Rostinawati T et al /J. Pharm. Sci. & Res. Vol. 10(3), 2018, 511-513

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Identification of Bacteria Causing Dental Caries through Genetic Testing and Activity Assay of Toothpastes

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Abstract

Dental caries is one of the most common dental problems in the world. Dental caries could happen because of lots of factors, from the host factor to microorganism activity. There are numerous bacteria that are associated with dental caries. One of prevention of dental caries is by maintaining the oral hygiene using toothpaste. The effectiveness of toothpaste in inhibiting the growth of bacteria that causes dental caries will also be influential in the prevention of dental caries. The bacteria that causes dental caries from clinical isolates was identified with experimental observations methods through the phenotype and genotype approach using Polymerase Chain Reaction (PCR) 16s rRNA. The phenotype observation through the observation of the colony and microscopic morphology (Gram stain) revealed that the bacteria had a rod shape and was a Gram-positive bacteria. The results of DNA sequence of 16S rRNA fragments homology with the DNA sequence of 16S rRNA of the BLAST database on the website http://blast.ncbi.nlm.nih.gov showed that the bacteria that cause dental caries had the highest similarity with *Bacillus licheniformis*. The test for the activity of toothpastes showed that the toothpastes were active against this bacteria.

Keywords: Dental caries, PCR 16s rRNA, Identification of bacteria, Toothpastes, activity

INTRODUCTION

Dental caries, commonly known as cavities, is a common dental problem in the world [1]. Dental caries is a chronic dental disease that damages the hard tissue of teeth and is formed from the accumulation of plaque on teeth surfaces formed by acid-producing bacteria from fermentable carbohydrates. Bacteria interact with carbohydrates that can be fermented in the long run, forming acids thereby lowering pH below critical and resulting in demineralization of hard tissue of teeth [2]. The prevalence of dental caries in Indonesia population showed that at age 12 year equal to 43.9%, age 15 year reached 37.4%, age 18 years 51.1% , age 35-44 years 80.1% and age 65 years and over reached 96.7% [3]. Meanwhile, according to Household Health Survey in Indonesia in 2004, caries rate in Indonesia was 90.05% of Indonesia population. The data of prevalence increasing of active caries population of Indonesia from year 2007 to year 2013 was 43.4% to 53.2% [4].

Dental caries is caused by many factors, such as host factors (teeth and saliva), food substances, microorganisms, and time. Known dental caries microorganisms are *Streptococcus* and *Lactobacillus* [5]. The most common cause of dental caries is *Streptococcus mutans* (*S. mutans*). *S. mutans* acts as an initiator of dental caries, while *Lactobacillus* sp, contributes to the developmental process and the continuation of caries [6]. However, it has recently been reported that *Veillonella, Bifidobacterium, Propionibacterium, Actinomyces spp.*, and *Atopobium spp*. bacteria also play an important role in the development and continuation of dental caries [7].

Various ways to prevent dental caries as early as possible, ranging from dental and mouth health education, to topical applications, and control of dental plaque. The most widely applied is the provision of topical applications as a precautionary measure of dental caries. One of the topical application materials that has been widely circulated among the public that is topical application with sodium fluoride material. Fluoride has been widely used as an additive on dental hygiene and dental products since 1950 because of its usefulness as a dental caries prevention. Millimolar concentrations of fluoride ions in a bacterial culture medium can inhibit the growth of bacterial cells [8]. So, this study was conducted to determine other bacterial species of dental caries in patient and also to determine the strength of activity of some toothpastes used in the community against this bacterium.

EXPERIMENTAL DETAILS

Bacterial sample

Sampling had earned ethical clearance from Medical Research Ethics Committee of Faculty of Medicine, Universitas Padjadjaran, Indonesia with ethics number 1073/UN6.C1.3.2/KEPK/PN/2016. Sample was taken from one of the Dental and Oral Polyclinics in Cileunyi, Bandung Regency, West Java, Indonesia. Sample was taken from the patient's plaque with dental caries using sterilized apparatus. The sample plaque was then incorporated into a transport medium containing Trypticasein Soy Broth (TSB) which had been sterilized. Sample was then taken to the laboratory of Microbiology, Faculty of Pharmacy, Padjadjaran University, to be incubated at 37°C for 18-24 hours.

Observation of the morphology of bacterial colony

Observations was done with a microscope to see the color, shape, edge, and elevation of bacterial colonies. Furthermore, Gram-stained bacteria was performed. One ose of bacterial isolate was suspended into sterile distilled water, then fixed on a clean glass object. The streaked bacteria was flooded with two drops of gentian violet carbolic, then left for a minute. The excess color was discarded, then the glass object was rinsed with running water. The bacteria was then flooded with two drops of 2% lugol solution, then left for one minute. Excessive Lugol is discarded, then rubbed with 95% alcohol. After rinsed with running water, smeared with 2-3 drops of 1% fukhsin solution, then left for one minute. Excess color was discarded, then rinsed with water and dried using filter paper. The sample was sprayed with emersi oil, then observed under a microscope at magnification 1,000 times [9].

Identification of bacteria with amplification of gene encoding 16S rRNA

Genetic identification of bacteria including isolation of bacterial chromosome and amplification of gene encoding 16S rRNA [9]. Isolation of bacterial chromosome followed the protocol in PROMEGA® DNA Purification System. The bacterial chromosome was used as template for amplification of gene encoding 16S rRNA using universal primers i.e 27F and 1492R. The component of Polimerase Chain Reaction (PCR) was consisted of 30 pmol primer 1492R; 30 pmol primer 27F; 10 pmol dNTP; 6 µl of ×10 PCR buffer (containing 20 mM $MgCl_2$); 0,002 U of Taq Polymerase; and 2 µl DNA template and added to 50 µl of nuclease-free water. Thermocycler was set with condition i.e

initial denaturation 94°C for 5 min, 30 cycles with denaturation 94°C for 1 min, annealing 55°C for 1 min, lengthening of DNA fragment 72°C for 1 min, then ended amplification at 72°C for 10 min.

DNA sequence determination of gene encoding 16S rRNA fragment

DNA sequence of gene encoding 16S rRNA fragment was determinated by Macrogen in Seoul, East Korea. Analysis of sequence result was performed using blast tool available in http://blast.ncbi.nlm.nih.gov.

Assay of toothpastes activity against bacterial growth

0,5 Mac Farland bacterial suspension was evenly spread on the surface of agar media in Petri dish. Paper discs were put on the surface of solid agar media subsequently was dripped with toothpaste diluted with 0,9 % NaCl (1:2) as much 10 µL. Then, Petri dish was incubated at 37°C for 18-24 hours. Inhibition zone of toothpaste was measured using a callipers.

RESULT AND DISCUSSION

Result of suspension of bacterial sample cultivated on Trypticasein Soy Agar (TSA) can be seen in Fig 1.

Fig 1. Bacterial sample cultivated on Trypticasein Soy Agar

Morphological observations of the colony revealed that colony was white, rod shape, irregular edge and flat elevation. Furthermore, Gram staining result of clinical bacterial-isolate performed that bacterial sample was Gram positive bacteria (Fig 2). So, lysozyme was used for chromosome isolation of Gram positive bacteria to lysis bacterial cell wall. Thus, isolation of bacterial chromosome becomes easier on Gram positive bacteria.

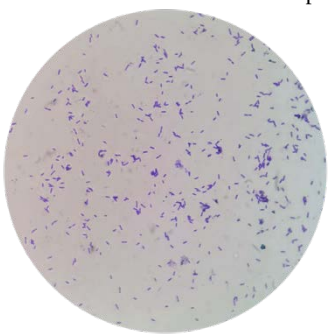

Fig 2. Result of Gram staining of bacteria causing dental caries

Result of bacterial chromosome sample isolated showed size over 10,000 bp. In general, bacterial chromosome can range from 130 kbp to over 14 Mbp. Hereafter, result of amplification of gene encoding 16S rRNA using bacterial chromosome performed DNA band in electroforegram with size ± 1500 bp (Fig 3). The DNA amplification using a primary 27F and a primary 1492F will produce DNA of that size [9].

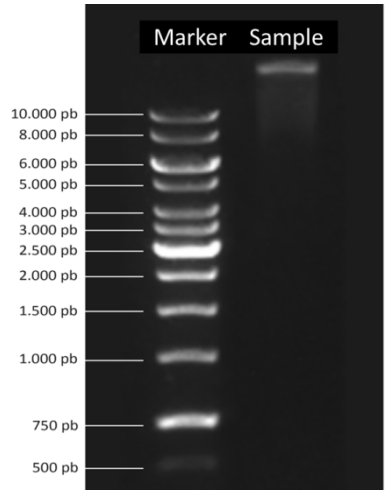

Fig 3. Electroforegram of bacterial chromosome causing dental caries

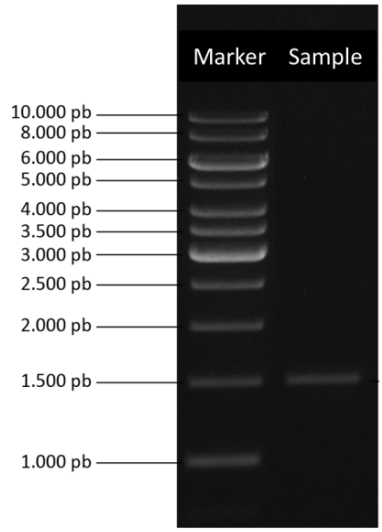

Fig 4. Electroforegram of amplification of gene encoding 16S rRNA

Then, the sequence of DNA encoding 16s rRNA indicated that sample was identified as *B. licheniformis* with homology 99% (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). *B. licheniformis* is a bacterium of the genus Bacillus. This bacteria is aerobic, Gram positive bacteria, can form spores, and can be found in almost all environments. *B. licheniformis* is increasingly known as pathogenic bacteria and causes serious infections, especially in patients with low immunity. This bacteria was been isolated in the case of bacteremia [10,11], peritonitis [12], food poisoning [13] and eye infections [14]. *B. lichiniformis* has never been reported as a bacteria that causes dental caries. Even two types of enzymes derived from *B. licheniformis* have a specific activity against *Streptococcus mutans* that cause dental caries [15]. *Streptococcus mutant* is a bacteria that causes the main factors caries teeth. Dental caries can also be caused by other bacteria such as *Enterococcus faecalis, Actinomyces naeslundii, A. viscosus, Rothia dentocariosa, Propionibacterium, Prevotella, Veillonella, Bifidobacterium, Lactobacillus* and *Scardovia* [16].

Figure 4. Result of DNA sequence fragment of gene encoding 16S rRNA

Table 1. Result of activity assay of toothpastes circulating in the market

Toothpaste	Inhibition Diameter (mm)		Average Diameter (mm)	Deviation Standard (mm)
	1	2		
1 (F)	11.4	14.7	13.05±2.333	2.333
2 (F)	10.6	12	11.3±0.99	0.990
3 (F)	14.1	16.2	15.15±1.485	1.485
4 (F)	11.8	12.6	12.2±0.566	0.566
5 (F)	15.06	14.16	14.61±0.636	0.636
6 (F)	9.12	9.08	9.1±0.028	0.028
7 (F)	17.1	17.14	17.12±0.028	0.028
8 (H)	13	12.18	12.59±0.58	0.580
9 (H)	19.52	19.6	19.56±0.057	0.057
10 (H)	16.96	17.72	17.34±0.537	0.537
11 (F)	15.2	15.2	15.2±0.61	0.000
12 (F)	8.93	8.89	8.91±0.028	0.028

F: toothpaste containing fluoride, H: toothpaste containing herbal

The main process of dental caries is localized demineralization of enamel, expression of degradation of hydroxyapatite in which the process is started in a bacterial biofilm and dental plaque that cover the tooth surface. Caries lesions develop where oral biofilms are allowed to flourish and stay on the tooth for a long time [16]. Bacteria that have been reported as a cause of dental caries also have the ability to form biofilm. Similarly, *B. licheniformis* was known as a bacterium that causes contamination of the pasterised product by forming biofilm [17].

The result of activity assay of 12 toothpastes circulating in the market can be seen in table 1. 12 such toothpastes still had anti-bacterial activity against *B. licheniformis* bacteria from samples of dental caries patient. This can be seen from the presence of inhibition zone diameter resulted from the 12 toothpastes. Toothpaste no. 9 with herbal formulations had the largest diameter inhibition with a diameter of 19.56 mm. This tootpaste contained major herbal composition potentially having anti-bacterial activity such as *Melaleuca alternifolia* leaf oil [18] and activated charcoal. While, toothpaste no. 12 with a fluoride formulation had the smallest diameter i.e 8.91 mm . This is probably due to the main composition of the toothpaste that is fluoride and zinc gluconate lacks anti-bacterial activity.

CONCLUSION

In the sample of dental caries patient was found *B. licheniformis* which was still sensitive to 12 toothpastes circulating in the market.

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