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Research Article

The *Streptococcus mutans* Genotype from Mother as Mark of Dental Caries Predictor to her Children

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Abstract

Background: *Streptococcus mutans* is the major causative bacteria of dental caries and thought to be spread through maternal transmission. This transmission indicated by the similarity of genotypes needs to be investigated widely.

Aim: To examine the relationship between dental caries in mothers and children aged 1-3 years old based on *S. mutans* genotype.

Methods: Thirty-five pairs of mothers and children who were visitors of the Integrated Health Service Post were clinically examined and their samples of saliva were collected. The identification of *S. mutans* in saliva was acquired by conducting Polymerase Chain Reaction (PCR). The positive samples were genotyped with Arbitrarily Primed-Polymerase Chain Reaction (AP-PCR) and compared visually. Statistical analysis for correlation was conducted.

Results: The relationship between the number of *S. mutans* colonies on the mothers' saliva with the number of colonies of *S. mutans* in children's saliva was quite strong and significant ($r = 0.517$ $p < 0.05$). There was a weak positive relationship but insignificant between the number of colonies of *S. mutans* in mothers' saliva with children's caries index ($r = 0.085$ $p > 0.05$). Among mothers-children pairs who had *S. mutans*, 60% have a similar genotype.

Conclusions: Vertical transmission of *S. mutans* may occur from mother to child.

Keywords: *Streptococcus mutans; Genotype; Transmission; Dental Caries*

Introduction

Recently, health problems in Indonesia are very diverse, including oral health problems. One of the oral health problems found in the majority of Indonesians is dental caries. Dental caries is a destructive process that causes enamel decalcification, which then reaches the dentin to form cavitation.

Dental caries is the most common chronic disease during childhood. Early childhood caries (ECC) in children under five years old in DKI Jakarta and its surrounding areas were reported to increase [1]. In Indonesia, the prevalence of ECC in children aged 12 - 60 months

is relatively high as it reaches 90% of the population [1]. Febriana (2017) reported that the ECC prevalence in children aged 6 - 24 months in five areas of DKI Jakarta in 2010 was 63.1%, with a def-t score of 3.3 [1]. Besides, dental caries in children, especially in pre-school children, could affect a child's growth, weight and quality of life [2,3]. Therefore, dental caries, especially in children, needs to be prevented [1-4].

According to The American Academy of Pediatric Dentistry (AAPD), ECC is the presence of one or more tooth decay (non-cavitated or cavitated), tooth loss (due to caries), or restoration of primary teeth in children aged ≤ 71 months.3 ECC is an infectious disease in which microorganisms play a significant role. *S. mutans* is the primary etiological agent in tooth decay, including ECC [2-4].

Early colonization of *S. mutans* indicates an increased risk of ECC [2-4]. Children tend to acquire mutans streptococcus in their oral flora from the mother due to postnatal maternal contact [5-6]. Recently, studies on the transmission of mutans streptococcus from mother to child have begun to develop in several countries, including in Argentina, Japan, and in several other countries in Central Asia whose subjects were pairs of mother-children under 36 months with the same genetic identity of *S. mutans* [7-10]. Until today, the study on the transmission of *S. mutans* from mother to children by analyzing the similarity of genotypes in children under 36 months has not been widely carried out in Indonesia. Therefore, the study aimed to analyze the relationship between maternal dental caries and children under 36 months in terms of the number of colonies and genotypes of *S. mutans*. From the results of this study, it is expected that information about the transmission of *S. mutans* can be obtained, so that ECC prevention can be carried out.

Materials and Methods

This was a laboratory study with a cross-sectional approach. After obtaining ethical clearance from the Dental Research Ethics Committee, Faculty of Dentistry, Universitas Indonesia (No.17/Ethical Approval/FKGUI/VIII/2020), an application for study permit was made to the Tugu Village, Depok City, West Java, to obtain subjects from Posyandu visitors.

This study consisted of 35 pairs of mothers and children who visited three Integrated Post Health in Tugu Village, Depok city were selected using a purposive sampling technique. Inclusion criteria for children were children aged 1 - 3 years, within the growth period of their primary teeth, and willing to participate in the study. Inclusion criteria for mothers were permanent resident in Tugu village and willing to participate in the study by signing informed consent. Exclusion criteria for both children and mothers were those who took antibiotics within three months before sampling.

Sample collection

The intraoral condition of mothers and children was examined using a standard oral examination tool (9 - 11 AM). After that, 1 - 2 ml of saliva was taken, each sample was collected in a container to be analyzed for the bacterial colonies and genotypes of *S. mutans*. Saliva was collected through a sterile funnel (without stimulation) and collected in an empty sterile 15 ml Eppendorf tube. For children, saliva was taken from the floor of the subject's mouth using a sterile cotton roll, then squeezed and collected in an empty sterile 15 ml Eppendorf tube.

The mother's saliva sample was labeled M and the child's saliva sample was labeled C. The samples were then sent to the Clinical Microbiology Laboratory, Faculty of Medicine, Universitas Indonesia, and the Oral Biology Laboratory, Faculty of Dentistry, Universitas Indonesia.

Bacterial culture

Before bacterial culture, the TYS20B selective medium was prepared in sterile disposable Petri dishes. The TYS20B formulation agar in one liter was 40 grams of trypticase-soy agar, 5 grams of bacto agar, 10 grams of yeast extract, 200 grams of sucrose, and 200 units of

bacitracin. Saliva samples were then vortexed and diluted with sterile PBS solution up to 104 times for children's saliva samples and 105 times for mothers' saliva samples.

Sample dilution

The steps of saliva sample dilution were as follows: 1) Four Eppendorf tubes for children's and five Eppendorf tubes for mothers' were filled with 9 ml sterile Phospat Buffered Saline (PBS) is a salt-water based solution containing sodium chloride, sodium phosphate, as a buffer that helps cells maintain their pH consistency; 2) Dilution was performed by taking 0.1 ml of saliva from each sample into Eppendorf tube I containing 0.9 ml of sterile PBS; 3) After that, 0.1 ml from Eppendorf tube I were extracted and being put into Eppendorf tube II; 4) The process were repeated until the samples were being put into Eppendorf tube IV; 5) Homogenization of Eppendorf tube I-IV were performed by pipetting technique after each sample was being put into the following tube; 5) For mothers' sample, the process were carried out till Eppendorf tube V which then were homogenized using a vortex tool.

50 µl of the diluted saliva sample was spread evenly over in agar tripticase soy-sucrose bacitracin (TYS20B) selective medium using an L-shaped stirring rod. Each plate was put into an anaerobic jar and given a gas pack, then incubated at 37 ° C for two days. At the end of this stage, the agar plates containing saliva-sourced colonies of *S. mutans* were ready. All colonies that grew on each plate were counted manually with the help of a colony counter.

Gram staining

Gram stain is a kind of differential stain. Based on gram staining, bacteria can be divided into two groups, namely the Gram-positive group and the Gram-negative group. Gram-positive bacteria do not release crystal-purple-iodine complexes when washed with alcohol. Thus, the coloring result is purple. Meanwhile, Gram-negative bacteria release crystal-purple-iodine complexes when washed by alcohol so that the staining results turn red due to safranin staining. *S. mutans* are Gram-positive bacteria. Gram staining was performed as follows: 1) The prep glass was marked with colored pencils; 2) Sterile NaCl was shed onto the glass preparation with a heated loop; 3) The colony were removed from the agar culture with a heated loop; 4) The bacteria and sterile NaCl on a glass slide were dissolved, then colored with purple crystals and for 1 minute; 5) The purple crystal solution was discarded and the Lugol solution were given for 45 seconds; 6) The glass were soaked in 95% alcohol for 20 seconds, then washed with water; 7) Coloring with safranin for 30 seconds; 8) The glass were washed with water and dried with filter paper; 9) The results of the stain were examined under a microscope.

Colony rejuvenation

One colony from each plate was rejuvenated. The colonies were taken using osse and then spread onto the selective agar TYS20B medium. After that, the entire agar plate was put into the anaerobic jar and given a gas pack inside it, then incubated at 37°C for 2 x 24 hours.

Extraction of bacterial DNA

The result of the rejuvenated *S. mutans* colony was prepared on a sterile worktable. The growth results were taken entirely by using osse then put into a 1.5 ml tube containing 1 ml of PBS, then homogenized by using a vortex. After that, the mixture was centrifuged at 12,000 rpm for 5 minutes, and then the supernatant was removed while leaving the pellets remained left. The DNA extraction process was carried out using the Qiagen QIAamp® DNA Mini Kit. The process was started by adding 20 µl of Qiagen protease (proteinase K) and 180 µl of ATL buffer, then vortexed. This mixture was incubated at 56°C for 3 hours, then spun down for 15 seconds. 200 µl AL buffer was added, then vortexed. The mixture was then incubated at 70°C for 10 minutes. Then the mixture was spun down for 15 seconds and 200 µl of 96 - 100% ethanol was added.

600 µl of the mixture was put into the QIAamp® DNA mini spin column. The column was centrifuged at a speed of 12,000 rpm for 1 minute. After that, 500 µl of AW I buffer was added, then centrifuged at a speed of 12,000 rpm for 1 minute. Another 500 µl of AW buffer was added and centrifuged at a speed of 12,000 rpm for 3 minutes. Container tubes were replaced after each centrifugation. Final centrifugation was then performed at a speed of 12,000 rpm for 1 minute. 1.5 ml epis were placed at the top column and 100 µl of AE buffer was added. The incubation was performed for 5 minutes at room temperature. Then, centrifugation was performed for 2 minutes at a speed of 12,000 rpm. The upper column was removed, and finally, 1.5 ml epis were obtained, which contained the DNA extraction results.

S. mutans identification by PCR

Before the PCR process, the concentration of each DNA extraction result was measured using a spectrophotometer at a wavelength of 280. In the spectrophotometric tube, five µl DNA extracts were taken and added 495 µl of aqua dest resulting in the total volume of 500 µl. Meanwhile, the control tube was filled with 500 µl of aqua dest. The result of the spectrophotometry reading was multiplied by 100. The DNA concentration unit was ng/µl and the minimum requirement for the PCR procedure was 100 ng /µl.

The PCR process used primers with sequences (5'-3') GTFB-F: ACTACACTTTCGGGTGGCTTGG, GTFB-R: CAGTATAAGCGCCAGTTTCATC (product size: 517 bp) and DreamTaq Green PCR Master Mix (Fermentas®) consisting of:

DreanTaq Green PCR Master Mix	10 µl
Primer 1	2 µl
Primer 2	2 µl
Template	(adjusted for DNA concentration)
Water, nucleus free	(adjusted for template volume)
Total volume	20 µl

PCR conditions for identification of *S. mutans* with GTFB primers: 1 x 95°C 1 minute

	95°C 30 seconds	
30x	59°C 30 seconds	}
	72°C 30 seconds	
1 x	72°C 4 minutes	

The PCR results were visualized using electrophoresis techniques. This technique used 1.5% agar concentrate. First, 750 mg of agarose powder (Fermentas®) was mixed with 50 ml of TAE electrophoresis buffer. The solution was heated for 2 minutes until the solution became clear. Then, the solution was cooled in a shaker for approximately 3 minutes. The red gel was added to the solution and mixed until homogeneous. Then, the solution was poured into the casting tray containing a comb and left at room temperature until it was hardened completely. After it was hardened, the comb was carefully removed.

The gel in the plastic container was placed in a horizontal position into the electrophoresis chamber so that all of the gel was submerged in a buffer. 5 µl of PCR products were piped into the well. DNA ladder (GeneRule, Fermentas®) 100 bp. Electrophoresis was performed at a device setting of 400 mA, 80 volts, for 30 minutes. The electrophoresis process that has been running was indicated by bubbles coming out of the electrodes. The negatively charged DNA would flow towards the positive pole, which was usually indicated in red. When finished, the gel was put into the documentation gel machine. DNA visualization was carried out by placing the gel on top of a UV light gel-doc (Biorad Lab Inc.). PCR results could be documented on paper printing or computer files, which were usually marked in red.

Genotyping of *S. mutans* through AP-PCR analysis

Specific genotypes were identified using the AP-PCR protocol on identified positive *S. mutans*. OPA-02 (5 'TGCCGAGCTG) was used for the amplification of the bacterial chromosomal DNA. All amplification reactions were carried out using Qiagen Hot Star Taq® DNA Polymerase in 25 µl volume consisting of 2.5 µl 10 x PCR, 1 µl MgCl2, 0.5 µl dNTP mix, 5 µl 5 x Q, 2.5 µl OPA primer, 0.2 µl enzyme, 11.3 µl distilled water, and 2 µl of DNA template. The amplification condition of the mixture was one cycle for 15 minutes at 95o. Then 45 cycles consist of 30 seconds at 94°C, 30 seconds at 36°C, and 1 minute at 72°C. The amplification production was visualized using the electrophoresis technique, which was preceded by making 8% polyacrylamide gel. The materials needed for this gel manufacture were 3.15 ml of distilled water, 30% Acrylamide 1.35 ml, 10 X TBE 0.5 ml, 10% Amper 30µl and TEMED 50µl. The mixture was then put into the combing tray. The gel hardened completely for ± 1 hour. The process of entering the AP-PCR results into agar wells was to mix 2 µl of loading dye for each sample. 10 µl of mixture was poured in each well. The electrophoresis process was then performed for 1 hour in TBE at a voltage of 100 volts, then stained with ethidium bromide for 5 minutes. When finished, the gel was put into the documentation gel machine. DNA visualization was carried out by placing the gel on top of a UV light gel-doc (Biorad Lab Inc.). AP-PCR results could be documented on paper printing data or in computer files. Then, the match between the genotypes of *S. mutans* in mother and child was compared visually.

Data analysis

Data were processed and analyzed using statistical software with computers. Correlation analysis were performed to investigate the relationship between the number of *S. mutans* colonies in mothers' saliva and the number of *S. mutans* in children's saliva and children's dental caries index.

Results

Characteristics of the subjects

In this study, 35 pairs of mothers and children under 36 months who were visitors of three Posyandu in Tugu Village, Depok City, West Java participated and completed the study procedure. Subjects consisted of 74.29% mothers in the 20 - 34 years age group and 25.71% mothers in the age group ≥ 35 years. Meanwhile, the child subjects consisted of 37.14% in the 13-24 months age group and 62.86% in the 25 - 36 months age group. The gender composition of the children was 48.57% girls and 51.43% boys.

Profile of dental caries and *S. mutans*

The mean DMF-S index in maternal subjects was 7.06 tooth surface/subject, significantly higher ($p < 0.05$) than the average DMF-S index for children. The mean number of *S. mutans* colonies in maternal saliva was 2.284 X 107 CFU/ml which was significantly higher than the mean number of *S. mutans* colonies in children's saliva 1.94 X 106 CFU/ml, with a p-value = 0.000. ($p < 0.05$). All subjects in this study had *S. mutans* in their saliva. This is known from the growth of bacterial colonies in all selective agar medium derived from the subjects' saliva.

There was a strong and statistically significant positive relationship between the number of *S. mutans* colonies in maternal saliva (CFU/ml) and the number of *S. mutans* colonies in children's saliva (CFU/ml). There was no significant positive relationship between the number of *S. mutans* colonies in maternal saliva. (CFU/ml) with child def-s index.

Identification of *S. mutans* with PCR (Polymerase Chain Reaction) procedure

The identification of *S. mutans* against bacterial DNA obtained from colonies growing on TYS20B selective media through the Polymerase Chain Reaction (PCR) procedure on 70 samples of bacterial DNA in this study is seen on the following results (Figure 1 and 2).

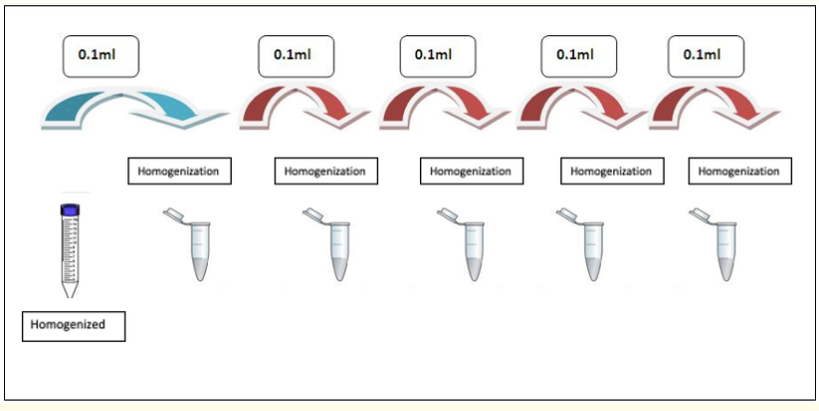

Figure 1: *Steps of Mother's Saliva Sample Dilution (10^5).*

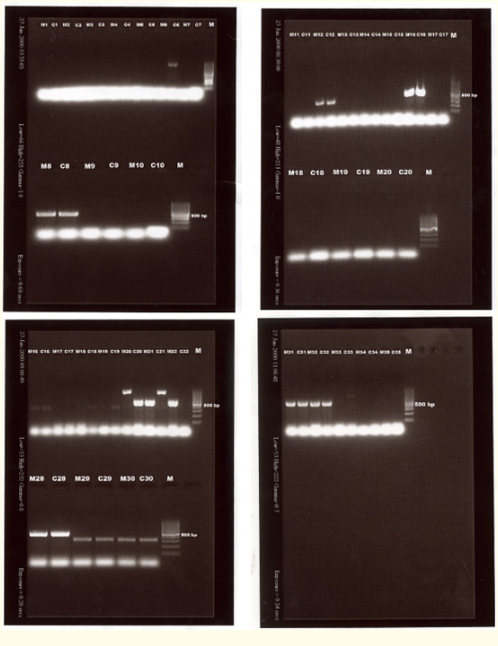

Figure 2: *Identification of Mutans streptococcus by PCR Procedure on Visualization with Agarose Gel There were 13 DNA bands visible at a product size of 517 bp (basepair) consisted of 7 bacterial DNA samples from maternal subjects (M8, M16, M21, M22, M28, M31, M32) and 6 bacterial DNA samples from child subjects (C8, C16, C20, C28, C31, C32).*

Figure 2 shows that of the 70 bacterial DNA samples that were sampled, there were 13 positive DNA samples of *S. mutans*. These 13 positive *S. mutans* bacterial DNA samples consisted of 7 bacterial DNA samples from mothers and six bacterial DNA samples from children.

Genotyping *S. mutans* using the AP-PCR (Arbitrarily Primed-Polymerase Chain Reaction) procedure

According to the identification of *S. mutans* by PCR procedure, it was found that five pairs of mother-children were positive for *S. mutans*. Based on these results, genotyping was carried out on ten positive DNA samples of *S. mutans* using the AP-PCR procedure. The results are shown in the figure 3 from the visualization of AP-PCR products with polyacrylamide gel.

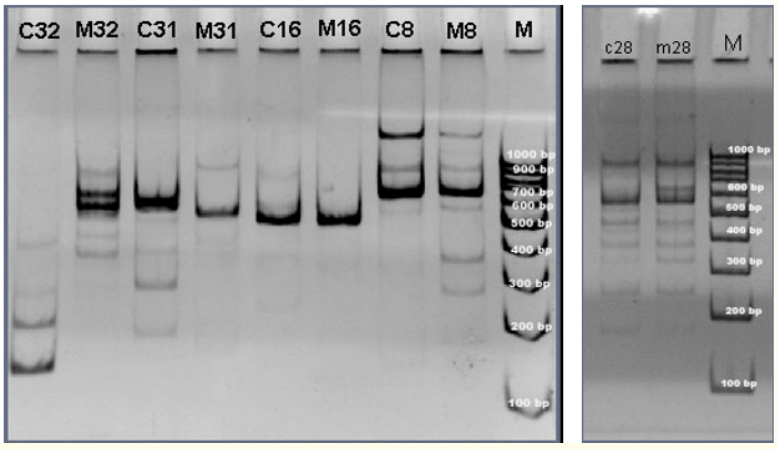

Figure 3: *Genotyping Results with the AP-PCR Procedure on Visualization with Polyacrylamide Gel*

Patterns of DNA strands resulting from genotyping with the AP-PCR procedure. Pattern A in the sample pair with identities: C8 and M8, pattern B on pairs C16 and M16, pattern C on C31, pattern D on M31, pattern E on C32, pattern F on M32 and pattern G in pairs M28 and C28.

It can be seen from the various patterns that there are similarities in the genotype of *S. mutans* bacteria in each of the three pairs of mothers and children of the five pairs of mother-children sampled (3/5). Meanwhile, there were differences in the genotype of *S. mutans* bacteria in 2 pairs of mother-children from 5 pairs of mother-children who were genotyped (2/5).

Discussion

Based on the bacterial culture results from saliva samples, all child subjects aged 14-36 months had *S. mutans* in their saliva, thus refuting Caufield's research on the window of infectivity period, which states that infants are susceptible to infection with *S. mutans* at age 19 - 31 months [11,12]. However, it is in line with research conducted by Zeng Y., *et al.* (2020), which states that infants aged 0 - 6 months can be infected with *S. mutans* [13]. Another study stated that *S. mutans* acquisition could occur before the primary tooth erupts (predentate period) [14,15].

There was a strong and significant positive between the number of *S. mutans* colonies in mothers' saliva and the number of *S. mutans* colonies in children's saliva. The results of this study are consistent with the results of a study conducted by Kishi., *et al.* (2009) and Riyadarshini (2013), which stated that the number of *S. mutans* in maternal saliva was significantly associated with the number of *S. mutans* in

children's saliva [14,15]. Meanwhile, there was a weak and insignificant positive relationship between the number of *S. mutans* colonies in maternal saliva and the dmf- index. s children. These results are in line with the results from several studies that stated that the number of *S. mutans* colonies in maternal saliva was positively related to children's dental caries index [16-23].

In this study, a PCR (Polymerase Chain Reaction) procedure was also carried out on bacterial DNA on TYS20B media. The results of positive images of *S. mutans* bacteria were less than 50% of the total bacterial DNA sample, possibly because there was only one colony from each sample selected to be rejuvenated and then extracted. Therefore, the probability of getting positive *S. mutans* bacteria is lower. This result is lower than the results of a study conducted by Mitchell., *et al*. (2009), where the results of their study showed that eight colonies isolated from selective agar media in each study sample, totaling fifty-four samples, were positive for *S. mutans* [24].

The results of the identification of *S. mutans* by PCR procedure showed that five pairs of mother-children were positive for *S. mutans*. Based on these results, genotyping was carried out on ten positive DNA samples of *S. mutans* with the AP-PCR procedure, which aimed to see *S. mutans* bacteria's genotype in mother with Mutans streptococcus bacteria genotype in children. The genotyping visualization of five mother-child pairs whose genotypes were seen showed that the three mother-child pairs had the same genotype. Thus, 60% of the mother-child pairs examined for the genotype of *S. mutans* bacteria had a genotype similarity, or 8.5% of the total mother-child pairs in this study had the same *S. mutans* genotype. The results of genotyping visualization proved the vertical transmission of *S. mutans* from mother to child. This transmission can occur because of frequent contact between mother and child, such as sharing eating utensils, trying children's food, and kissing children [25,26]. The results of this study are in line with research conducted by Mitchell., *et al*. (2009), which stated that maternal transmission occurred in 41% mother-child pair who were the sample of their study and a study conducted by Fabiana., *et al.* (2010) which stated that 58% or ten of the seventeen mother-child pairs studied had a genetic similarity to the *S. mutans* bacteria [14,24].

Conclusion

In conclusion, the number of *S. mutans* colonies in the mother's saliva is correlated with the number of *S. mutans* colonies in children's saliva and children's dental caries index. Vertical transmission of *S. mutans* may occur from mother to child.

Bullet Points

*Why this paper is important to paediatric dentists

- This paper revealed the relationship between the number of *S.mutans* colonies in mothers' saliva and the number of *S. mutans* colonies in children's saliva.
- This paper revealed the similarity between *S. mutans* genotype of mothers and children.
- The results of this study suggest that vertical transmission of *S. mutans* may occur from mother to child.

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