

Research Report

Horizontal transmission of *Streptococcus mutans* in children attending kindergarten

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ABSTRACT

Background: Transmission of *Streptococcus mutans* (*S. mutans*), the main pathogen found in dental caries, is particularly prevalent during the first two years of infancy. Numerous children are reportedly infected with *S. mutans* by their mothers with early initial acquisition of the condition considered to carry a higher risk of subsequent dental caries. **Purpose:** This research aimed to examine the possibility of horizontal transmission of *S. mutans* in children attending Surabaya-based kindergartens. **Methods:** The number of subjects who satisfied the inclusion criteria totaled 146. Dental plaque was collected for one minute with a sterile toothbrush. After completion of an isolation process, 25 *S. mutans* colonies were identified in Trypticase Cysteine Yeast (TYC) media by means of morphological, microscopic and biochemical tests using API 20 Strept (bioMerieux France). A polymerase chain reaction with OPA-2 and 13 was subsequently used to determine the genotype of *S. mutans*. Primary data collection was completed by the administering of a questionnaire intended to elicit information regarding the gender, age, diet and medical history of subjects. **Results:** An arbitrarily primed polymerase chain reaction (AP-PCR) fingerprint profile of the strains isolated from the subjects indicated similarities in five genotypes of *S. mutans* and differences in 18 genotypes. **Conclusion:** The study indicated that certain children are similarly infected by *S. mutans* bacteria which might be due to horizontal transmission between classmates.

Keywords: children; dental caries; horizontal transmission; kindergarten; *Streptococcus mutans*

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INTRODUCTION

During the last 20 years, a considerable body of research has confirmed that the prevalence of childhood dental caries, especially in developing countries, has increased and is considered to represent a significant child health problem. *Streptococcus mutans* (*S. mutans*) constitutes the main bacterium causing dental caries whose prevalence in Indonesia increases year-on-year. According to Basic Health Research (RISKESDAS), the national community based research which conducted by the Indonesian Ministry of Health, the rise was one from 43.4% in 2007 to 53.2% (a total of 93 million individuals) in 2013.¹

Dental caries in children impede appetite, eating patterns and appropriate sleep regimes, can cause discomfort and negatively affect health. Berkowitz (2006) reported that

S. mutans can be transmitted from one person to another due to the similarity of its strains in children and parents, especially mothers.²

The transmission of *S. mutans* is frequently reported in the research conducted as a condition probably induced by altered social relationships leading to changes in the pattern of family life, especially in large cities.³ Child care, PAUD (Education Program for infants), and kindergartens are all locations where children spend the majority of their time, sharing toys and eating/drinking utensils contaminated with *S. mutans*.^{4,5}

Global changes in behavior have been triggered by the boom in daycare centers (TPA) where children of pre-school age spend 5-10 hours on weekdays, leading to changes in *S. mutans* infection patterns. Research conducted by Klein showed that with regard to children as many as

71% in America, 60% in Sweden and 45% in China are at high risk of dental caries.⁶

The emergence of molecular tools over the past few decades has substantially promoted an understanding of microbial dental caries pathogenicity, thereby improving the detection of bacteria and genotypes. In addition, the etiology of dental caries currently focuses on the host and physical behavior linking dental caries with changes in microbial ecology based on physiological imbalances.⁶ Nowadays, the arbitrarily primed polymerase chain reaction (AP-PCR) molecular method can be employed to evaluate *S. mutans* transmission. Characterization of the *S. mutans* genotype by means of AP-PCR can provide the practical and reproducible data necessary to compile a catalog comprising a large number of longitudinal *S. mutans* isolates. The genotype data collected is expected to provide an important basis for tracking *S. mutans* colonization in populations at risk of caries.⁷ This research was intended to examine horizontal transmission of *S. mutans* in children attending kindergarten in Surabaya through the *S. mutans* genotype equation.

MATERIALS AND METHODS

The research reported here constituted an observational laboratory study whose subjects were randomly selected kindergarten students in Surabaya who met the inclusion criteria of belonging to either gender, aged 4-6 years old and with a dmf-t \geq 4. Subjects also had to be of sound health as confirmed by their responses to a questionnaire indicating their general medical condition. Moreover, they could not have undergone any treatment which had suppressed their immune response. Each subject was examined by a dentist using dental operatory lights, a dental mirror and an explorer. The def-t number (d = decay, e = exfoliation, f = restoration, t = teeth) used was based on WHO criteria.⁸

Isolation of Streptococcus mutans bacteria

The dental caries of subjects were examined and recorded. Plaque samples were taken with a sterile toothbrush which was subsequently inserted into a sterile tube containing 10ml of Brain Heart Infusion (BHIB) media and transferred to the laboratory within two hours. Thereafter, the plaque samples were vortexed for 30 seconds to ensure that all plaque present on the surface of the toothbrush was dissolved in the BHIB media. Serial depletion was then carried out with liquid media yeast numbering between 1/10 and 1/10,000. Ten μ l of plaque was planted on selective TYC agar media before being incubated in an anaerobic jar at 37°C for 48 hours using an anaerobic kit (An Aerogen Thermo Science, Japan) in order to facilitate microscopic identification of *S. mutans*.

One colony, suspected of being *S. mutans*, was extracted from the TYC culture, re-planted in BHI media and incubated at 37°C in an anaerobic jar for 24 hours using an anaerobic kit. The results of isolation were subsequently identified microscopically. Colonies suspected of being *S.*

mutans were stained with gram dyes and examined under a light microscope at 100x magnification. Macroscopic and microscopic test results were further analyzed by means of an hemolysis test. One *S. mutans* colony was planted in blood agar media and incubated in an anaerobic jar at 37°C for 48 hours using an anaerobic kit. A biochemical and enzymatic reaction test was then conducted with an API 20 Strep test to determine the validity of the previous isolation results.

An API 20 Strep test (bioMerieux, Lyons, France) was conducted in successive stages. Firstly, the results of an *S. mutans* culture on blood agar were harvested, placed in 2ml of aquadest, agitated until homogeneous and, finally, adjusted to McFarland standard 4. Secondly, the incubation box was prepared with the wells being filled with 5ml of aquadest to maintain moisture levels. Thirdly, the test strip was placed on top of the wells and 100 μ l of bacterial suspension including: VP (sodium pyruvate), HP (hippuric acid), ESC (esculin), PYRA (pyroglutamic acid), α GAL (α D-galactopyranoside), β GUR (naphthol ASBI-glucuronic acid), β GAL (2-naphthyl β D-galactopyranoside), PAL (2-naphthyl phosphate), LAP (L-leucine- β -naphthylamide) and ADH (L-arginine) placed into ten enzymatic test wells.

The 2 ml of media available in the API 20 Strep kit was added to 0.5 ml of bacterial suspension and mixed until homogeneous. One hundred μ l of suspension, namely: RIB (D-ribose), ARA (L -arabinose), MAN (D-mannitol), SOR (D-sorbitol), LAC (D-lactose), TRE (D-trehalose), INU (inulin), RAF (D-raffinose), AMD (starch (2) and GLYG (glycogen) was then deposited in each of the ten fermentation test wells. In order to prevent penetration by air, mineral oil was deposited in the wells which were then incubated for 4.5 hours at 37°C (in accordance with the manufacturer's instructions).

A single drop of VP1 and VP2 reagent was introduced into the VP and NIN wells, while two drops were also added to the HIP well. The enzymatic results were then reviewed after an incubation period of 4.5 hours. The reaction caused by the addition of a drop of Zym A and Zym B reagent subsequently added to the PYRA, α GAL, β GUR, β GAL, PAL and LAP wells lead to discoloration. Color changes produced by the enzymatic test were recorded after ten minutes, while the fermentation test results were reviewed after 24 hours. The resulting color changes were identified with Fire-Web software and assigned a score according to their nature. The number of scores was then read with the Analytical Profile Index software, the results confirming the bacteria as belonging to the Streptococcus species. All identification procedures outlined above were carried out using the *S. mutans* comparison (ATCC 25175) as a positive control. The *S. mutans* isolation results were taken from one colony and replanted in BHI 37°C for 24 hours, before 500ul was extracted and stored in 20% glycerol media at -80°C for later AP-PCR examination.⁹

The culture of *S. mutans* was incubated for 16 hours (Logarithmic phase) in 5 ml BHIB. The number of bacteria

in culture tube was measured using the McFarland standard. After the number of bacteria reaching standard 4, the culture was centrifuged at 7,000 rpm for five minutes. After discharge the culture liquid, 5ul of lysozyme and 200 ul of PBS were added to the remaining pellets and incubated for 15 minutes at 37°C. Two hundred ul binding buffer was added and 40 ul proteinase K was agitated and incubated at 70°C for ten seconds. One hundred ul isopropanol was subsequently added and vortexed for ten seconds. The sample was then inserted into a filter tube placed above the collection tube and centrifuged at 12,000 rpm for one minute.

At that point, the collection tube was replaced with a new one. The samples to be used were those in the filter tube which were washed twice, first with 500 ul washing buffer centrifuged at 12,000 rpm for one minute and, subsequently, with 500 ul washing buffer before being centrifuged at 12,000 rpm for 10 seconds to remove the remaining washing buffer. The filter tube was then inserted into a sterile micro centrifuge tube, 200 ul elution buffer (previously heated to 70°C) was added, centrifuged again in 12,000 rpm for one minute. Microcentrifuge tubes contained isolated DNA to be stored at -20°C until used (in accordance with the High Pure Template Preparation Kit Roche-Germany procedure).¹⁰

Determination of bacterial DNA

Up to 10ul of the purification results of DNA samples were added to 100 ul of aquadest, homogenized, and deposited in quart cuvettes to measure its absorption with ultraviolet spectrophotometers at wavelengths of 260nm and 280nm. Aquadest was used as a blank. The DNA purity value obtained from the calculation of the ratio A260/A280 DNA was categorized as pure if the A260/A280 ratio ranged from 1.8-2.0. Twenty five *S. mutans* strains resulting from isolation with API-20 Strep were employed for genotyping.¹¹

AP-PCR fingerprinting was performed with two random primers, OPA-02 (5'-TGCCGAGCTG-3 ') and OPA-13 (5'-CAGCACCCAC-3 '). PCR Beads Ready-To-Go was added to DNA template, primer and sterile water producing a final volume of 25ul. A PCR tube was then inserted into the Master Cycler machine with Hotstart at 94°C for five minutes, denaturation at 94°C for one minute, turning at 35°C for two minutes, extension at 72°C for two minutes, and 35 cycles, followed by an extra extension at 72°C for five minutes. Amplification products in 2% agarose gel were analyzed by electrophoresis. Staining with ethidium bromide was performed for 30 minutes prior to the gel being photographed with a red filter under UV310 Illuminators and Polaroid 667 black and white film.^{11,12}

Electrophoresis results can be detected in the form of a band in different lanes and will appear after completion of the coloring process. A lane can be considered as the direction of movement of the sample from the "well" gel. Bands that are equidistant from the gel well in electrophoresis contain molecules that move at the same speed indicating that they are of the same size. Markers

which are a mixture of molecules of different sizes can be used to determine the size of a molecule in a sample band by electrophoresing the markers in a strip in the gel parallel to the sample. The bands in the mark lanes can be compared with the sample bands to determine their size. The distance of the band from the gel well is inversely proportional to the logarithm of the molecular size. The molecular weight and mobility of electrophoresis in polyacrylamide gels can, consequently, be measured using the Mobility rate (Mr) or RF formula in which the distance traveled by the compound is divided by the distance traveled by the solvent to enable calculation of the band distance from the well as follows: $Y = 0.322 (x) + 4.753$ ($Y = \log \text{ bp}$. $X = \text{tape distance from the well}$).^{13,14}

RESULTS

Twenty five samples were identified as *S. mutans*. The genotyping results of the 25 sample analyzed using the AP-PCR method can be seen in Figure 1. As positive control we use *S. mutans* ATCC 1275 as a guide (lane 15 and 16). In Figure 3 samples 12 and 13 were exclude from the results due to lack of *S. mutans* detection accuracy (API Strep 20 showing only 50%).

Twenty five *S. mutans* samples were isolated from students attending 15 kindergardens in Surabaya. In Figure 2, there were three types of *S. mutans* strains that are similar based on visual determination, type A (samples 3 and 8), B (samples 4, 6 and 9), and C (samples 12 and 13), while in Figure 3 two types of *S. mutans* were shown based on visual determination, types D (samples 6 and 7) and E (samples 9, 10, and 11). Based on the type of similarity, it is known that in type A, samples 3 and 8 came from kindergartens in one area of East Surabaya, while type B sample 4 had similarities with samples 6 and 9, with the *S. mutans* isolated originating in the same area in the west of the city. *S. mutans* suspected was transmitted horizontally between children who were classmates. *S. mutans* also featured type C with which sample 12 demonstrated similarity to sample 13. *S. mutans* was isolated from kindergarten students in the same class in South Surabaya. Similarly, Type D (samples 6 and 7) indicated that *S. mutans* was isolated from students in the same class in central Surabaya. Meanwhile, type E (samples 9, 10 and 11) showed that *S. mutans* was isolated from kindergarten students in the same class in North Surabaya (Figure 4).

In addition, the bands 1, 2, 5, 7, 10, 11 and 14 in Figure 1 and bands 1, 2, 3, 4, 5 and 8 in Figure 3 are *S. mutans* derivative species which are not similar on the basis of visual comparison. Thirteen *S. mutans* strains came from different kindergartens and classes. Except for those in sample 11 and 14, kindergarten students based in South Surabaya are shown in Figure 1. Samples 2 and 5, featuring students from the Surabaya center, are shown in Figure 2. The students in samples 11 and 14, and samples 2 and 5 were all members of the same class.

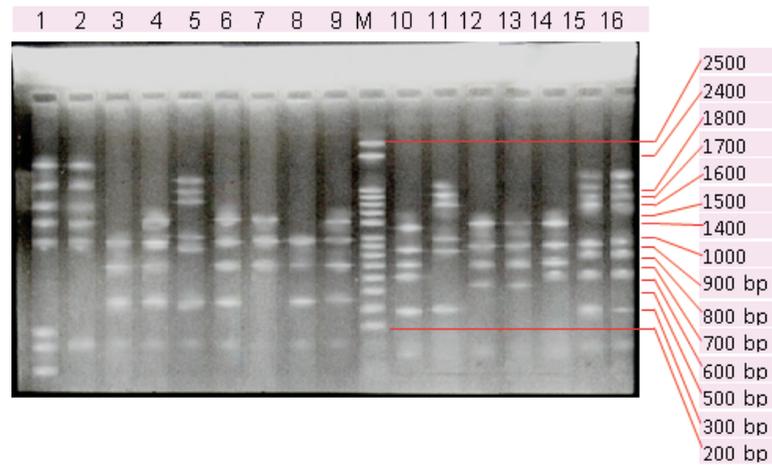


Figure 1. AP-PCR fingerprint profile of the 14 *S. mutans* strains that demonstrated the same *S. mutans* genotype: A (samples 3 and 8), B (samples 4, 6 and 9) and C (samples 12 and 13).

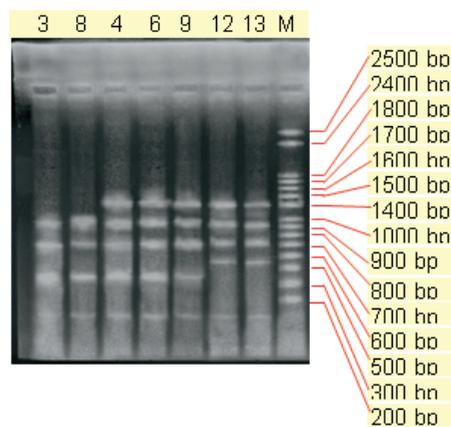


Figure 2. Grouping results of AP-PCR fingerprint profiles of the samples of *S. mutans* strains that demonstrated the same three genotypes of *S. mutans*: A (samples 3 and 8), B (samples 4, 6 and 9) and C (samples 12 and 13).

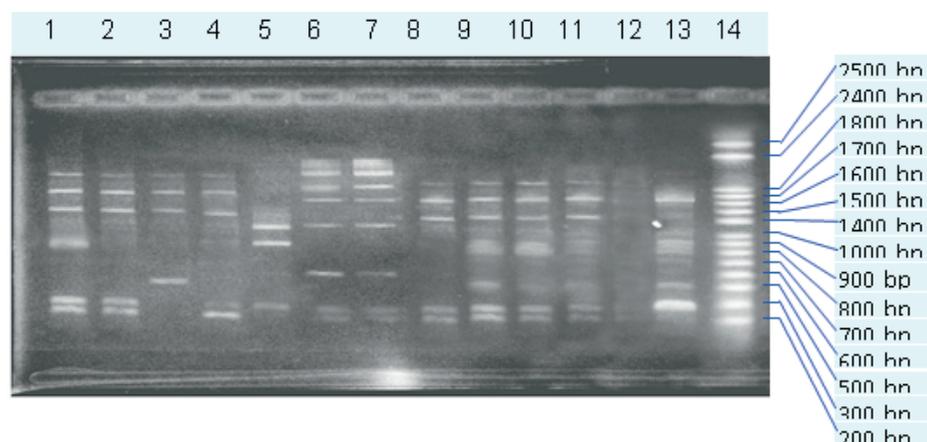


Figure 3. AP-PCR fingerprint profiles of the 11 *S. mutans* strains that demonstrated the same two *S. mutans* genotypes; D (samples 6 and 7), E (samples 9, 10 and 11). Samples 12 and 13 were exclude from the results due to lack of *S. mutans* detection accuracy (API Strep 20 showing only 50%).

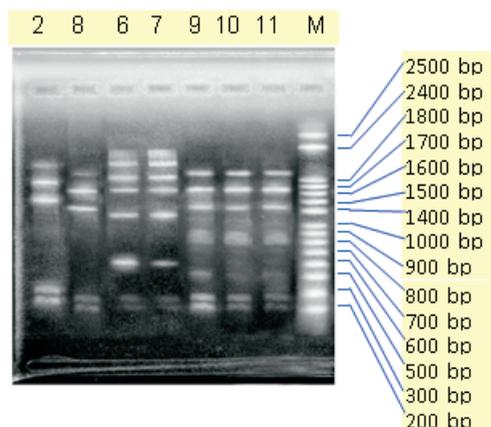


Figure 4. Grouping AP-PCR fingerprint profiles of the *S. mutans* strains demonstrating the same two *S. mutans* genotypes, D (samples 6 and 7), E (samples 9,10 and 11).

DISCUSSION

Dental caries constitute a communicable disease. Considerable research has been conducted into the initial acquisition of *S. mutans* and its role as the main cause of dental caries. Mother to child transmission has been identified as the predominant means of early acquisition of *S. mutans* in infants. However, mothers are not the only source of *S. mutans* transmission. According to Tedjosongko, *S. mutans* can be transmitted to children through intra- and extrafamilial transmission.⁵

In this research, 18 different strains of *S. mutans* were identified. The visual data depicting the comparison results of AP-PCR bands indicated horizontal transmission between non-genetically related 4 to 6-year-olds at kindergarten in Surabaya. Eighteen strains of *S. mutans* were identified. There were five similar strains in five pairs of children who were classmates at 15 schools. The discovery of five pairs of children who demonstrated the same *S. mutans* strain confirmed the occurrence of horizontal transmission.

The kindergarten students from South Surabaya forming samples 12 and 13 are shown in Figure 1. The kindergarten students from Central Surabaya forming samples 6 and 7 who, it transpired, were classmates are shown in Figure 3. Based on visual comparison, there were no similar *S. mutans* strains present in the electrophoresis tape, strongly suggesting the absence of horizontal transmission of *S. mutans*. Gamboa (2010) argues that low transmission rates can occur due to short contact time and less intimate contact between individuals.^{13,15}

Furthermore, research conducted by Klein found that *S. mutans* transmission can be transitory or permanent, strongly influenced by various factors, including: behavior, the frequency of salivary contact, *S. mutans* levels present in individuals, culture, environmental conditions (socio-economic and/or education), individual vulnerabilities (e.g. sucrose consumption) and the window for potential infection occurring at the age of 8.64 months.^{5,6} Another piece of previous research conducted by Berkowitz which

involved 786 one-year olds also identified high caries risk factors for such children such as *S. mutans* infection, fluoride exposure, eating habits and oral hygiene (OHI).² The results of this research can be detected from cross-tabulation between the Oral Hygiene Index (OHI) and age, indicating that the OHI is in poor category (no data shown). The AP-PCR method was then applied to investigate *S. mutans* transmission and genotypic determination. The primary choices (OPA 05 and OPA 13) were used in this study since these two AP-PCR primers, based on previous investigations, can increase sensitivity and specificity to identify various streptococci.¹³

S. mutans transmission will indirectly increase the prevalence of dental caries and other problems. Cases of dental caries in children such as Early Childhood Caries (ECC) will have an impact on various aspects of a child's life, including: physical, psychological and social. Consequently, ECC must be addressed immediately and requires cooperation from the child's parents since this case requires comprehensive, high quality care. Unfortunately, in this research, no data or samples were collected from mothers, teachers or caregivers. As a result, the possibility of *S. mutans* infection from these sources remains unknown. This study confirmed the possibility of horizontal transmission between kindergarten students in Surabaya aged 4-6 years. However, further research is required to identify several risk factors strongly associated with horizontal transmission of cariogenic bacteria among children of different ages in order to break the infection chain in children as part of the prevention of dental caries in Indonesia.⁵ The study showed that some children share similar *S. mutans* bacteria possibly resulting from horizontal transmission between classmates.

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