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Research Report

Glucosyltransferase B/C expression in Streptococcus mutans of rampant and caries-free children

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ABSTRACT

Background: *Streptococcus mutans (S. mutans) as specific bacteria causing dental caries have cariogenic characteristic related to glucosyltransferase (gtf) B/C that can change sucrose into insoluble glucan. Insoluble glucan functions as an attachment media and bacteria colonization, and also as a source of extracellular polysaccharide which is needed for the bacteria and may lead to caries formation.* ***Purpose:*** *The aim of this study was to find out the gtf B/C expression in isolated S. mutans from dental plaque of rampant and caries-free children.* ***Methods:*** *An observational study was done on 96 isolated bacteria grown in sucrose and bacitracin containing media, which include S. mutans INA 99, S. Mutans EU3, S.mutans EU7, S.EU10a, and S.mutans 10b. PCR technique was used as amplification technique for gtf B/C.* ***Result:*** *This study showed that gtf B/C gene was found in S. mutans, S. constellatus, S. bovis, S. anginosus, L. fermentum, L. salivarius, and Kleibsiella oxytoca. The presence of gtf B/C gene was found in 9 of 10 samples identified in the sample of rampant caries children.* ***Conclusion:*** *The gtf B/C enzyme was found not only in S. mutans, but also in other bacteria.*

Key words: *cariogenic bacteria, S. mutans, Glucosyltransferase*

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INTRODUCTION

Dental caries was not a new disease and has become an important problem in all around the world. It has been the focus of researchers for decades. The researchers tried to identify the bacteria which cause dental caries at the end of the nineteenth century. They found that *L. acidophilus* and *S. mutans* are specific cariogenes which trigger dental caries. These cariogenes called as lactic acid bacteria are the main causes.[1] They are considered as specific agents that produce primary acid for dental caries.[2,3] To ensure the influence of *Lactobacilllus* toward dental caries, a study by Byun *et al*.,[4] showed that the increased amount of *Lactobacillus* has correlation with the increase of carbohydrate on teeth. The average amount of carbohydrate found on *L.gaseri* and *L.ultunens* was higher than that on other *Lactobacillus*.

Another observation indicates that *S. mutans* was the main factor causing dental caries because of its characteristics which can change sucrose into glucan, produce lactic acid by homofermentation, form colony on teeth surfaces, be more aciduric than other Streptococcus. As consequence, *S.mutans* was considered as the main etiology organism because it was potential to be virulent in triggering dental caries.[5]

Munson *et al*.,[6] divided the role of *S. mutans* and Lactobacilus in the process of dental caries. Their emphasized that *S. mutans* was responsible in the initiation of caries lesion which was attached on dental surfaces by glucan production or retention of pit and fissure.

Based on above statements, all oral bacteria may cause caries, but the level of their potential needs to be researched. If the previous researchers have already identified that *S. mutans* was cariogenic, the presence of characteristics found in other bacteria should also be observed. As a result, researchers can prove that the most cariogenic bacteria are those which have the highest potential. The purpose of this study was to find out the gtf B/C that was expressed due to the exceeded gtf B/C gene in bacteria isolated from dental plaque of rampant and caries-free children.

MATERIAL AND METHODS

Samples of plaque were taken from Student Clinic in Department of Dentistry, Padjadjaran University, Kindergarten and Elementary School of Bale Indah, Bandung Regency, Elementary School of Sukasari I and Elementary School of Sukasari II, Bandung Regency. Samples of *S. mutans* were gained from laboratory in Faculty of Dentistry, Trisakti University, Jakarta, and laboratory in Faculty of Dentistry, Airlangga University, Surabaya.

Ninety six strained bacteria that were separated from the material in the form of plaque and 5 strained bacteria which have been identified as *S. mutans* which were *S. mutans* INA 99, *S.mutans* UE3, S.mutans EU7, *S.mutans* EU10a, and *S.mutans* EU10b. The separation was identified by applying a test on MSA, TYCSB, the coloring of Gram, and Biochemistry. The characteristic was taken based on Streptococcus morphology, positive Gram, positive mannitol, positive sorbitol, positive aesculin, negative arginin, positive melibiose, positive raffinose which was taken by fermentation test.

Mitis Salivarius Agar plate was used after incubated anaerobically for 2 × 24 hours. The strained bacteria generally identified by the coloring of Gram forming colony which turned into blue. Then, their morphology was observed by microscope. By Gram coloring it can be seen from microscope that *Streptococcus* was purple. If the coloring of Gram and morphological identification are appropriate with the characteristic owned by *Streptococcus*, the positive Gram will be in the form of chained or paired coccus.

After applying incubation on TYCSB by 15 g/l Bacto-Casitone (Difco), 5 g/l Yeast extract (Difco), 0.2 g/l L-cystine, 0.1 g/l Na_2SO_3, 1 g/l NaCl, 2 g/l Na_2HPO_4. 12 Aq, 2 g/l $NaHCO_3$, 20 g/l Na-acetate, 15 g/l Bacto agar, the colony will turn into white and the size was 0.5–1.0 mm.

Further identification of biochemistry found in the bacteria will be done by adding proliferated bacteria on the tube which contains arginin solution, aesculin, sucrose, lactose, mannitol, sorbitol, raffinose, melibiose 1%. To add the proliferated bacteria on the tube, an ose which has been heated was used.

DNA Extraction and PCR

DNA of anaerob bacteria was isolated by applying Wizard DNA isolation Purification Kit with a half composition of reaction. Cell was taken 10 ml from culture and dissolved with 240 ml, 50 mM of EDTA, and 60 ml of lysozyme 10 μg/ml. After that, it was incubated for 30–60 minutes at 37° C and spun for 2 minutes with 13,000 rpm rotation.

Then, 300 ml of Nuclei Lysis Solution was added. The supernatant was removed and incubated at 80° C. Next, it was stored at room temperature and added with 1.5 ml of RNAse, and incubated at 37° C for 30–60 minutes. Then, it was added with 100 ml of vascor protein, and stored for 5 minutes. Next, it was taken with 13,000 rpm for 5 minutes. After that, supernatant was put in the Eppendorf containing 300 ml of isopropanol. It was spun back and forth with 13,000 rpm rotation for 2 minutes. Then, the supernatant was removed and the pellet was washed by ethanol 70%. Later, the same process was repeated while DNA was dried by concentrator. When the DNA was completely dried, it was dissolved with 50 ml of DNA rehydration.

Amplification using Primer universal gene 16s rDNA was applied in: double denaturation at 94° C for 2 minutes, annealing at 48° C for 1 minute, elongation at 72° C for 1 minute, and post-elongation at 72° C for 10 minutes, and amplification as many as 30 cycles. The genes which were used include: Forward: 5' AGAGTTTGATC(A/C)TGGCTAC3' (19 pairs of alkali); Reverse: 5' GGTTC(G/C)TTGTTACGACTT3' (18 pairs of alkali)

Amplification was applied by using Primer gene gtf B/C. Forward: 5' AGATTT CCGT CCCTT ACTG 3'; Reverse: 5' ATCA TATTTGT CGCCAT CATA 3' and Tegenerated Primer.[6] Both Primer genes were used in early denaturation at 94° C for 2 minutes. The cycle consists of denaturation at 94° C for 1 minute, Primer attachment at 50° C for 1 minute, and extension process at 72° C for 1 minute as many as 35 cycles. At the last cycle, the extension was applied at 72° C for 10 minutes. Optimization of PCR was completed by setting the template of PCR, concentration of magnesium chloride, Primer concentration, temperature of Primer attachment, and concentration of dNTP. Based on optimization, the proper compositions of PCR were 40 printings, 20 genes of forward, 20 genes of reverse, 9 μl of magnesium chloride 25 mM, 5 μl of Tag polymerase dapar, 1 μl of Tag polymerase enzyme, and 1 μl of dNTP. At last step, sterile aquabidest was added as much as 50 μl.

PCR product was confirmed with electrophoresis of agarose gel 1% (w/v) by comparing DNA token, positive control, and negative control. Agarose gel 1% was made by dissolving 400 mg of agarose and 40 ml of TAE 1X *dapar* (Trwas-base, EDTA 0.5 M pH 8.0, glacial acetic acid and natrium hydroxide. Electrophoresis was applied in 90 Volt for 45 minutes. After the electrophoresis was completed, PCR product was observed by using transluminator UV.

Nucleotide was arranged based on dideoxy method of Sanger. The compound to set nucleotide in order was PCR product that was purified by 10 ng/3 μl of Primer forward, Tag polymerase *dapar* 10×, Tag polymerase enzyme, DNPT, ddTP, and stop solution.

RESULT

Based on the research through PCR technique using Primer universal gene 16s rDNA by token of DNA pUC19/HinfI, it was known that 18 samples can be identified to produce DNA with band sized 1,500 pb (Figure 1) and 1,400s pb (Figure 2).

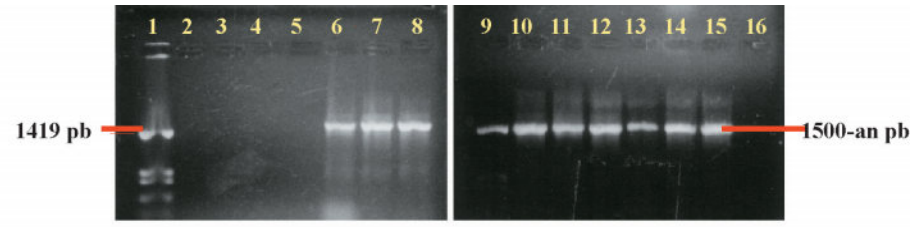

Figure 1. Amplification result of gene 16s rDNA with band length 1,500s pb. 1) pUC- *Hinf*I; 2) Isolate BK48; 3) Isolate BK47; 4) Isolate K24; 5) Isolate K32; 6) Isolate BK42; 7) Isolate BK45; 8) Isolate BK43; 9) Isolate BK46; 10) Isolate K21; 11) Isolate K19; 12) Isolate K18; 13) Isolate BK44; 14) Isolate K20; 15) Isolate K17; 16) Isolate K16.

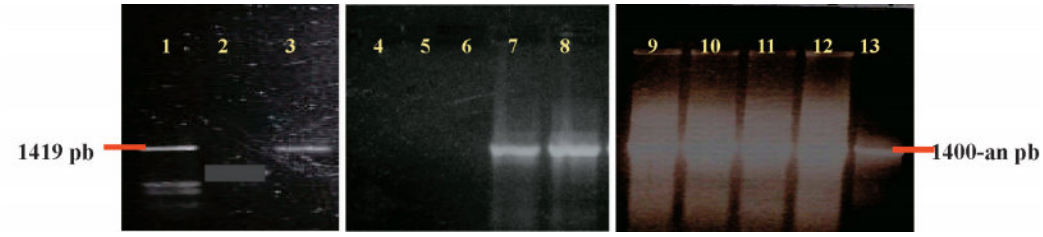

Figure 2. Amplification result of gene 16r sDNA with band length 1,400s pb. **1) Token of molecule weight** pUC-*Hinf*I; 2) Isolate K31; 3) Isolate K12; 4) Isolate 10aEU; 5) Isolate BK22; 6) Isolate BK25; 7) Isolate 7EU; 8) Isolate 3EU; 9) Isolate K39; 10) Isolate BK55; 11) Isolate K40; 12) Isolate K41; 13) Isolate BK54.

The result showed that the bacteria which were isolated include: *S. mutans*, *S. anginosus*, *S. constellatus*, *S. bovis*, *L. salivarius*, *L. fermentum*, dan *K. oxytoca* (Table 1).

In this study the Fragment amplification result of gene gtf B/C using specific Primer showed that the band was 600 pb (Figure 4), and less than 600 pb (Figure 5).

Complete amplification result of the band length of gtf B/C on various bacteries can be seen on Table 2.

Table 1. Result of bacteria identification by 16s rDNA approach

Num	Sample	Sample category	Name of bacteria	Homology (%)
1	K12	Rampant caries	*Streptococcus mutans*	93%
2	K17		*Lactobacillus fermentum*	94%
3	K18		*Klebsiella oxytoca*	96%
4	K19		*Streptococcus anginosus*	97%
5	K20		*Streptococcus constellatus*	85%
6	K21		*Streptococcus bovis*	96%
7	K39		*Streptococcus bovis*	85%
8	K40		*Streptococcus anginosus*	96%
9	K41		*Lactobacillus salivarius*	99%
10	7EU		*Streptococcus mutans*	100%
11	BK46	Free Caries	*Kleibsiella oxitoca*	94%
12	BK54		*Streptococcus anginosus*	96%
13	BK55		*Streptococcus constellatus*	97%
14	BK42		*Streptococcus anginosus*	99%
15	BK43		*Lactobacillus salivarius- subsp.*	99%
16	BK44		*Lactobacillus salivarius-subsp.*	95%
17	BK45		*Lactobacillus salivarius-subsp.*	95%
18	3EU		*Streptococcus mutans*	92%

Description: Identified bacteria are homologized based on the data in Gene Bank, and percentage (%) shows the homology level.

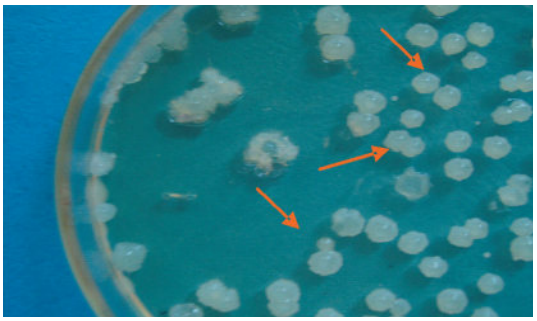

Figure 3. Colonies of *S.mutans* on TYCSB media. The colonies of *S.mutans* are white, glossy, not flat, cauliflower like, in the form of crystal, and sticky on media.

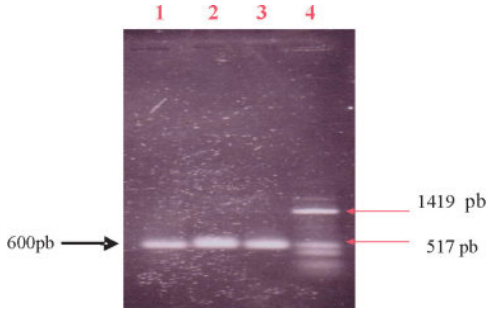

Figure 4. Fragment amplification of gtf B/C with band length 600 pb. 1) PCR product: Isolate K12, S. mutans; 2) PCR product isolate K18, K. oxitoca; 3) PCR Product Isolate K39, S. Bovis; 4) Molecule token pUC-HinfI.

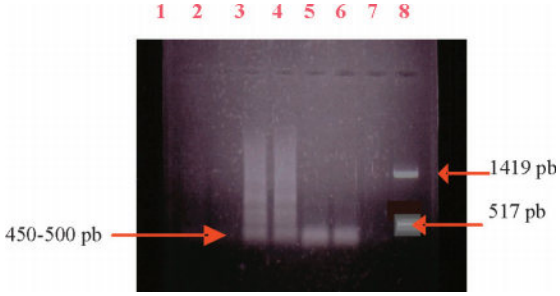

Figure 5. Fragment amplification of gtf B/C with band length less than 600 pb. 1) Isolate BK42 (no amplification); 2) Isolate BK55 (no amplification); 3) PCR Product isolate K21 (*S. bovias*); 4) PCR product isolate K40 (*S. angunisus*); 5) PCR product isolate K46 (*K. oxitoca*); 6) PCR product isolate K46 (*K. oxitoca*); 7) Isolate 7EU (no amplification); 8) Molecule token pUC-HinfI.

Table 2. Fragment amplification result of gtf B/C on various bacteria

Num	Sample	16s rDNA	Band length amplification	Sample category
1	K21	*Streptococcus mutans*	600 pb	Rampant caries
2	K17	*Lactobacillus fermentum*	700 pb	
3	K18	*Klebsiella oxytoca*	600 pb	
4	K19	*Streptococcus anginosus*	500 pb	
5	K20	*Streptococcus constellatus*	700 pb	
6	K21	*Streptococcus bovis*	500 pb	
7	K39	*Streptococcus bovis*	600 pb	
8	K40	*Streptococcus anginosus*	600 pb	
9	K41	*Lactobacillus salivarius*	600 pb	
10	7EU	*Streptococcus mutans*	No band	
11	BK46	*Kleibsiella oxitoca*	450 pb	Caries-Free
12	BK54	*Streptococcus anginosus*	600 pb	
13	BK55	*Streptococcus constellatus*	No band	
14	BK42	*Streptococcus anginosus*	No band	
15	BK43	*Lactobacillus salivarius subsp.*	No band	
16	BK44	*Lactobacillus salivarius subsp.*	No band	
17	BK45	*Lactobacillus salivarius subsp.*	No band	
18	3EU	*Streptococcus mutans*	No band	

Description: The band length that can be identified shows amplification of gtf B/C, if there was no band, there will be no amplification of gtf B/C

DISCUSSION

Several *S.mutans* which can be identified are K12 and 7EU (taken from dental caries plaque) and 3EU (taken from free dental caries) (Table 1). In the process of proliferation all samples have *S.mutan* characteristics: which has blue color on MSA, glossy, sticky on proliferation media, has diameter of 0.1–1 m or could reach 1–1.5 mm.

On TYCSB media, their color was white and their surface was not flat. Their form was crystal like cauliflower and attached on media (Figure 3). These findings have reconstructed the previous opinions which stated that MSA and TYSCB are selective media for *S.mutans*. The reason was that after PCR was applied with Primer gene 16s rDNA, some colonies besides *S.mutans* have similar characteristics.

On this basis, it was predicted that MSA and TYCSB media are not selective for *S.mutans* proliferation and those characteristics appeared because the bacteria used sucrose as substrate to produce glucan. However, further research needs to be conducted to study these findings.

A little amount of *S.mutans* which can be identified can contribute to the development of microbiology study focusing on characteristics of biochemical bacteria. Some bacteria have particular characteristics; forming ammonia from arginin, fermenting mannitol, sorbitol, aesculin, melibiose, and raffinose. These characteristics are similar to the biochemistry of *S.mutans*.

Enzyme that forms caries was gtf B/C. Insoluble glucan is indicated by gft B by while gtf C indicates the soluble and insoluble glucan.[7,8] gtf B and C enzyme has high homology when forming operon. The operon has strong promoter at the top of gtf B. While for grf C, its operon was at the bottom of gtf C.[9]

*S.mutans*gtf B/C enzyme creates pathogenic insoluble glucan because polimer glucose produced by these two enzymes was aggregation mediator for bacteria on dental surface.[10,11] This can contribute to the intensity of thickness and structure integrity of dental plaque which later can form caries.

The presence of gtf B/C enzyme was expressed by the presence of gtf B/C coding the two enzymes. Gene gtf B/F can be amplified by PCR techniques using Primer gene gtf B/C.

The presence of gtf B/C enzyme was one characteristic owned by cariogenic bacteria.[13,14] Bacteria which are isolated from the plaque of dental caries have gtf B/C.

Based on the result of the study and the discussion. It is concluded that the presence of caries was closely related to the presence of gtf B/C gene, since it was one of characteristics owned by cariogenic bacteria. This implies that the presence of glucosyltransferase enzyme was expressed by the presence of gtf B/C. This study indicates that bacteria with gtf B/C have one of the characteristics owned by cariogenic bacteria. Besides *S. mutans*, other oral bacteria which indicate glucosyltransferase enzyme can trigger caries. Bacteria like *S. mutans*, *S. anginosus*, *S. constellatus*, *L. salivarius*, and *K. oxytoca* are not only found in rampant caries children, but also in free-caries children. All bacteria (*S. mutans*, *S. bovis*, *S. anginosus*, *S. constellatus*, *L. salivarius*, *L. fermentum*, and *K. oxytoca*) found in rampant caries children contain gene gtf B/C. *S. mutans* which does not contain gene gtf B/C was found either in rampant caries or caries-free children. If gene gtf B/C was found in caries-free children, it was suggested that prevention to eradicate the bacteria causing caries should be applied as soon as possible. *S. mutans* was classifed as cariogenic bacteria although it has no gtf B/C, acidogenic bacteria funtion as cariogenic. Therefore, further studies need to be conducted to identify other causes.

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