately after removal from incubator, then 300 µl of trizol reagent (Invitrogen, Carlsbad, California, USA) was added to the cell pellets and the solution was homogenised by vortex, followed by adding 700 µl trizol (Invitrogen, Carlsbad, California, USA) and incubated 55°C for 30 min. next, centrifuged at 4°C for 10 min. Then, 0.2 ml chloroform (Nacalai Tesque Inc., Kyoto, Japan) was added to the solution, which then was shaken vigorously, and was followed by incubation about 3 min. at room temperature. Next, solution was centrifuged for 15 min. at 11.000 rpm at 4°C. Aqueous supernatant upper phase was transferred into new tube. Next, isopropanol (Nacalai Tesque Inc., Kyoto, Japan) 0.5 ml was added, and then incubated the solution for 10 min. at room temperature. Followed by centrifugation 11.000 rpm for 15 min. at 4°C. After that, supernatant was removed and washed the sample in 1 ml 70% ethanol (Nacalai Tesque Inc., Kyoto, Japan) by flicking. Continue with

centrifugation 9.000 rpm for 5 min. at 4°C, then supernatant was removed and sample was air dried for 5 -10 min. RNA pellets was dissolve by 50 µl of DEPC treated water.

### 2.3.1.2 Reverse transcription and quantitative PCR

The reverse transcription (RT) reaction and quantitative PCR (qPCR) assays were conducted to analyze *gtfB*, *gtfC* and *gtfD* gene expression. PCR was performed using a PTC-200 thermal cycler and real-time fluorescence monitoring by a Chromo 4 optical detector (MJ Research, Inc, Bio-Rad, Waltham, Massachusetts, USA) with One step SYBR® Prime script® RT-PCR Kit II (Takara, Otsu, Japan). The reaction mixture 25 µl contain 0.2 µl of the RNA sample and 0.4 µM of the appropriate primer. After RT step 42°C for 5 min. and 95°C for 10 sec. The cycles profile was as followed: 95°C for 5 sec., followed by 40 repeats of 54°C for 30 sec and 60°C for 1 min. Dissociation curve analysis was performed at the end of 40 cycles to verify PCR product identity. Each sample was tested on three occasions to increase the reproducibility of the data.

The primers for mRNA expression of *gtf* genes are listed in table <sup>(28)</sup> <sup>(29)</sup>.

Primers	Sequence	Size (bp)
<i>gtfB</i>	F: GCCTACAGCTCAGAGATGCTATTCT	114
	R: GCCATACACCACTCATGAATTGA	
<i>gtfC</i>	F: GACAACACCTTACTTCCTAAATCG	119
	R: GCTTGGTTACCACTCGTTGAATAA	
<i>gtfD</i>	F: TGACAGGTAGCCAACGCATTAA	85
	R: GCTCATCATAAGCAACATCACCTT	
16S rRNA	F: CGCTAGTAATCGTGGATCAGAATG	69
	R: TGTGACGGGGGGTGTGTA	

### 2.3.2 Statistic analysis

Data were statistic analysis using *Student t-test* to evaluate the difference mRNA expression in *gtf* genes between the transmitted genotypes with non-transmitted genotypes. Data was performed as mean  $\pm$  Standard Deviation.

### 2.3.3 Result

The mRNA expression of *gtf* genes between the transmitted and non-transmitted genotypes is shown in fig.15. There were three *gtf* genes (*gtfB*, *gtfC*, *gtfD*) that were analyzed in this study. Figure 15 showed that there were differences of mRNA expression for each *gtf* gene between the transmitted and non-transmitted genotypes. However, the expression of *gtf* genes analysis showed that there were statistically significant differences in *gtfC* gene ( $p < 0.05$ ), while the expression of *gtfB* and *gtfD* genes showed no significant differences (Fig.16). The reactive value of the *gtfB* in transmitted genotypes were range 0 – 0.006 ( $0.003 \pm 0.002$ ) and from non-transmitted were range 0 – 0.003 ( $0.001 \pm 0.001$ ). The reactive value of the *gtfC* in transmitted genotypes were range 0 – 0.013 ( $0.006 \pm 0.004$ ) and from non-transmitted were range 0 – 0.004 ( $0.001 \pm 0.002$ ). The reactive value of the *gtfD* in transmitted genotypes were range 0 – 0.009 ( $0.003 \pm 0.003$ ) and from non-transmitted were range 0 – 0.003 ( $0.001 \pm 0.001$ ).

### 2.3.4 Summary

There were differences found on the mRNA expression of *gtf* genes in transmitted and non-transmitted genotypes. From analysis of each *gtf* genes, it was

found that the *gtfC* gene mRNA expression between the transmitted and non-transmitted genotypes showed the significant difference ( $p<0.05$ ).

## Figures Chapter 2

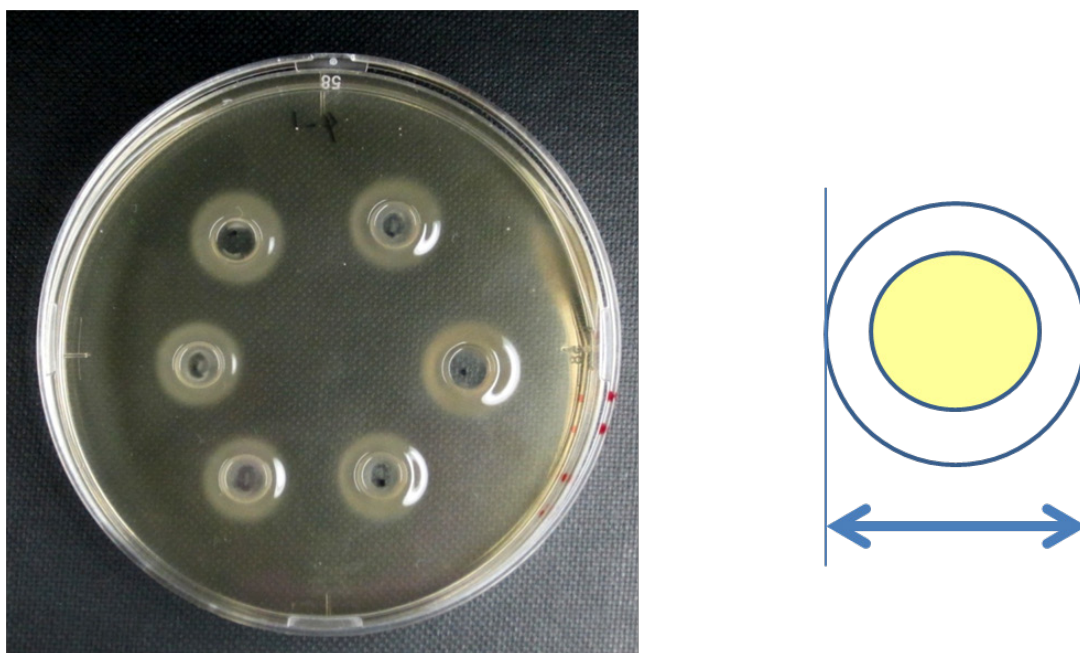


Fig.12      Figure showed the way to measure the bacteriocin inhibition zone activity in transmitted and non-transmitted genotypes of *S. mutans*.

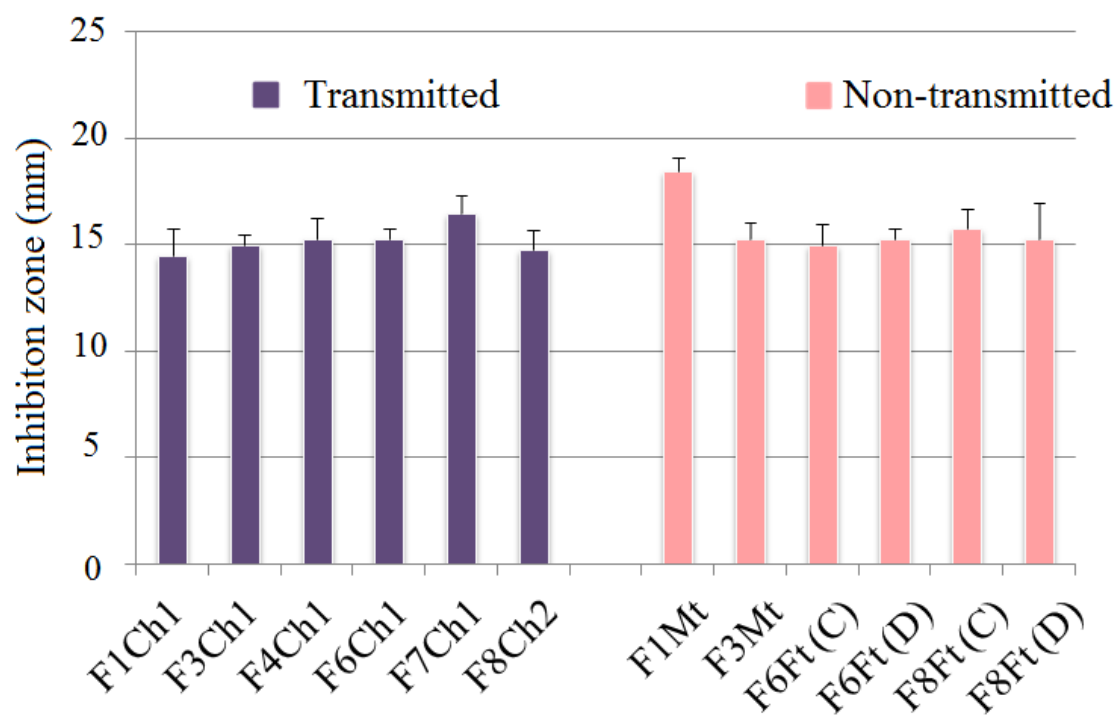


Fig.13. The bacteriocin activity assay between the transmitted and non-transmitted genotypes of *S. mutans*.

Abbreviation : F = Family, Ch = Child, Mt = Mother, Ft = Father.

Parentherized for different genotypes in one individual.

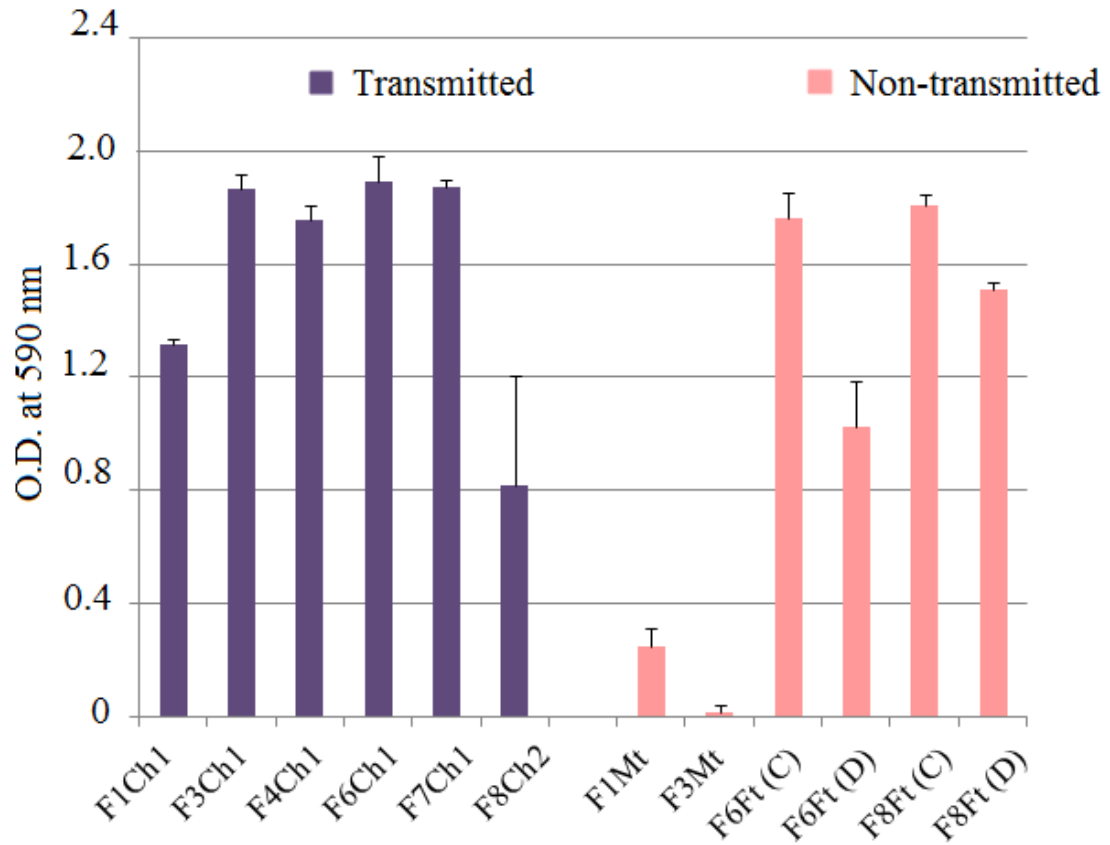


Fig.14. The biofilm formation assay between the transmitted and non-transmitted genotypes of *S. mutans*.

Abbreviation : F = Family, Ch = Child, Mt = Mother, Ft = Father.

Parentherized for different genotypes in one individual.



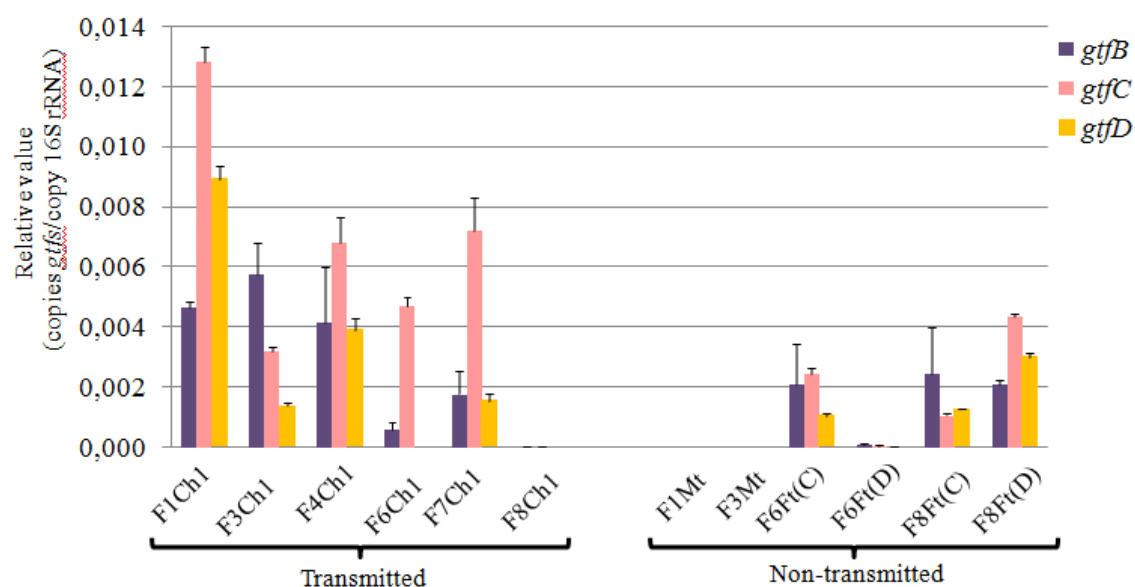


Fig.15 mRNA expression between the transmitted genotypes with non- transmitted genotypes of *S. mutans*.

Abbreviation : F = Family, Ch = Child, Mt = Mother, Ft = Father.

Parentherized for different genotypes in one individual

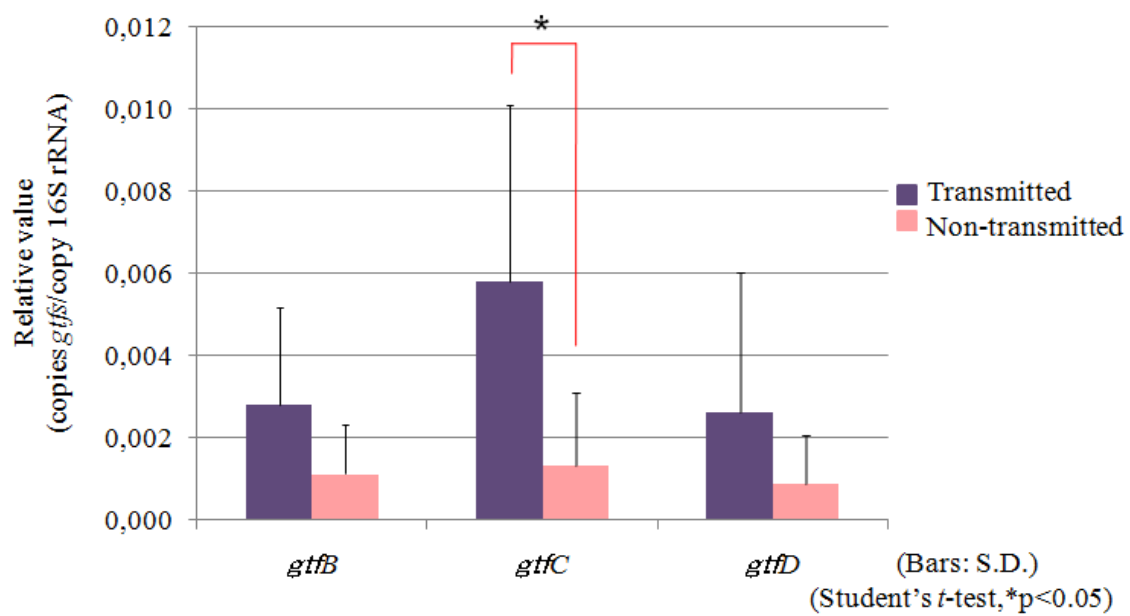


Fig.16 mRNA expression of *gtf* genes from transmitted and non-transmitted *S. mutans* genotypes.

## Discussion

### Chapter 1

#### Detection of mutans streptococci and genotypes of *S. mutans*

##### Experiment 1-1. Detection of *S. mutans* and *S. sobrinus* (PCR)

The detection of *S. mutans* and *S. sobrinus* from caries free children in this research found that there were children who harbor *S. mutans*, *S. sobrinus* and both *S. mutans* and *S. sobrinus*. From the prevalence of these bacteria in age of groups, it was found that most of the children in this population of study harbor for *S. sobrinus* only about 70.3%, then followed by harboring both, *S. mutans* and *S. sobrinus* which were about 27% from the population study. There were 2.7% children from this population of study who harbor *S. mutans* only. The new conducted study from Okada *et al* <sup>(30)</sup> showed the same result as in this study, in which the prevalence of *S. sobrinus* was higher than *S. mutans*. It was suggested that prevalence of MS from subjects who were caries free 7.7% were positive for *S. mutans* only, 53.9% were positive for *S. sobrinus* only and 38.5% were negative for both. The result that obtained in this study could possibly due to the more sensitive detection by PCR for *S. sobrinus* compared to the conducted study in the past, in which the detection in the past was performed using cultural methods <sup>(30)</sup>. Probably, subjects in this study received application fluoride which could also affect the adherence of the *S. mutans*. The fluoride might disrupt the permeability of the *S. mutans* membrane and F-ATPase activity. F-ATPase which was expressed at high level, function as proton-extruding when the pH values 5.0 or below, allowing *S. mutans* to

maintained adequate gradient pH when external pH falls to 4.0 or lower <sup>(31) (32)</sup>. The disruption of the membrane cells leads to an inhibition of the overall intracellular metabolism of the cells, which possibly include the inhibitory on GTF secretion by *S. mutans*. Thereby the *S. mutans* adherence was interfered, since GTF influenced the formation of water insoluble glucans. Water insoluble glucans create a sticky environment, so that the prevalence of *S. mutans* was lower in this population of study. On the other hand, there was possibility that *S. sobrinus* thrive in acid condition not related to F-ATPase activity. F-ATPase activities from *S. sobrinus* grown at pH 5.0 showed no significant increase compared with cells grown at pH 7.0. It is also should be noted that the enhanced acid tolerance of *S. sobrinus* was not correlated with levels of F-ATPase activity, or differences in the optimum pH for enzyme activity <sup>(31) (33)</sup>. Clearly, further studies about the acid tolerance from *S. sobrinus* are needed, because *S. sobrinus* might possess other mechanism to reduce proton permeability or to prevent acid damage the cells.

The result in this study showed that children aged below 3 until 4 years old harbored whether *S. sobrinus* only or both *S. mutans* and *S. sobrinus*. None of the children harbor *S. mutans* only. This result was corresponding with the time children could be colonized by MS. Previous study suggested that children average age 26 months was in the window infectivity to harbor MS <sup>(5)</sup>. Furthermore, another studies suggested a wider range of children colonized by MS in which, age 8 until 52 months coinciding with eruption of the primary teeth <sup>(7)</sup>. This could be explained since the MS acquired by children related to the eruption of the tooth, then window infectivity could occur when coinciding the permanent teeth were erupted <sup>(4)</sup>. The eruption of permanent teeth was probably related to the result that children above 5

years old in this study harbor *S. mutans* only, *S. sobrinus* only and also both *S. mutans* and *S. sobrinus*.

### **Experiment 1-2. *S. mutans* isolation and DNA fingerprinting from children and parents (Restriction endonuclease analysis)**

In this study showed that there were 26 genotypes from 27 subjects of population study. There were up to 3 number of genotypes harbor in individual, in which 21 subjects has one genotype (77.8%). Number of genotypes that harbor by family showed that in one family harbor 2 to 5 genotypes, whereas most of the families in this population study harbor 4 genotypes (37.5%). All of the children in this population study harbour only one genotype. Previous study stated that, individual could harbor up to 4 genotypes. Moreover, genotypes that harbor by family could up to 7 genotypes, whereas 4 genotypes was also the number that mostly harbor by family <sup>(9)</sup>. Children in this population study were caries free and harbor only one genotype. Study from Braga et al. <sup>(34)</sup> found that children who were caries free had only one genotype than children with dental caries who showed more genotypes diversity (5 genotypes).

The fingerprinting pattern analysis in this study, found that there were families which showed similar fingerprint patterns between children and parents. The genotypes harbor in children which showed similar genotypes with the parents, suggested that mother and father could act as the source of transmission <sup>(35) (8) (9)</sup>. Furthermore, the result in this study showed the similar fingerprint patterns between children in the same family. Similar genotypes could occur between mother and

children, followed between siblings, and between father and children. Thus, inside family intimacy beside parents, other persons such as siblings or other relatives could participate in transmission of *S. mutans* <sup>(6)</sup>. This study showed that, the children from different families who attending the day nursery had similar fingerprint pattern. Previous study found that there were similar genotypes between children who attend the same day nursery <sup>(8) (7)</sup>. The similar fingerprint patterns between parents were also found. Study that had been conduct showed there were similar genotypes found between parents <sup>(9)</sup>. This condition suggested factors that might related to transmission, such as intimate or frequent contact with *S. mutans* carrier could be the source of transmission. In this case could be mother, father or other people in the family or outside of the family. Others factor that also related is host susceptibilities, such as the window infectivity, number of erupted teeth, and presence of enamel hypoplasia, sucrose consumption, salivary and mucosal immune systems <sup>(8) (11)</sup>. The transmission of *S. mutans* might also relate to the bacterial properties such as, biofilm formation and adherence ability to colonize <sup>(11)</sup>. Another study also supported that bacterial properties related to the transmission, in which mother with genotypes diversity, showed that only one or some of the genotypes could be transmitted to the children, this suggested distinct capacity or infectivity in different genotypes <sup>(8)</sup>.

## Chapter 2

### Factors involved in transmission of *S. mutans*

#### Experiment 2-1. Bacteriocin activity assay (Agar diffusion)

The result from the bacteriocin activity assay between the transmitted genotypes and the non-transmitted genotypes showed inhibition zone produced against the indicator strains. The inhibition zone of *S. mutans* bacteriocins (mutacin) between the transmitted and non-transmitted genotype showed no significant differences. However, most of the transmitted genotype showed slightly lower inhibition zone with inhibition zone range 14.5 – 16.5 mm ( $15.2 \pm 0.7$ ), while contrary result obtained for the non-transmitted genotypes with the mm range 15 – 18.5 mm ( $15.8 \pm 1.3$ ). These condition could probably caused by transmitted genotypes were caries free subjects. On the other hand, the non-transmitted genotypes in this study were caries affected subjects. Study achieved that *S. mutans* genotypes of caries affected individuals had a higher mutacin production and broad inhibitory spectrum against *S. mutans* indicator strains. The genotypes from caries free individual showed higher inhibitory against initial colonizers (*S. sanguis*, *S. oralis*, *S. mitis*) and low antagonist activity against *S. mutans* indicator strains<sup>(36)</sup>.

In correlation with the bacteriocin activity against strains of the same or closely related species, condition in this study might occur because the non-transmitted genotypes were caries affected subjects. Caries affected subjects predominantly harbor cariogenic streptococci, in which this might elucidate the slightly higher mutacin activity against the predominant colonized bacteria. On the

other side, the caries free subjects may harbor higher prevalence of *S. sanguis* and other streptococci of the mitis group, which could be an explanation the low intense bacteriocin activity against *S. mutans* indicator strain.

## **Experiment 2-2. Biofilm formation assay**

The biofilm formation analysis showed that there were biofilm formation differences between transmitted and non-transmitted genotypes of *S. mutans*, however statistically not significant. The process of biofilm formation begins with adhesion. The adhesion occurs since there were sticky environment which assist the adherence of *S. mutans*. Sticky environment was the condition resulted from the production of water insoluble glucan. Water insoluble glucan was produced from the GTF enzymes when there was presence of sucrose, in which these GTF enzymes was modulated by *gtf* genes<sup>(17) (15) (14)</sup>. Study used smooth glass surfaces stated that there were correlation between water insoluble glucans to the adherence of the *S. mutans*<sup>(37)</sup>. The result from this study it is shown that transmitted genotypes had ability to make biofilm slightly higher rather than the non-transmitted genotypes. This showed that some genotype might be able to colonize better than other genotypes and variation in genotypes may also exhibit variation in virulence character, which was their ability to grow as biofilm.



### **Experiment 2-3. mRNA expression of glucosyltransferase (*gtf*) genes (Reverse transcriptase and quantitative PCR)**

The mRNA expression of *gtf* genes between the transmitted and non-transmitted genotypes showed that there were three *gtf* genes: *gtfB*, *gtfC* and *gtfD* that were analyzed in this study. In these study, showed that there were differences of mRNA expression for *gtf* genes between the transmitted and non-transmitted genotypes. This was due to the *gtf* genes, having a function to encode GTF enzymes that serves for the attachment of *S. mutans* <sup>(17)</sup>. GTF enzyme produces glucans associated with the attachment of *S. mutans* to the tooth surface resulting biofilm formation, in which from the transmitted genotype in this study showed slightly higher biofilm formation. The expression of each *gtf* gene analysis from the transmitted and non-transmitted showed that there were differences and statistically significant in *gtfC* gene ( $p<0.05$ ), while the expression of *gtfB* and *gtfD* genes showed statistically no significant differences. The low mRNA expression of *gtfB* and *gtfD* in this study could probably due to the possibility whether *gtfB* and *gtfC* have independent promoter, even though the coding sequences of *gtfB* and *gtfC* were only 198 bp apart and located in operon like arrangements. On the other hand *gtfD* gene is located upstream of *gtfB* and *gtfC* loci, which presents an independent promoter and may be regulated in a manner opposite from *gtfB* and *gtfC* <sup>(15)</sup>. GTFC encoded by *gtfC* gene produces mixture of soluble and insoluble glucans <sup>(15) (17)</sup>. GTF C enzyme that produce water insoluable glucan, could create sticky environment resulting intial adherence which induce colonization related to transmission of *S. mutans*. High expression of *gtfC* genes, induce the water insoluble

glucans produced also increased, thus enhanced *S. mutans* adherence. So the virulence of transmitted *S. mutans* genotypes elevated and transmission occurs.

## Conclusion

The conclusion from this study is that *gtfC* gene which encoded GTFC to produce water insoluble and water soluble glucans, might affect the factors involved in transmission of *S. mutans* genotypes.

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