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Level and Strain Number of Streptococcus Mutans in Toddlers after The Initial Acquisition

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Abstract: Introduction. *Streptococcus mutans* (S *mutans*) are known as major bacteria in human dental caries. Previous experiments reported that S *mutans* could transmit between persons. Purpose. The purposes of this study were to determine the level and number of strain type of S *mutans* after the initial acquisition in children. Methods. Samples were the plaque of 39 children in range of age 0-5 years old of a day nursery in Hiroshima City Japan which were taken with toothbrush every month for 30 months. The S *mutans* isolate were taken by inoculation using mitis salivarius agar and BHI broth respectively. Identification was done by mannitol, sorbitol fermentation test and insoluble glucan synthesis test. The strain types of S *mutans* were determined by comparing the chromosomal DNA fingerprint of S *mutans* using restriction endonuclease *Eco*RI and *Hae*III. Results. Twenty-one children (54%) acquired S *mutans* and 18 children (46%) remain free. The level of S *mutans* were increased gradually and changed irregularly; rapidly increased and remained at high level; and remained at low level. Seventeen children acquired 1 strain type, 3 children acquired 2 strain types, and 1 child acquired 3 strain types of S *mutans* from different sources. Conclusion. ThE study showed that the children could possessed more than one strain type of S *mutans* from different sources and the level of S mutans after initial acquisition period changed variously.

1 INTRODUCTION

Streptococcus mutans bacteria are the main bacteria causing dental caries (de Soet, JJ., de Graaff, 1998). Many previous researches have shown that *S mutans* bacteria are not obtained from birth, but acquired later due to age development. *S mutans* bacteria are also considered as opportunistic commensal bacteria that can be transmitted from one person to another through saliva. To form a stable colony in oral cavity, these germs require the presence of permanent teeth or surfaces. Thus, *S mutans* bacteria are reported only to be found after tooth eruption due to the use of obturator or denture (Berkowitz RJ, Turner J, 1980; Berkowitz RJ, 1985; Kulkarni GV, Chan KH, 1989)

Hence, knowledge of child's age at the onset of *S mutans* preliminary colonization is important for understanding the caries process, knowing the exact moment of taking precautions, and predicting the risk of a child with caries (Li Y., Caufield, 1995). The age of children at the onset of *S mutans* colonies found in oral cavity is then associated with the risk of dental caries. The earlier the child's age has *S mutans* colony, the higher the risk of caries occurrence in the child is (Alaluusua S., 1991; Köhler B, Andréen I, 1988).

Caufield PW1, Cutter GR, (1993) reported that the child's age at the time of the initial acquisition is called as "window of infectivity", ranging in age from 19 to 31 months. Many researches have reported that mother is the main source of *S mutans* transmission in her child (Berkowitz RJ, 1985; Davey AL, 1984; Li Y., Caufield, 1995). This is based on the similarity of strains of *S mutans* found between mother and child. Nevertheless, both of how many strains of *S mutans* a child receives and how high the level of *S mutans* is after the initial acquisition period have not been widely reported. Therefore, this research aimed to determine the level

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and strain number of *S mutans* in children after the initial acquisition. By knowing the level and strain number of *S mutans* after the initial acquisition, the pattern of bacterial growth can be obtained, as well as the strain number of *S mutans* that can be acquired by a child can be detected. However, further research should focus on efforts that can inhibit the acquisition and growth of *S mutans* bacteria in children by looking for transmission characteristics of each strain of *S mutans*. The purposes of this study were to determine the level and number of strain type of S *mutans* after the initial acquisition in children.

2 METHODS

This research was an analytic observational study with restriction endonuclease analysis approach (Kozai et al., 1999). Samples of this research were plaques collected from 39 children aged 0-5 years, parents (mothers and fathers of those children), as well as caregivers at a daycare in Hiroshima City Japan. Plaque samples were taken using a toothbrush once a month for 2 years and 6 months (30 months). Sample criteria of this research were plaques taken from children who had no *S mutans* in their oral cavity and no dental caries due to clinical examination.

2.1 Isolation of Streptococcus Mutans

The samples of plaque and toothbrush were put into 3 ml buffered glycerol saline transport medium (NaCl, K2HPO4, KH2PO4, glycerin, distilled water). Processing then was conducted within 30 minutes after the sampling. Sixty µl of the solution was inoculated on a specific medium for Mitis Salivarius (Difco) with addition of bacitracin (MSB), and then incubated an aerob of 37^{0}C for 48 hours. Next, the *S mutans* colony was taken and inoculated on BHI (Difco) medium. The culture was repeated until the isolates of *S mutans* were obtained. The fermentation test then was performed using mannitol and sorbitol. Afterwards, insoluble glucan synthesis test was carried out on the *S mutans* isolates as confirmation. The initial acquisition of *S mutans* in those children then was determined when the presence of the bacteria in two consecutive sampling processes. Subsequently, the number of colonies of *S mutans* on MSB media was calculated semi-quantitatively and divided into six levels, ie 0 = if no colony obtained; 1 = 1 ~ 50 colonies; 2 = 51 ~ 500 colonies; 3 = 501 ~ 5000 colonies; 4 = 5001 ~ 10,000 colonies; 5 = 10.001 ~ n colony. Colony measurement was conducted every sampling process. The type of *S mutans* strain then was determined by comparing chromosomal DNA fingerprints of S mutans through Restriction Endonuclease Analysis (EcoRI and HaeIII).

2.2 Preparation of chromosomal DNA

S mutans cultures were incubated overnight, inoculated in 4.5 mL BHI broth, and then incubated aerobically at 37^{0}C using a water bath shaker. After bacterial growth reached the optimum density of 0.4 on measurement with spectrophotometer (550 nm), 0.25 g of glycine was added to the cultures and incubated for 45 min. The cultures then were centrifuged at 3000 rpm for 15 minutes, and the supernatant was discarded. Next, cell washing was performed with 0.75 ml of 0.1 M / liter Tris-HCl (pH 8.0) (Trizma base, Sigma Chemical Co., St.Louis, Mo., USA). After the centrifugation of 15000 rpm for 10 min, the supernatant was discarded and the cell was stored at – 20 ^{0}C.

Subsequently, 0.15 ml of lysis buffer was added to Eppendorf containing cell, and sonication then was performed for 5 sec. Next, 0.141 ml of lysis buffer containing lysozyme was added, and then incubated aerobically at 37^{0}C for 30 minutes using the water bath. Afterwards, nine µl of 5 M Sodium Fluoride was added and followed by addition of 0.15 ml of 10% SDS. After left out at room temperature for 10 minutes, they were added with 0.45 ml of phenol-chloroform and then vortexed. Next, the centrifugation was carried out for 10 minutes, and then the supernatant was taken carefully and transferred to the new Eppendorf. Cold ethanol as much as 0.9 ml was added and centrifuged for 10 min. The supernatant was removed again, and then cold ethanol was added again as much as 0.75 ml. After that, the suspension was centrifuged for 10 minutes and the supernatant was continually removed. Ultra-pure water then was added as much as 0.1 ml to chromosomal DNA precipitations and stored in temperatures –20°C until use.

2.3 DNA Cutting and Electrophoresis

Two µl of restriction endonuclease enzymes (EcoRI and HaeIII), 7 µl of chromosomal DNA, and 11 µl of incubation buffer were mixed. The DNA cutting process was carried out at 37 ° C in a dry thermo unit for 6 hours. After that, five µl of loading buffer was added to each sample and put back on the dry

thermo unit at 55 ° C for 5 minutes. Electrophoresis then was performed on 0.7% agarose gel in Tris-borate-EDTA (TBE) buffer solution. Electrophoresis was run at 33 volts for 16 hours at room temperature. Next, gel staining was conducted with ethidium bromide and then washed with pure water. Afterwards, the gels were photographed using Polaroid MP3 and Polaroid 667 films. The band comparison of electrophoretic results then was performed on the same gel. This was conducted several times with various combinations of samples, and monitored by two operators visually. The type of two strains would be considered as the same one when the two strains had the same gel band pattern of electrophoresis.

3 RESULTS

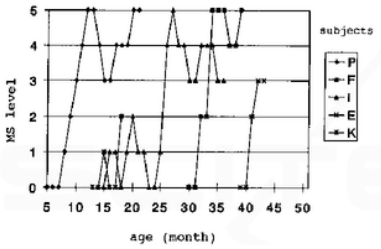

Figure 1: The first pattern of *S mutans* level changes.

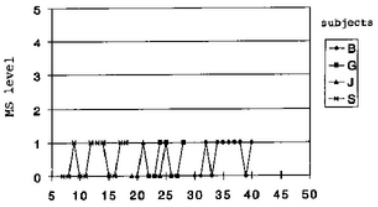

Figure 2: The second pattern of *S mutans* level changes.

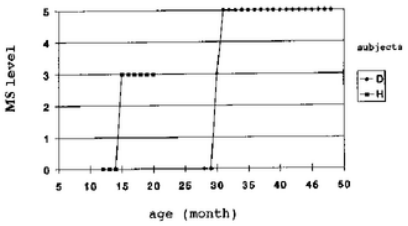

Figure 3: The third pattern of *S mutans* level changes.

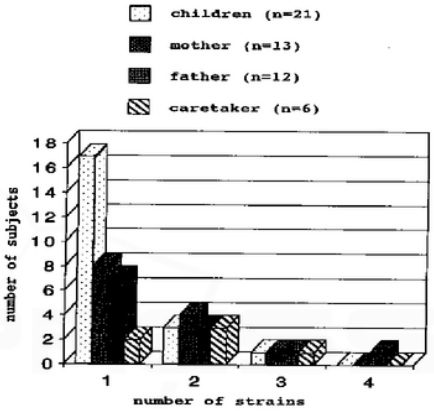

Figure 4: The number of *S mutans* strains.

Twenty-one children (54%) acquired *S mutans,* while 18 children (46%) remained free of S mutans. The initial age of *S mutans* acquisition ranged from 8 months to 4 years and 4 months with an average age of 2 years 0 months. *S mutans* levels in those children after the initial acquisition were divided into three patterns: gradually increased, but then fluctuated (see Figure 1); increased sharply. but then stabilized at high level (see Figure 2); and remained at low level (see Figure 3). Seventeen child samples had one *S mutans* strain, three child samples had two *S mutans* strains, and one child sample had three *S mutans* strains from different sources (see Figure 4). The one *S mutans* strain was found in 8 mothers, 7 fathers, and 2 caregivers, while the two *S mutans* strains were found in 4 mothers, 3 fathers and 3 caregivers. Besides, the three 3 *S mutans* strains were found in one mother, one father, and one caregiver, while the four *S mutans* strains were only found in one father.

4 DISCUSSION

Not all positive child samples acquired *S mutans* until the end of the research period. Eighteen children remained free of *S mutans*. The initial age of the *S mutans* acquisition in the toddler samples in the daycare ranged from 8 months to 4 years and 4 months with an average age of 2 years 0 months. This average age is approximately similar to the average age of *S mutans* acquisition in a research conducted by (Caufield PW1, Cutter GR, 1993), about 2 years 2 months.

The result of this research, moreover, also indicates the variation of *S mutans* levels in children after the initial acquisition period. There were two prominent variations, namely increasing gradually and then fluctuating - pattern 1, and relatively small and stable change at low level - pattern 3. There were two child samples with a sharply increased *S mutans* level, and then it stabilized at high level - pattern 2. In the child samples with pattern 3 level variation, *S mutans* bacteria were not found although those samples had been found to be positive during the previous sampling process. This is probably due to the level of *S mutans* at such a time so low that it is not detected by the hatchery medium used.

This result is consistent with a research conducted by Masuda, Tsutsumi, Sobue, & Hamada, (1979) stating that micro flora on the surface of baby tooth has not been stable, especially regarding the ecology of *S.mutans*. Several factors may affect the variation of these levels, such as sugar intake (Minah GE, Solomon ES, 1985; Wennerholm K, 1995); antibiotic use (Maltz, M, Zickert, 1982); bacterial factors (Kozai et al., 1999); as well as immune factors and food composition (Van Houte & Green, 1974). Sucrose is commonly reported as the most dental caries food. This is because *S mutans* form extracellular polysaccharides derived from sucrose, so the bacteria can attach to the enamel surface.

In this research, a child is also known to be able to acquire *S mutans* more than one strain from different sources. The number of strains that the parents (mom and dad) and the caregivers of the child samples at the daycare can provide an overview of the child's daily environment. Factors reported to affect the transmission of *S mutans* include the serotypes of bacteria, the number of bacteria belonging to the transmitting source, the number of bacteria that move in each contact, the frequency of contact, as well as the dietary factors and immune status of children (Germaine, 1984; Grindefjord M, Dahllof G, Wikner S, Hojer B, 1991; Kozai et al., 1999; Newbrun, 1992; Van Houte & Green, 1974). Besides, these factors are also related to habit and day-to-day behavior (life style) that can be different in different populations. As the levels of *S mutans* vary, the number of strains the child acquires may also change. This is because of the unstable colonies in the oral cavity as well as the nature of *S mutans* which is difficult to compete with other microorganisms that first colonized.

The research reveals that a child can acquire *S mutans* more than one strain from different sources. In general, there are three patterns of level changes due to the initial acquisition. However, further research needs to be focused on attempts to inhibit the acquisition and growth of these bacteria in children by looking for transmission characteristics of each type of *S mutans* strain.

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