HYBRIDOMA
Volume 31, Number 3, 2012

DOI: 10.1089/hyb.2011.0114

A Feasible Enzyme-Linked Immunosorbent Assay System Using Monoclonal and Polyclonal Antibodies Against Glucosyltransferase-B from *Streptococcus mutans*

Noriko Shinozaki-Kuwahara,[1] Tomomi Hashizume-Takizawa,[2] Masatomo Hirasawa,[1] and Kazuko Takada[1]

Streptococcus mutans has been considered the principal etiological agent of dental caries in humans. *S. mutans* can secrete three kinds of glucosyltransferases (GTFs). One of these, GTF-B, which synthesizes water-insoluble glucans from sucrose, has been considered to be one of the most important factors of cariogenic dental plaque formation. Therefore, determination of whether GTF-B is present in plaque and saliva samples may contribute to the evaluation of individual virulence potential (caries risk). The aim of this study was to develop a feasible enzyme-linked immunosorbent assay (ELISA) for the routine quantification of GTF-B in plaque-derived cultures and clinical samples, and to apply this assay to an epidemiological study. To determine the presence of GTF-B in plaque samples, a sandwich-ELISA was devised, consisting of mouse monoclonal and rabbit polyclonal antibodies against GTF-B and a horseradish peroxidase-conjugated anti-rabbit antibody. The developed ELISA allowed for quantification of the amounts of purified GTF-B with satisfactory sensitivity and specificity; this method was not affected by other components such as plaque and saliva. Plaque samples from healthy volunteers were examined using this ELISA method and microbial analysis to apply the assay to an epidemiological study. A correlation was observed between the amount of extracted GTF-B and *S. mutans* levels as determined by ELISA and cultivated with Mitis Salivarius Bacitracin agar plates derived from plaque samples, although there were some exceptions. In this regard, this ELISA system has the advantage of estimating both the individual numbers of *S. mutans* and the productivity of GTF-B, namely, the cariogenic potential of *S. mutans* simultaneously. These results indicate that this ELISA method is a useful tool for the diagnosis of caries risk.

Introduction

Oral gram-positive mutans streptococci are the principal etiological agents of human dental decay.[1] Among these streptococci, *Streptococcus mutans*, which is isolated as the predominant species from human saliva and dental plaque, can secrete three kinds of glucosyltransferases (GTF-B, C, and D) that catalyze the synthesis of water-insoluble and water-soluble glucans from dietary sucrose.[2] An important virulence factor of *S. mutans* that promotes caries development is its ability to firmly colonize on the tooth surface in the presence of sucrose, and to produce a cariogenic dental plaque *in situ* through the action of these GTFs. Therefore the levels of mutans streptococci in saliva and plaque are a means of predicting the risk of caries. There are several commercially available kits to measure an individual's risk of caries; however, most of them require more than 24 h because of the cultivation of *S. mutans* and/or other oral bacteria present in plaque and saliva samples with appropriate media. To speed up the process of the detection procedure, various methods have been developed to measure the levels of mutans streptococci using monoclonal antibodies (MAbs) and oligonucleotide probes.[3,4] Furthermore, several nucleotide-based detection systems of mutans streptococci, such as real-time PCR, have been reported and are increasingly used.[5,6] However, epidemiological and microbiological studies have revealed different cariogenic potential and genetic diversities among fresh *S. mutans* isolates (i.e., GTFs productivity is different in each *S. mutans*[7,8]), and it is not clear whether the expression levels of mRNA of *gtf* genes and translation levels of GTF proteins are correlated. Thus, we focused on the GTF-B protein, which is considered to be one of the most important factors of cariogenic dental plaque formation. Directly measuring GTF-B present in plaque and saliva samples by ELISA may contribute to the swift evaluation of an individual's caries risk. For this reason, hybridoma cells producing mouse MAb and rabbit polyclonal antibodies (PAb) against *S. mutans* GTF-B were produced in our laboratory.[9] The aim of this

Departments of [1]Oral Microbiology and [2]Oral Immunology, Nihon University School of Dentistry at Matsudo, Chiba, Japan.

study was to develop a feasible, specific, and sensitive ELISA to routinely quantify the amount of GTF-B from *S. mutans* in plaque samples. Also the advantages of this ELISA system for the diagnosis of caries risk applied to epidemical studies using healthy volunteer plaque samples are described.

Materials and Methods

Bacterial strains and culture media

S. mutans PS14 and *Streptococcus anginosus* KSB8[10] were used in this study and grown in Todd-Hewitt broth (THB; Becton Dickinson, Franklin Lakes, NJ). Mitis Salivarius (MS) agar (Becton) supplemented with 0.2 U/mL of bacitracin and 15% sucrose (MSB agar) was used as a selective medium for microbial analysis.

Collection and preparation of clinical plaque samples

Oral samples were collected from 31 healthy volunteers (aged 21 to 23 years) with different *S. mutans* levels on tooth surfaces. All volunteers gave informed consent for the use of their samples in research, and the study protocol was approved by the Ethics Committee of the Nihon University School of Dentistry at Matsudo (EC02015). Clinical brushing-plaque samples from each subject were collected as previously described.[11]

Preparation of anti-GTF-B monoclonal and polyclonal antibodies

MAb P136, which was raised against GTF-B purified from *S. mutans* PS14, was prepared previously[9] and was used for coating of 96-well microtitration plates as the first antibody. Anti-GTF-B PAb was also prepared previously.[9]

Extraction of GTF-B from cultured cells or samples

Cultured cells in THB or clinical plaque samples (1 mL) were centrifuged, and the precipitate was washed with distilled water. This sample was dissolved in 200 μL of 0.5 N NaOH, and left for 1 min at room temperature. Subsequently, 400 μL of 1 M MOPS buffer containing 0.1% ProClin300 (Sigma-Aldrich, St. Louis, MO) for the neutralization of alkaline was added, and diluted with 400 μL of phosphate buffered-saline (PBS) containing 0.05% Triton X-100, 0.2% bovine serum albumin (BSA), and 0.1% Proclin300. Insoluble materials in this solution were removed by centrifugation and the supernatant was used as an extracted GTF-B.

Procedure of ELISA system

The ELISA was based on a sandwich method involving MAb and PAb against GTF-B. MAb P136 was diluted with PBS (10 μg/mL) and applied onto microtiter plate wells (70 μL/well) for immobilization of the microtiter plate and left at 4°C overnight. After three washes with PBS containing 0.05% Tween-20 (PBS-T), the plate was blocked with BSA, left at 4°C overnight, dried at 25°C for 24 h, and stocked at 4°C. Extracted GTF-B samples were pipetted into microtiter plates (70 μL/well) coated with MAb P136, and left at 25°C for 2 h. After three washes with PBS-T, the plates were allowed to react with anti-GTF-B PAb (10 μg/mL diluted with PBS), incubated at 25°C for 1 h, and washed with PBS-T. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Zymed, South San Francisco, CA) was then diluted with PBS to the working concentrations recommended by the manufacturer and added to each well. The plates were incubated at 25°C for 1 h and washed with PBS-T. HRP color development reagent (0.3% *o*-phenylenediamine dihydrocloride and 0.003% H_2O_2) was added and left for exactly 15 min. The reaction was terminated by the addition of 1.5 N H_2SO_4, and the optical density (OD) was measured at 492 nm using a microplate reader.

Microbial analysis

The collected clinical plaque samples were dispersed by sonication (50 W, 10 s), serially diluted, and inoculated on the MSB agar plates. After anaerobic incubation for 48 h, *S. mutans* colonies on MSB agar were counted under a stereomicroscope and the colony-forming units (cfu)/mL of *S. mutans* were calculated for each subject.

Purification of GTF-B expressed in S. anginosus *transformant*

An extracellular recombinant GTF-B (rGTF-B) protein was prepared from the culture fluids of *S. anginosus* KSB8 grown anaerobically at 37°C for 20 h in 1 L of THB containing erythromycin (25 μg/mL) and 1 mM EDTA. Cells were removed by centrifugation (10,000 *g*, 15 min), and the supernatant was mixed with an equal volume of chilled ethanol and then left at 4°C for 3 h. The precipitate was collected by centrifugation (20,000 *g*, 20 min), and was dissolved in distilled water. Insoluble materials were removed by centrifugation and the supernatant was used as the crude enzyme preparation. The purification procedure was described previously,[7] except that lithium dodesyl sulfate was used instead of sodium dodecyl sulfate. Eluted fractions were subjected to Western blot analysis using MAb P136. Immuno-positive fractions were pooled and yielded as purified rGTF-B preparations. Protein estimation was carried out using a DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard protein.

Results

Standardization of ELISA for GTF-B concentration

The purified rGTF-B was used for calibration of the developed ELISA system. The calibration curve was constructed from a set of seven points of the rGTF-B protein. Figure 1 shows the plots of purified rGTF-B concentrations and the OD value measured with this ELISA. A correlation was observed between the amounts of rGTF-B protein and the OD value of this ELISA from 0 to 40 ng/mL of protein. The minimum detection limit for purified GTF-B was 2 ng/mL, and the practical measurement range of this sample was from 4 to 40 ng/mL of protein. Furthermore, the minimum detection limit for extracted GTF-B protein from cultured *S. mutans* cells was 10^4 cfu, and the practical measurement range of this sample was from 10^5 to 10^7 cfu (data not shown).

Specificity of sandwich-ELISA system

The specificity of this sandwich-ELISA for extracted GTF-B from *S. mutans* was examined further in the presence of *S. mutans*-free human saliva, which is a mixture of many other

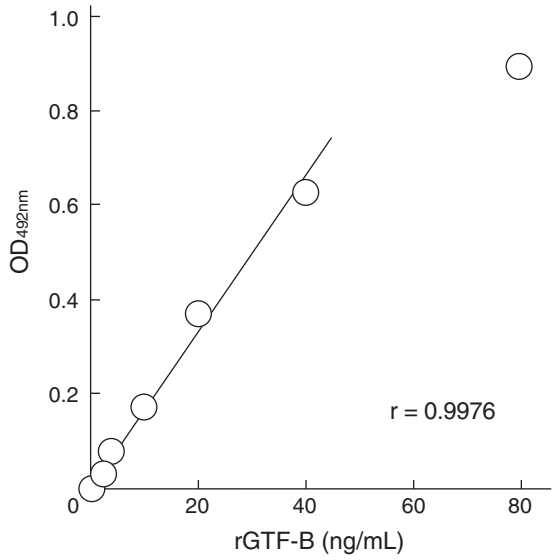

FIG. 1. Calibration curve for purified rGTF-B protein. The means of triplicate samples are shown at each concentration.

oral substances. *S. mutans*-free saliva was prepared by filtration (0.22 μm). One milliliter of *S. mutans*-free saliva or PBS, including various amounts of cultured *S. mutans* cells ($OD_{550nm} = 1.0$), had the GTF-B protein extracted and was subsequently subjected to ELISA. As shown in Figure 2, the present ELISA system is not affected by the many other components of saliva. When other oral streptococci were cultured, extracted protein, and subjected to ELISA, only *S. mutans* was strongly recognized and no other oral streptococci were detected, even *S. sobrinus*, which is another cariogenic mutans streptococci in humans (Table 1). These results strongly suggest that this ELISA system was highly specific against *S. mutans* GTF-B.

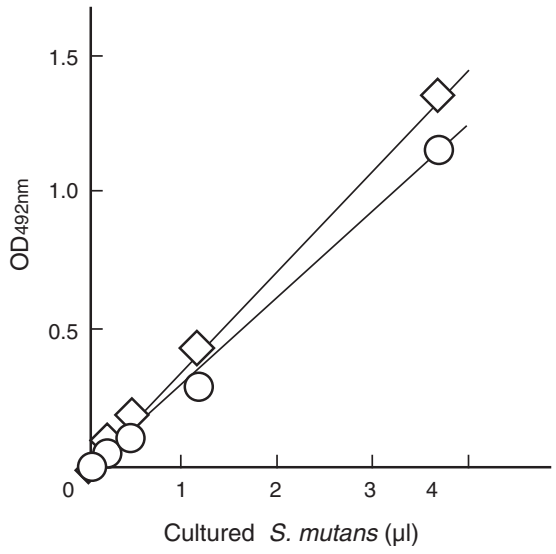

FIG. 2. Specificity of this ELISA system. GTF-B extracted from cultured *S. mutans* cells in *S. mutans*-free human saliva (◇) and PBS (○).

Table 1. Streptococcal Species to Confirm the Specificity of Oral the ELISA System

Species	*Strain*	*Detection*
Streptococcus mutans	PS14	+
Streptococcus sobrinus	6715	–
Streptococcus mitis	9811	–
Streptococcus sanguinis	ATCC10556	–
Streptococcus oralis	ATCC10557	–
Streptococcus gordonii	ATCC10558	–
Streptococcus salivarius	9222	–
Streptococcus anginosus	NCTC10709	–

Application of ELISA system to clinical plaque samples

Previously, three kinds of oral samples, stimulated saliva, swabbing plaque, and brushing plaque, were prepared to evaluate the caries risk.[11] Among these, preparation of the brushing plaque sample is simple and may be useful as a means for assessment of an individual's caries risk. Therefore, the brushing plaque sample was subjected to ELISA in this study. Brushing plaque samples from 31 healthy volunteers were collected, and GTF-B was extracted and subjected to the ELISA test. At the same time, the number of *S. mutans* in collected samples was determined by the cultivation method using selective medium MSB agar plates and calculated cfu of *S. mutans*. Figure 3 shows the result of the ELISA and microbial analysis plot. A positive correlation was observed between the amount of GTF-B estimated by sandwich-ELISA and *S. mutans* levels determined by cultivation with MSB agar ($r = 0.95$). This result strongly suggested that the sandwich-ELISA system would be very useful for epidemiological studies.

Discussion

Since the infectious level of mutans streptococci is largely different from individual oral cavities, each of them possesses a different caries risk.[1,13] It is essential to measure the exact caries risk for effective caries prevention. The quantification of

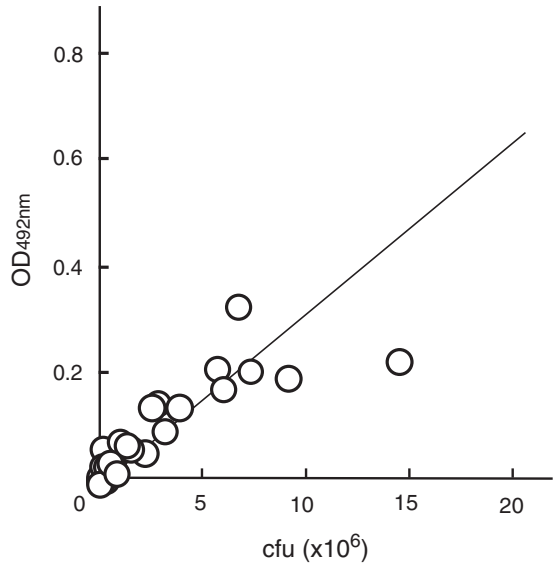

FIG. 3. Relationship between the amount of GTF-B and *S. mutans* levels of 31 clinical plaque samples from volunteers.

mutans streptococci using MSB agar is laborious but necessary for the morphological discrimination of colonies to identify the species of mutans streptococci. In general *S. mutans* has resistance against bacitracin; however, when bacitracin sensitive *S. mutans* exists in oral samples, it is unable to accurately evaluate *S. mutans* levels. For a solution to this problem, several improved MSB media, and the development of genetic detection systems of *S. mutans*, such as quantitative real-time PCR (qPCR), has been reported.[5,6] Indeed, qPCR is a powerful tool for the detection and quantification of bacteria; however, qPCR depends on the efficacy of the cell lyses in RNA extraction procedures. In the case of *S. mutans*, all RNA extraction is very difficult due to the gram-positive bacterial hard cell walls, and it is not clear whether the expression levels of mRNA of *gtf* genes and translation levels of GTF proteins are correlated or not.

Thus, in this study we have established a feasible sandwich-ELISA assay using MAb and PAb targeting the GTF-B protein of *S. mutans*, because this bacterium is able to isolate from almost human saliva and dental plaque, and determination of GTF-B should contribute to the evaluation of an individual's caries risk. This ELISA routinely quantified GTF-B in plaque-derived cultures and clinical samples, was highly sensitive to quantify the amount of the GTF-B protein, was highly specific against *S. mutans*, and was not affected by any other components present in plaque and saliva (Fig. 2). Furthermore, when this ELISA system was applied to clinical plaque samples from 31 healthy volunteers with different *S. mutans* levels, a correlation was observed between the amount of GTF-B determined by this ELISA system and *S. mutans* levels examined by cultivation with MSB agar plates, with some exceptions (Fig. 3). For example, there were no *S. mutans* colonies on MSB agar; however GTF-B was detected by ELISA. In contrast, many *S. mutans* colonies were detected on MSB agar, but GTF-B was barely detected by ELISA. This result suggested that the ability of GTF-B production may be different in each *S. mutans* strain, which is supported by a previous report.[14]

There are several commercially available kits to measure an individual's caries risk; however most of them require more than 24 h because of the cultivation of *S. mutans* and/or other oral bacteria present in plaque and saliva samples with appropriate media. In addition, plural 96-well plates coated with MAb P136 were prepared and stocked at once; only 4 to 5 h operations are sufficient to process up to 25 samples compared to conventional assay methods, which require more than 24 h. Therefore, our sandwich-ELISA system is a useful tool for the diagnosis of caries risk in epidemiological studies. Furthermore, correlativity of the quantity of GTF-B, *S. mutans* levels, and a Decayed Missing Filled Teeth index would be assessed in clinical samples.

Acknowledgments

This study was supported in part by a Grant-in-Aid for SBFSR 2008-2012 from MEXT and a Grant-in-Aid for Young Researchers of Ninon University School of Dentistry at Matsudo, 2011.

Author Disclosure Statement

The authors have no financial interests to disclose.

References

1. Loesche WJ: Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986;50:353–380.
2. Kuramitsu HK: Virulence factors of mutans streptococci: role of molecular genetics. Crit Rev Oral Biol Med 1993;4: 159–176.
3. Cangelosi GA, Iversen JM, Zuo Y, Oswald TK, and Lamont RJ: Oligonucleotide probes for mutans streptococci. Mol Cell Probes 1994;8:73–80.
4. de Soet JJ, van Dalen PJ, Pavicic MJ, and de Graaff J: Enumeration of mutans streptococci in clinical samples by using monoclonal antibodies. J Clin Microbiol 1990;28:2467–2472.
5. Yano A, Kaneko N, Ida H, Yamaguchi T, and Hanada N: Real-time PCR for quantification of *Streptococcus mutans*. FEMS Microbiol Lett 2002;217:23–30.
6. Yoshida A, Suzuki N, Nakano Y, Kawada M, Oho T, and Koga T: Development of a 5′ nuclease-based real-time PCR assay for quantitative detection of cariogenic dental pathogens *Streptococcus mutans* and *Streptococcus sobrinus*. J Clin Microbiol 2003;41:4438–4441.
7. de Soet JJ, van Loveren C, Lammens AJ, Pavicić MJ, Homburg CH, ten Cate JM, and de Graaff J: Differences in cariogenicity between fresh isolates of *Streptococcus sobrinus* and *Streptococcus mutans*. Caries Res 1991;25:116–22.
8. Caufield PW, and Walker TM: Genetic diversity within *Streptococcus mutans* evident from chromosomal DNA restriction fragment polymorphisms. J Clin Microbiol 1989;27: 274–278.
9. Fukushima K, Okada T, and Ochiai K: Production, characterization, and application of monoclonal antibodies which distinguish three glucosyltransferases from *Streptococcus mutans*. Infect Immun 1993;61:323–328.
10. Fukushima K, Ikeda T, and Kuramitsu HK: Expression of *Streptococcus mutans gtf* genes in *Streptococcus milleri*. Infect Immun 1992;60:2815–2822.
11. Neta T, Inokuchi R, Shinozaki-Kuwahara N, Kouno Y, Ikemi T, and Fukushima K: Investigation of microbiological methods to estimating individual caries risk. Evaluation of sampling methods and materials. Int J Oral-Med Sci 2002;1:29–30.
12. Hayakawa M, Hosogi Y, Takiguchi H, Saito S, Shiroza T, Shibata Y, Hiratsuka K, Kiyama-Kishikawa M, and Abiko Y: Evaluation of the electroosmotic medium pump system for preparative disk gel electrophoresis. Anal Biochem 2001;288:168–175.
13. Hamada S, and Slade HD: Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980;44: 331–384.
14. Tomita Y, Zhu X, Ochiai K, Namiki Y, Okada T, Ikemi T, and Fukushima K: Evaluation of three individual glucosyltransferases produced by *Streptococcus mutans* using monoclonal antibodies. FEMS Microbiol Lett 1996;145:427–432.

Address correspondence to:
Dr. Noriko-Shinozaki Kuwahara
Department of Oral Microbiology
Nihon University School of Dentistry at Matsudo
2-870-1, Sakaecho-Nishi
Matsudo
Chiba 271-8587
Japan

E-mail: kuwahara.noriko@nihon-u.ac.jp

Received: December 27, 2011
Accepted: March 5, 2012