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Technical note

Rapid detection of the *Streptococcus mutans cnm* gene by loop-mediated isothermal amplification

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ABSTRACT

This study investigated a method using loop-mediated isothermal amplification (LAMP) for the rapid detection of *cnm*-positive *Streptococcus mutans* (*S. mutans*) associated with cerebral microhemorrhage. LAMP amplified the *cnm* gene plasmid vector, but not human or microbial genomic DNA. The *cnm* DNA of the *cnm*-positive *S. mutans* strain was detected in saliva without DNA extraction after 1 day of culture. This method resulted in a *cnm*-positive rate of 26.4% in 102 samples, which was higher than that obtained with conventional PCR. In conclusion, LAMP may be used for the detection of *cnm*-positive *S. mutans* in a large number of samples.

1. Introduction

Streptococcus mutans (*S. mutans*) is a pathogen that causes dental caries, which may result in tooth loss. Recent studies have reported that *S. mutans* is associated with systemic diseases in addition to periodontal disease [1,2]. Specifically, *S. mutans* with cnm collagen-binding ability is associated with cerebral microbleeds [3,4]. Furthermore, infection with *cnm*-positive *S. mutans* is a potential risk factor for cerebral hemorrhagic stroke [5]. Therefore, it is important to evaluate whether a patient is infected with *cnm*-positive *S. mutans*.

The conventional method for detecting *S. mutans* DNA is to culture saliva or dental plaque on Mitis-Salivarius agar containing bacitracin (MSB) for 48 h. Colonies are subsequently incubated in brain heart infusion broth (BHI) for 1 day. DNA is extracted from the bacteria, and the target *S. mutans* gene is amplified by the polymerase chain reaction (PCR) [3]. This time-consuming and labor-intensive process typically takes up to 3–4 days to complete.

The loop-mediated isothermal amplification (LAMP) method was developed to ensure the specific, efficient, and rapid amplification of DNA under isothermal conditions [6–8]. Furthermore, loop primers, which hybridize to the stem-loops, and prime strand displacement DNA synthesis reduce the reaction time to less than half that of the original LAMP method [9]. Additionally, calcein, the main component of the fluorescence visual detection reagent, binds manganese ions that quench fluorescence. Thus, when a manganese ion is deprived of the pyrophosphate ion generated by LAMP, fluorescence is emitted [10]. The LAMP method can be applied for the direct detection of pathogens in crude samples without DNA purification steps [11,12], and has been used to detect the causative agents of tuberculosis, mycoplasma, influenza, and severe acute respiratory syndrome [13–16]. The rapid detection of causative bacteria and viruses leads to prompt treatment and prevention, even in chronic diseases.

The present study investigated a method using LAMP for the rapid detection of *cnm*-positive *S. mutans* associated with cerebral microhemorrhage.

2. Material and methods

All experimental protocols were approved by the epidemiological research ethics review committee of Hiroshima University (no. E−786-2). All subjects provided written informed consent.

Vector construction: The *S. mutans cnm* gene fragment was amplified from a PCR product using the Expand Long Template kit (Roche, Toyo, Japan), as previously described [3], and cloned into pGEM-T Easy (Promega, Tokyo, Japan), a thymidine adenine cloning vector. The resulting product was named pGEMcnm. The primer set used for the *cnm* gene was as follows: forward primer 5′-CAGACTGAATGTCATCTT CAA-3′ and reverse primer 5′-GCAGTAACATTTCATCGCTG-3’.

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Table 1
Primers used in the present study.

Primer name	5′-primer sequence-3′	conc. (nM)
FIP	AACCATTAAGCTGGAGGTTCAGGAACTGCTTTGTCTTGCGT	1600
BIP	CGTATAACCTGTTCCTCTGACTGTAATATTAAAGCAGGCGACAC	1600
F3	CCAGTAATACTGTCATTGAAAGT	400
B3	CGCTTTGAGTTTGATGAGC	400
loop F	GCAAGTATGTTGGTGATTTG	800
loop B	CCTGAATTCTGCCAGTTAAC	800

Bacterial strains: Clinical isolates of *S. mutans* and *Candida species,* and the standard strains *S. sobrinus* (OMZ176a) and *Porphyromonas gingivalis (P. gingivalis,* W83), were used in this study.

Collection and processing of clinical isolates: Saliva was collected from 102 volunteers following *paraffin wax chewing* stimulation. Saliva samples (20 μl) were added to 60-mm Mitis-Salivarius agar (Becton Dickinson, NJ, USA) plates containing bacitracin (Sigma-Aldrich, St. Louis, USA). The samples were cultured at 37 °C for 24 h. Subsequently, 50 μl BHI medium (Nissui Pharmaceutical, Tokyo, Japan) was added to the MSB agar, and all the colonies were collected. Analyses of *cnm* expression were performed after all samples were confirmed to be *S. mutans.* The samples were stored at −80 °C until use.

LAMP reaction: LAMP primers for the *S. mutans cnm* gene were designed using PrimerExplorer V5 software (Fujitsu, Tokyo, Japan). The primer sequences and concentrations are presented in Table 1. The LAMP reaction was performed using DNA Amplification Reagent D (Eiken Chemical, Tokyo, Japan), the specific primers, Fluorescent Detection Reagent (Eiken Chemical), 0.8 M betaine, and 1 μl of the BHI broth containing colonies from the MSB agar. The mixture was incubated at 64 °C for 2 h. The human genomic DNA template was purchased from Millipore Sigma (St. Louis, MO, USA).

Limit of detection: The pGEMcnm plasmid vector (10^4, 10^3, 10^2, and 10 molecules) and OD_{600} *cnm*-positive strains (10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) were prepared in separate LAMP reaction tubes. *cnm* gene expression was confirmed by LAMP.

3. Results and discussion

Specificity and sensitivity test: To examine specificity, the LAMP reaction was performed using human genomic DNA and the pGEMcnm plasmid vector as templates. The pGEMcnm plasmid vector was amplified by LAMP, but human genomic DNA was not (Fig. 1A). This suggested that the primer set used did not amplify DNA derived from human oral cells in the salivary samples. In the sensitivity test, *cnm* was detected with 10^3 pGEMcnm plasmid vector molecules, but not with 10^2 molecules (Fig. 1B). In the diluted solution of *cnm*-positive strains, *cnm* was detected with 10^{-5} OD_{600}, but not with 10^{-6} OD_{600}, suggesting that the LAMP method may be used to detect the *cnm* gene if ~10^3 bacterial cells are present (Fig. 1C). For detection in a fewer number of cells, direct detection in saliva may still be possible though judicious primer design. *S. sobrinus*, *P. gingivalis,* and *Candida* spp., which are present in the oral cavity of several mammals, do not have the *cnm* gene and were not detected (Fig. 1D). Therefore, the novel method described in the present study can accurately detect *cnm*.

Comparison of culture conditions: Saliva containing *cnm*-positive *S. mutans* was cultured on MSB agar, and LAMP was subsequently performed. A 1-day culture in MSB agar is required to selectively culture *S. mutans*, because 1 mL of human saliva from a healthy adult contains approximately 100 million bacterial cells [17]. The results showed that *cnm* DNA was detected without DNA extraction after 1 day of culture. However, when the same saliva was cultured in BHI medium alone, *cnm* DNA was not detected, even after 1–2 days of growth (Fig. 1E). This result suggested that LAMP enabled the detection of *cnm* following a short culture period, even when DNA was not extracted.

Positive rates for the cnm gene in 102 samples: The positive rates for the *cnm* gene were examined in saliva from 102 volunteers. With LAMP, 27 positive cases were identified, with a positive rate of 26.4% (Fig. 1F). The distribution frequencies of *cnm*-positive *S. mutans* in Japan, Finland, and Thailand were previously reported to be 10–20% using PCR [18–20]. In the present study, the *cnm* positive rate in the same samples using PCR was 12.7% (13/102) (data not shown).

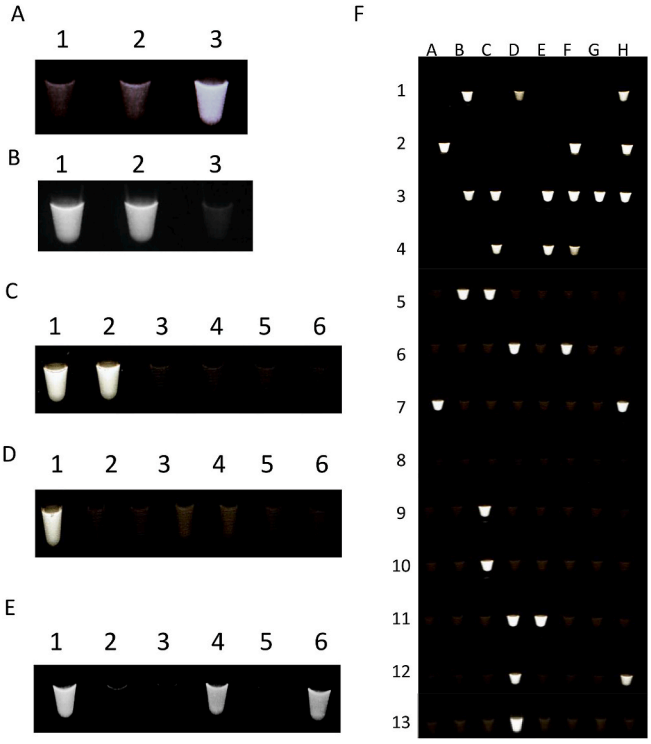

Fig. 1. LAMP detection of the *cnm* gene. (A) Specificity test. 1: negative control (no DNA), 2: human genomic DNA, and 3: *cnm* plasmid DNA. (B) Sensitivity test with *cnm* plasmid DNA. 1: 10^4 molecules, 2: 10^3 molecules, and 3: 10^2 molecules. (C) Sensitivity test using the diluted solution of the *cnm*-positive strain 1: 10^{-4} OD, 2: 10^{-5} OD, 3: 10^{-6} OD, 4: 10^{-7} OD, 5: 10^{-8} OD, and 6: negative control. (D) Detection of the *cnm* gene in *S. sobrinus*, *P. gingivalis,* and *Candida* spp. 1: positive control (*cnm*-positive strains), 2: *S. sobrinus* (cells) 3: *S. sobrinus* (DNA), 4: *P. gingivalis* (cells), 5: *P. gingivalis* (DNA), 6: *Candida* spp. (cells), and 7: *Candida* spp. (DNA). (E) Comparison of culture differences. 1: positive control (*cnm* plasmid DNA), 2: negative control, 3: saliva cultured in BHI medium for 1 day, 4: all colonies cultured on MSB agar for 1 day, 5: saliva cultured in BHI medium for 2 days, and 6: all colonies cultured on MSB agar for 2 days. (F) Positive rates of the *cnm* gene in 102 samples. The *cnm* gene expression in 102 samples was examined by the LAMP method and 27 samples were positive. Positive control (*cnm*-positive DNA, H12). Negative control (no DNA, H13). LAMP, loop-mediated isothermal amplification; BHI, brain heart infusion; MSB, Mitis-Salivarius agar containing bacitracin.

Conclusion: A conventional method for detecting *cnm*-positive *S. mutans* is to increase the number of colonies in BHI medium after selectively culturing *S. mutans* on MSB agar and subsequent DNA extraction. The target gene is amplified by PCR, and agarose gel electrophoresis is performed to confirm the expression of the target gene

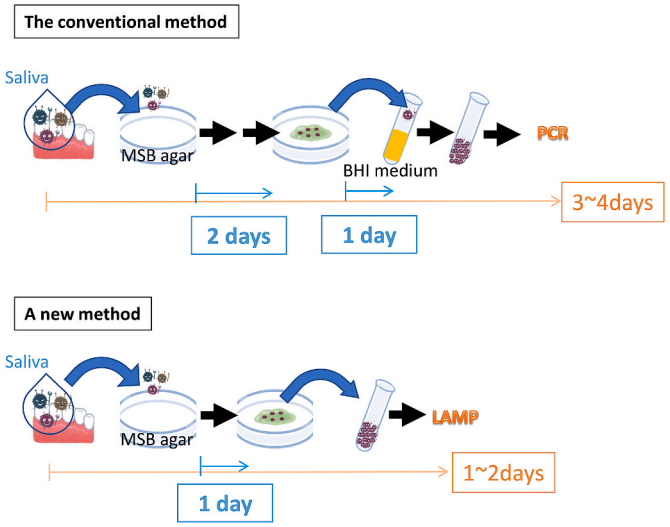

Fig. 2. Schematic representation of *Streptococcus mutans* detection methods. Upper panel: conventional PCR. This process takes 3–4 days from saliva collection. Lower panel: new method. The results can be obtained within 2 days.

[3]. This process takes 3–4 days from saliva collection. With LAMP, all colonies are collected in BHI medium after culturing *S. mutans* on MSB agar. BHI was used to collect the bacterial colonies; however, tris-ethylenediaminetetraacetic acid buffer or distilled water may also be used. Since *cnm*-positive *S. mutans* can be directly detected using the liquid, the results can be obtained within 2 days (Fig. 2). Thus, LAMP appears to be effective in screening *cnm*-positive *S. mutans* carriers and these patients can be forewarned of the risks for cerebral microbleeds and cognitive decline. Early detection enables proactive treatment, thereby diminishing the risk of deterioration in quality of life.

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Author contributions

The study was conducted and designed by Masae Kitagawa, Kentaro Nagamine, and Hidemi Kurihara. The clinical samples were recruited and collected by Hiroko Oka, Kazuhisa Ouhara, Kentaro Nagamine, and Masae Kitagawa. Kentaro Nagamine and Masae Kitagawa acquired the data. The results were analyzed and interpreted by Ikuko Ogawa and Masae Kitagawa. Masae Kitagawa wrote the manuscript. Kentaro Nagamine, Hitoshi Komatsuzawa and Hidemi Kurihara reviewed and edited the manuscript. All authors read and approved the final manuscript.

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