Detection of cariogenic bacteria genes by a combination of allele-specific polymerase chain reactions and a novel bioluminescent pyrophosphate assay

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Abstract

We developed a novel bioluminescent assay for detection of pyrophosphate in polymerase chain reaction (PCR) product. The principle of this method is as follows: pyrophosphate released by PCR is converted to adenosine 5′-triphosphate (ATP) by pyruvate phosphate dikinase in the presence of the substrate pyruvate phosphate and the coenzyme adenosine 5′-monophosphate; subsequently, ATP concentration is determined by firefly luciferase reaction. The detection limit of pyrophosphate is $1.56 \times 10^{-12}$ mol assay. Additionally, luminescent intensity reached a maximum at $\sim 100$ s and remained elevated beyond 10 min. This approach is applicable to the detection of cariogenic bacteria in dental plaque. Thus, the allele-specific PCR products of \textit{Streptococcus mutans} and \textit{Streptococcus sobrinus} developed in this study were measured via the proposed bioluminescent assay. This protocol, which does not require expensive equipment, can be utilized to rapidly monitor cariogenic bacteria in dental plaque.

Keywords: Bioluminescence; Pyrophosphate; Cariogenic bacteria; PCR; Dextranase gene

Maintenance of tooth health, which is important to the health of the entire body, enhances the quality of life. Removal of dental plaque by tooth brushing following meals is effective in the prevention of caries. Bacteria comprise 75% of dental plaque. One gram of dental plaque contains $2 \times 10^{11}$ bacterial cells \cite{1}. \textit{Streptococcus mutans} and \textit{Streptococcus sobrinus}, which are the primary cariogenic species, play a role in the generation of caries; consequently, these bacteria have been vigorously examined \cite{2-6}. Previous data clearly demonstrated that cariogenic ability is promoted when two species coexist \cite{7}. Therefore, identification and determination of the habitation ratio of these species as a diagnostic method for caries prevention in a clinical setting are essential.

Currently, identification of \textit{S. mutans} and \textit{S. sobrinus} is conducted with biochemical and immunological techniques or via analysis of a bacillus gene involving allel-specific PCR, restriction enzyme fragment length polymorphism, and hybridization methodologies, etc. However, these approaches generally employ slab gel electrophoresis; as a result, considerable time and skill are required for proper utility of these distinct procedures. In addition, results are occasionally unsatisfactory. Furthermore, application of these techniques in a clinical setting presents problems.

Previously, we developed a bioluminescent detection method for the O157 VT gene, which involved the
luciferin–luciferase reaction following transformation of pyrophosphate produced during PCR to ATP by adenosine 5'-phosphosulfate (APS) and ATP sulfurylase. However, the sensitivity of this technique was insufficient due to the slight light emission of APS during the luciferin–luciferase reaction, leading to elevation of the blank value. Furthermore, APS and ATP sulfurylase, which are expensive, display poor stability for utility in routine analysis.

Pyruvate phosphate dikinase (PPDK) catalyzes the conversion of phosphoenolpyruvate (PEP) to ATP, phosphate, and pyruvate in the presence of AMP and pyrophosphate. PPDK catalyzes ATP synthesis in microorganisms lacking pyruvate kinase such as Entamoeba histolytica and Clostridium symbiosum. Furthermore, significant work on this enzyme has been conducted in plants, including Propionibacterium shermanii, Acetobacter xylinum, photosynthetic bacteria, rice, and corn with respect to the glycolytic pathway, i.e., PEP synthesis. Generally, PPDK occurs in low concentrations; moreover, this protein is unstable upon freezing and under alkaline conditions.

Eisaki et al. isolated PPDK in the heat-resistant microorganism Microbispora rosea aerata (IFO 14047); subsequently, the PPDK gene was transfected into Escherichia coli. This PPDK displayed molecular weight of 230,000. In addition, PPDK is stable in a wide pH range; it also exhibits thermal stability and stability with regard to chemical modification. PPDK produced by the recombinant technique was also applied to the high-sensitivity measurement of ATP, AMP, and RNA.

In this study, the dextranase gene (dex) in S. mutans was selected as a marker gene for preventive diagnosis of cariogenicity. Allele-specific PCRs, which consisted of mutans PCR and sobrinus PCR, were developed relative to slightly distinct dex genes in S. mutans and S. sobrinus. Furthermore, a bioluminescent assay for pyrophosphate functioned as the detection methodology of PCR amplification.

**Materials and methods**

**Reagents**

PPDK from M. rosea aerata (EC2.7.9.1) and thermostable Luciola cruciata firefly luciferase (EC 1.13.12.7) were obtained from Kikkoman (Chiba, Japan). Pyrophosphate, lysozyme, and proteinase K were purchased from Wako Pure Chemical Industries (Osaka, Japan). dNTP mixture, Taq DNA polymerase, and 10× PCR buffer were acquired from Takara Shuzo (Osaka, Japan). Primers were synthesized by Takara Shuzo (Osaka, Japan). Luciferin and mutanolysin were obtained from Sigma Chemical (St. Louis, MO). Perfect match (PCR Enhancer) was manufactured by Toyobo. Other reagents were of analytical grade.

**Apparatus**

The Luminescence Reader BLR-301 (Aloka, Tokyo, Japan), Wallac 1420 ARVOx multilabel counter, and Gene Amp PCR System 9600 (Perkin–Elmer, Norwalk, CT) were employed.

**Extraction of DNA from S. mutans and S. sobrinus in dental plaque**

The dex genes of S. mutans and S. sobrinus consist of 2550 and 4011 bp, respectively. DNA extraction of bacterial plaque samples (dental plaque) was conducted following protein degradation with lysozyme. Collected dental plaque was suspended in 500 μL of 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA (TE buffer). The suspension was heated at 100°C for 10 min. After cooling, DNA extraction was effected via addition of 40 μL of lysozyme (50 mg/mL), 1000 U of mutanolysin, and 3 μL of proteinase K (20 mg/mL) to S. mutans and S. sobrinus cells in dental plaque. All reagents were dissolved in a final concentration of 1.3% SDS. Following phenol–chloroform extraction, DNA was refined by ethanol precipitation.
Isolated DNA samples were dried, followed by dissolution in TE buffer. Samples were stored at −40°C until use.

**Allele-specific PCR and common PCR**

Strains of *S. mutans* and *S. sobrinus* were derived from dental plaque samples following clinical isolation. PCR conditions with regard to dNTP concentration and improvement of PCR specificity were assessed to obtain specific strong signals with respect to bioluminescence detection. Two allele-specific PCR methods (*mutans* and *sobrinus* PCR), involving individual primers specific to *mutans* and *sobrinus*, and common PCR, involving a primer common to *mutans* and *sobrinus*, were developed. In allele-specific PCR, sequences of the sense (MF) and antisense (MR) primers for amplification of *S. mutans* (*mutans* PCR) were 5'-GACCTTAAGGTGCAAGAGAA-3' and 5'-GGTTCTGTGCAGTTTCTAGC-3', respectively, and sequences of the sense (SF) and antisense (SR) primers for amplification of *S. sobrinus* (*sobrinus* PCR) were 5'-GGGTTATCCGAGCAACAAGT-3' and 5'-GGTTGACATCTTCACCCTGA-3', respectively. In common PCR, sequence of the sense (CF) and antisense (CR) primers for amplification of *S. mutans* and *S. sobrinus* was 5'-CTGTTTTGCCAGAGACGGAATAC-3' and 5'-GTATCACCCTGCCAACCATCAAA-3', respectively. These PCR protocols were performed as follows. Template DNA (1 μL), which corresponded to 20 ng as bacterial DNA and 1 μL of Taq polymerase (2.5 U), was introduced to 48 μL of mixed solution containing 5 μL of 10× buffer, dNTP (dATP, dCTP, dGTP, and dTTP), 1 μL of each primer (20 pmol), and H₂O. The reaction was conducted at 94°C after a 5-min heating process (94°C for 1 min) at 45°C for common PCR or at 55°C for allele-specific PCR for 1 min and at 72°C for 1 min. The number of PCR cycles was 25. Following the final PCR cycle, the reaction was completed at 72°C for 7 min. Allele-specific and common PCR products were determined with the bioluminescent assay.

**Bioluminescent detection of PCR products and pyrophosphate**

Pyrophosphate solution or PCR product (10 μL), which was diluted 10 times with H₂O, was introduced to a test tube; subsequently, 100 μL of PPDK luciferin–luciferase solution (PPDK reagent) (2.34 U/mL PPDK, 0.2 mM luciferin, 5.5 U/mL luciferase, 0.0125 mM AMP, 0.04 mM PEP, 0.005 U/mL apyrase, 0.05 mM di-thiotheritol, 5% trehalose, 1 mM EDTA, 7.5 mM MgSO₄, 30 mM 2-bis[2-hydroxyethyl]amino)ethanesulfonic acid, pH 8.0) was added. After 150 s, emission intensity was measured for 10 s in the luminescence reader (Aloka).

**Results and discussion**

**Measurement of pyrophosphate via bioluminescence detection**

PPDK–luciferin/luciferase reaction has been employed for measurement of AMP and RNA. To apply the PPDK–luciferin–luciferase reaction to measurement of pyrophosphate in the present investigation, conditions of the AMP measurement system involving this reaction were improved and the optimal conditions for measurement of pyrophosphate based on the presence of excess AMP were determined as PPDK–luciferin–luciferase solution described under Materials and methods.

**Kinetics of light emission for measurement of pyrophosphate**

To obtain optimum reaction time for the measurement of pyrophosphate, PPDK–luciferin–luciferase reagent (100 μL) was introduced to 10 μL of pyrophosphate; subsequently, light emission intensity was measured from the initiation of the reaction. As a result, light emission intensity reached a plateau after 100 s; the reading remained elevated beyond a 10-min period. Kinetics of light emission is presented in Fig. 2.

**Calibration curve of pyrophosphate**

The calibration curve of pyrophosphate was obtained via this method. Pyrophosphate from 1×10⁻¹² to 1×10⁻³ M was examined. The calibration curve of pyrophosphate is illustrated in Fig. 3. The detection limit of pyrophosphate was 1.56×10⁻¹⁵ mol/assay (as blank +2 SD).

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**Fig. 2.** Time course of bioluminescent intensity. Pyrophosphate concentrations of 1×10⁻⁷, 1×10⁻⁸, 1×10⁻⁹, and 0 M (H₂O) were measured. The 10-μL samples were measured according to the procedure under Materials and methods.
The underlying principle of this approach involves the determination of pyrophosphate released during allele-specific amplification of genes of cariogenic species via the bioluminescent PPDK–luciferin–luciferase reaction. Previously, we developed allele-specific PCRs for *S. mutans* and *S. sobrinus* with regard to detection of cariogenic bacterial genes, which are analyzed by microchip electrophoresis. However, excess dATP used in PCR and nonspecific PCR amplification observed in electrophoresis may raise the blank value during light emission detection based on this principle, which influences the sensitivity and specificity. Therefore, dNTP concentration, *Taq* DNA polymerase amount, PCR cycle number, and annealing temperature were reexamined.

**Effect of dNTP concentration.** Pyrophosphate is formed in the PCR extension reaction; pyrophosphate was released during thermal decomposition of dNTP during PCR. In addition, use of high concentrations of dATP leads to elevation of the blank value of the bioluminescence reaction following PCR (data not shown). Luciferin/luciferase utilized in this study reacts with pyrophosphate released during thermal decomposition of dNTP. Therefore, to obtain optimum light emission intensity, high S/N ratio and rapid completion are required; 25 cycles appear optimum.

**Effect of cycle number.** dNTP decomposes upon heating above 90°C; slight pyrophosphate release occurs. Therefore, limiting the number of PCR cycles associated with heating is highly desirable. In addition, PCR characterized by increased cycle number generally produces nonspecific PCR product. Consequently, reduced cycle number is important to enhance specificity. As a result, cycle number was examined. Light emission intensity increased with cycle number. However, increases in the number of PCR cycles exceeding 30 diminished light emission intensity of the PCR product; in contrast, the blank value increased due to decomposition consequent to extended heating and nonspecific amplification of dNTP. Therefore, to obtain optimum light emission intensity, high S/N ratio and rapid completion are required; 25 cycles appear optimum.

**Effect of annealing temperature.** This investigation was conducted employing optimum conditions (dNTP 0.4µL, *Taq* polymerase 1µL, PCR, 25 cycles). Annealing temperature was examined subsequently. The experiment was performed at temperatures of 45, 50, and 55°C. However, changes in the S/N ratio could not be observed at these temperatures. 55°C was selected for specific duplex allele-specific PCR; on the other hand, 45°C was selected for common PCR to achieve equal amplification of *S. mutans* and *S. sobrinus*.

**Measurement of PCR product**

Allele-specific PCRs (*mutans* and *sobrinus* PCRs) employing a set of MF and MR primers for the *dex* gene in *S. mutans* and a set of SF and SR primers for the *dex* gene in *S. sobrinus* can be amplified specifically; consequently, *S. sobrinus* and *S. mutans* can be identified readily. Common PCR is not specific for *S. sobrinus* and *S. mutans*; therefore, identification of these bacteria was conducted via allele-specific PCR (*mutans* and *sobrinus* PCRs). Results of bioluminescent detection for the two allele-specific PCR techniques are exhibited in Fig. 4A. Light emission intensity was presented as the S/N ratio (S, signal of PCR product using template DNA; N, noise of PCR product without DNA). When sharply. Therefore, 0.8μL of dNTP was selected for PCR conditions characterized by a S/N ratio at emission intensity in excess of 10 and a smaller reduction of the PCR product.

Effect of *Taq* DNA polymerase quantity. Optimum conditions for *Taq* polymerase consist of pH 8.3–8.8 and temperature of 70–75°C; moreover, unit amount in PCR is the usual 1–2.5U. In this study, *Taq* polymerase quantity of 0.25–5U was examined. As a result, the steady state was present upon addition of 1µL of *Taq* polymerase (data not shown). Therefore, polymerase quantity of 1µL (5U) was utilized in the following experiment.

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S. mutans and S. sobrinus DNA which corresponded to 20ng as bacterial DNA were used, the S/N ratio of S. mutans was 13.7 and that of S. sobrinus was 13.0. This finding indicates that S. mutans and S. sobrinus were clearly identified by the respective bioluminescent allele-specific PCR approaches.

**Bioluminescence detection of PCR product obtained by common PCR**

As described above, identification of S. mutans and S. sobrinus is challenging due to nonspecific amplification of both bacteria by common PCR. However, types of fungi, which inhabit the human mouth, approximate S. mutans and S. sobrinus with regard to the dex gene; consequently, detection of one or the other bacterium is sufficient for cariogenic prevention. Therefore, to identify S. mutans and S. sobrinus, common PCR with CF and CR primers was conducted; subsequently, PCR products were determined by bioluminescent PPDK–luciferin–luciferase reaction. As a result, supersetitive detection of both genes of S. mutans and S. sobrinus was possible; S/N ratios were 21.6. This finding indicates that common PCR is suitable for sensitive determination of cariogenic species. The result is presented in Fig. 4B.

**Specificity of allele-specific PCRs and a common PCR**

The specificity of allele-specific PCR (mutans and sobrinus PCR) was determined with DNA extracted from bacteria. These bacteria were divided into three groups (Table 1): group 1, mixed sample of S. mutans and S. sobrinus; group 2, mixed sample of dex(+) bacteria (3–7) in Table 1; and, group 3, mixed sample of dex(−) bacteria (8–13) in Table 1. DNA extracted from these groups was mixed and amplified by duplex PCR, which consisted of mutans PCR and sobrinus PCR, and by common PCR following bioluminescent pyrophosphate assay.

As a result, bioluminescent assay of duplex PCR composed of mutans PCR and sobrinus PCR demonstrates bioluminescent intensity exclusively for a mixture of S. mutans and S. sobrinus in group 1. In contrast, bioluminescent assay involving common PCR reveals bioluminescent intensity with respect to DNA of groups 1 and 2 (dex(+)); however, group 3 (dex(−)) exhibits no bioluminescent intensity. Therefore, the two allele-specific PCR techniques (mutans and sobrinus PCRs) were specific for S. mutans and S. sobrinus; moreover, common PCR, which amplified DNA involving dex(+), did not amplify specific DNA with dex(−). Consequently, allele-specific PCR can function as a specific detection method for S. mutans and S. sobrinus, whereas common PCR is applicable to fungi characterized by dex(+). These results are depicted in Fig. 5.

**Detection limit of dex gene of S. mutans and S. sobrinus**

The detection limit of the bioluminescent pyrophosphate assay of dex genes of S. mutans and S. sobrinus was examined; 20, 2, 0.2, 0.02, 0.002, and 0.0002ng/μL DNA of S. mutans and S. sobrinus served as a template. As a result, 20pg of DNA of both S. mutans and
S. sobrinus was detectable by both allele-specific PCR protocols consisting of 25 cycles. The detection limit was expressed as an S/N ratio exceeding 1.5 (data not shown). This sensitivity corresponded to that of agarose gel electrophoresis involving nondiluted PCR product (5 μL) and ethidium bromide as an intercalating agent.

Reproducibility of bioluminescence detection method

The reproducibility of the proposed method was examined using allele-specific PCR products of S. mutans and S. sobrinus. Reproducibility (CV%, n = 6) demonstrated high accuracy, 4.65 and 3.28%, respectively.

Application to dental plaque samples

Bioluminescent detection of allele-specific PCR (mutans and sobrinus PCRs) was applied to the detection of S. mutans and S. sobrinus in dental plaque with regard to a preventive diagnosis of cariogenicity. DNA was extracted from dental plaque samples (YBK1~YBK11); subsequently, mutans and sobrinus PCRs were performed followed by bioluminescent pyrophosphate assay.

The specificity of this method was examined previously using the gene of 13 types of dex(+) and dex(−) bacteria. Those findings demonstrated that these PCR approaches afforded great specificity for S. mutans and S. sobrinus (Fig. 5). However, specificity of PCR with respect to dental plaque samples declined consequent to the large number of bacteria, usually 300–400 types, that exist in dental plaque; that is, allele-specific PCR nonspecifically amplified other DNA. Subsequently, PCR conditions (annealing temperature, PCR cycle number) for specific amplification of S. mutans and S. sobrinus genes in DNA extracted from dental plaque specimens were reexamined.

As a result, PCR conditions were modified as follows: addition of 5 μL of dimethyl sulfoxide, annealing temperature of 57°C, and PCR cycle number of 30. Under these conditions, nonspecific PCR amplification of dental plaque samples was not observed.

Measurement of S. mutans and S. sobrinus in dental plaque samples by bioluminescent detection of allele-specific PCR

S. mutans and S. sobrinus in dental plaque samples (YBK1~YBK11) were identified via allele-specific PCR (mutans and sobrinus PCRs). In this experiment, 96-well microtiter plates and a light emission measurement instrument (Wallac 1420 ARVOsx multilabel counter) were utilized. As shown in Fig. 6, DNA of S. mutans and S. sobrinus from all 11 specimens was detected. In each sample, the abundance ratio of S. mutans and S. sobrinus is distinct (Fig. 6); furthermore, fungus quantities may vary also. The size of dental plaque samples is different; therefore, accurate quantitative determination of S. mutans and S. sobrinus is not possible. Consequently, several methods for correct dental plaque
sampling are necessary. At present, a technique for amplification of mutans streptococcus DNA employing PCR, which simultaneously detects normal oral cavity bacteria in dental plaque as a control, and a correction method related to sampling amount utilizing pigment to dye dental plaque are under development. In addition, the improvement in sensitivity of allele-specific PCR employing the longer PCR product, which generates larger quantities of pyrophosphate, was examined.

Conclusion

A novel bioluminescent pyrophosphate assay utilizing the PPDK–luciferin–luciferase reaction was established to quantitatively measure PCR product. Detection of pyrophosphate \( (1.56 \times 10^{-15} \text{ mol/assay}) \) characterized by enhanced sensitivity was possible with the proposed method. Furthermore, this bioluminescent assay in association with allele-specific PCR was applied to the analysis of the dex gene of mutans streptococcus. Allele-specific PCR for S. mutans and S. sobrinus involving mutans and sobrinus primers and common PCR employing the primer common to both S. mutans and S. sobrinus were developed for this analysis. Consequently, detection and identification of S. mutans and S. sobrinus were possible; moreover, combination with common PCR afforded supersensitive detection of S. mutans and S. sobrinus. Based on the aforementioned data, analysis of mutans streptococcus via the novel bioluminescent assay for PCR product appears to afford a technique suitable for diagnosis and prevention of cariogenic bacteria.

References