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TECHNICAL NOTE

CRIMINALISTICS

Hiroaki Nakanishi,[1,3] *Ph.D.; Takeshi Ohmori,*[2] *Ph.D.; Masaaki Hara,*[3] *Ph.D.; Aya Takada,*[3] *M.D.; Hideki Shojo,*[4] *Ph.D.; Noboru Adachi,*[4] *M.D.; and Kazuyuki Saito,*[3] *M.D.*

A Simple Identification Method of Saliva by Detecting *Streptococcus salivarius* Using Loop-Mediated Isothermal Amplification

ABSTRACT: We previously reported that detection of *Streptococcus salivarius* is feasible for proving the presence of saliva in a forensic sample. Here, a simple and rapid method for the detection of *S. salivarius* in forensic samples was developed that uses loop-mediated isothermal amplification (LAMP). The LAMP primer set was designed using *S. salivarius*-specific sequences of glucosyltransferase K. To simplify the procedure, the sample was prepared by boiling and mutanolysin treatment only, and the entire analytical process was completed within 2.5 h. The cut-off value was set at 0.1 absorbance units, measured at 660 nm, upon termination of the reaction. *S. salivarius* was identified in all saliva samples, but was not detected in other body fluids or on the skin surface. Using this method, *S. salivarius* was successfully detected in various mock forensic samples. We therefore suggest that this approach is useful for the identification of saliva in forensic practice.

KEYWORDS: forensic science, saliva identification, saliva stain, *Streptococcus salivarius,* loop-mediated isothermal amplification, glucosyltransferase K

In forensic science, discrimination of body fluids is important in unraveling the details of a crime. For example, detection of semen provides evidence of a sexual crime, and detection of urine at a crime scene suggests that the victim was incontinent. Detection of saliva can provide evidence in sexual crimes, as it is important for identifying bite marks and useful for narrowing down possible test samples before DNA typing. Conventional methods for identification of saliva rely on the detection of the activity of the salivary enzyme α-amylase (1,2). A recent evaluation of commercial kits based on detection of α-amylase activity for saliva screening concluded that these kits are easy to use to screen for saliva in forensic samples (3). However, as α-amylase can be present in other body fluids, such as urine and semen (4,5), it can be used only as a preliminary test and not as a specific marker for saliva.

Another assay that does not depend on α-amylase activity, which is based on RNA and targets saliva-specific gene expression products, was reported previously (6,7). Additionally, we previously reported an assay system that targets saliva-specific bacteria using a PCR-based method (8). We concluded that the bacterium *Streptococcus salivarius* is particularly useful as a novel marker of saliva. However, although reliable, the method that we described previously was relatively complicated and time-consuming, largely because the detection of bacterial DNA relied on gel electrophoresis. Moreover, the size of the amplified product was *c.* 500 bp, and products of this size might be difficult to obtain from extremely degraded saliva samples.

Recently, Natomi et al. (9) developed the loop-mediated isothermal amplification (LAMP) method, which amplifies and detects DNA in a single step using four different primers specifically designed to recognize six distinct regions on a target gene. The reaction process proceeds by strand displacement and is carried out at a constant temperature. Thus, LAMP amplification is both much more efficient and less time-consuming than traditional PCR amplification. The method does not require sophisticated equipment, and a positive result is signaled by the increased opacity of the reaction mixture, which is visible to the naked eye. These merits have recently led to the application of LAMP to the detection of pathogenic oral bacteria (10,11) and of human-specific DNA in various forensic specimens (12), as well as sex determination from dental pulp (13).

In the present study, we exploited the LAMP method to develop a simple and rapid technique for revealing the presence of saliva in forensic samples. This method depends on LAMP-based detection of *S. salivarius*, a bacterium specific to the oral cavity.

Materials and Methods

Samples

Saliva, semen, and urine samples were collected from 10 healthy donors, and saliva stain samples were produced by licking a cotton swab. Skin bacteria were collected from 10 healthy donors by wiping

[1]Forensic Science Laboratory of Yamanashi Prefectural Police H.Q., 312-4 Kubonakajima, Isawa, Fuefuki, Yamanashi 406-0036, Japan.
[2]National Research Institute of Police Science, 6-3-1 Kashiwanoha, Kashiwa, Chiba 277-0882, Japan.
[3]Department of Forensic Medicine, Faculty of Medicine, Saitama Medical University, 38 Morohongo, Moroyama, Saitama 350-0495, Japan.
[4]Department of Legal Medicine, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan.

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the skin with a wet cotton swab, and vaginal fluid samples were collected from nine healthy donors using a cotton swab. Informed consent was obtained from all participants who provided samples. In the case of samples on cotton swabs, we used a 2 × 2 mm piece of cotton swab for the examination.

The bacterial strains used in this study were *S. salivarius* ATCC 13419, *S. mutans* ATCC 35668, *S. mitis* ATCC 6249, *S. sanguinis* ATCC 10556, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 8100, and *Staphylococcus aureus* ATCC 25923. These strains were purchased from Microbiologics (St. Cloud, MN). The streptococci were cultured on Mitis-Salivarius Agar (BHI, Difco Laboratories, Detroit, MI), and the remaining bacteria were cultured on Nutrient Agar (BHI).

Preparation of Samples for LAMP

Stain samples submerged in 30 μL of distilled water were boiled for 10 min and then incubated with 20 U mutanolysin (Sigma, St. Louis, MO) at 50°C for 60 min. Liquid samples were treated similarly with the exception that the incubation with mutanolysin was omitted. The prepared stain and liquid samples were used as the template for the LAMP reaction. For the analysis of bacterial samples, DNA was extracted as we described previously (8) and was used as the template for the LAMP reaction. Briefly, bacterial samples were suspended in 50 μL of water and then boiled at 98°C in microcentrifuge tubes. To lyse the bacterial cells, the samples were incubated with 100 μL of 200 U/mL mutanolysin (Sigma) and 20 μL of 100 mg/mL lysozyme (Sigma) at 50°C for 60 min. The DNA was then extracted and purified using a QIAamp® Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

LAMP Method

The *S. salivarius*-specific LAMP primer set was designed using the Primer Explorer program http://primerexplorer.jp/e/index.html to amplify the *S. salivarius* glucosyltransferase K (*gtfK*) gene (GenBank accession no. Z11872). The primers are shown in Table 1.

LAMP reactions were performed using a Loopamp DNA Amplification Kit (Eiken Chemical, Tokyo, Japan). A reaction mixture (25 μL) containing 1.6 μM of each inner primer (FIP and BIP), 0.2 μM of each outer primer (F3 and B3), 0.8 μM of each loop primer (LF and LB), 12.5 μL of 2× reaction mix, 1 μL of *Bst* DNA polymerase, and 2 μL of sample was incubated at 63°C for 60 min in a Loopamp Real-time Turbidimeter (RT-160C; Eiken Chemical). The reaction was terminated by heating at 80°C for 2 min. In the LAMP reaction, the turbidity increases as a result of the production of magnesium pyrophosphate, which is the by-product of the amplification reaction http://loopamp.eiken.co.jp/e/index.html (14). In the present study, the cut-off value was set at 0.1 absorbance units, measured at 660 nm upon termination of the reaction, based on our preliminary experimental data. For visual fluorescence detection (15), 1 μL of Loopamp Fluorescent Detection reagent (Eiken Chemical) was added to the reaction mixture, and a positive result was defined as the observation of green fluorescence of stronger intensity than the cut-off value, as determined using a real-time turbidimeter.

TABLE 1—*Primer sequences used in this study.*

Name	Sequence (5′–3′)
F3	GTTACTGCTGACAAACCAG
B3	CCTTAATTTCGGCTTCAGAA
FIP	CAGGTTTGGCTTCAACCTCTACGTTCAACCAAATTCAGGAAC
BIP	GTTGCTACTAAACCAGAAACAGCACTTCTCAGTCGTTGGAG
LF	TGCTGCAGCTCTATCACTAG
LB	AGAAGTCGCTGCAAATGCTG

The LAMP products show ladder-like amplification bands on agarose gel electrophoresis, reflecting the various sizes of the amplified fragments http://loopamp.eiken.co.jp/e/index.html. To confirm amplification of the intended product, the amplified products were digested with the restriction enzyme *Cel*II (Roche Diagnostics, Mannheim, Germany), which has a recognition/digestion site at nucleotides 386–392 of the target *gtfK* gene. The digested reaction mixture was then analyzed using 2.0% agarose gel electrophoresis with SYBR Green I staining (TaKaRa, Ohtsu, Japan).

Forensic Applications

The evaluation to diluted saliva was performed using saliva serially diluted from 2-fold to 1000-fold. As mock forensic samples, we employed five used cigarette butts, five cotton swabs wiped against licked skin, five pieces of used chewing gum, five cotton swabs wiped against the mouths of used water bottles, seven saliva stains stored for 6 years on filter paper, and cotton swabs stained with saliva mixed with a 10-fold excess (v/v) of semen or urine. For the mock forensic stain samples (cigarette paper, cotton swab, and filter paper), we used 5 × 5 mm of substrate for analysis. For analysis of chewing gum, the gum was rinsed with distilled water to extract the saliva, and the rinsed solution was concentrated to a volume of 30 μL. We also used 2 × 2 mm saliva swabs from three dogs and one cat to determine whether our method detects *S. salivarius* in the saliva of common household pets.

Moreover, to establish whether carrier materials (cigarette paper, cotton gauze, filter paper, and cotton swab) might cause false positives using the LAMP detection method in the absence of saliva, we performed control LAMP reactions with these carriers alone.

Results

Evaluation of the LAMP Method

When we performed the LAMP procedure using various concentrations of purified *S. salivarius* DNA, an increase in turbidity was observed with the Loopamp Real-time Turbidimeter (Fig. 1). As expected, no evidence of amplification was observed in the reaction mixture lacking template DNA (negative control). For more than 50 pg (corresponding to the amount of DNA in *c.* 2.4×10^4 bacteria) of purified *S. salivarius*, DNA could be amplified in all five samples, and 25 pg of the DNA could be amplified in two of five samples, whereas 10 pg of the DNA was not amplified in all five samples. Under visual fluorescence detection, positive or negative results were able to be easily judged (figure not shown).

We confirmed amplification of the target region by observing that *Cel*II converted most of the LAMP products into fragments of the expected size. Figure 2 shows that the amplification products from purified *S. salivarius* DNA (lane 1) and saliva (lane 3) were converted into the expected fragments (lanes 2 and 4, respectively). No ladder-like amplification band was observed in body fluid samples other than saliva (Fig. 2, lanes 5–9).

Forensic Applications

S. salivarius was identified in all saliva and saliva stain samples tested in this study using the LAMP assay and was not detected in semen, urine, vaginal fluid, or samples taken from the surface of

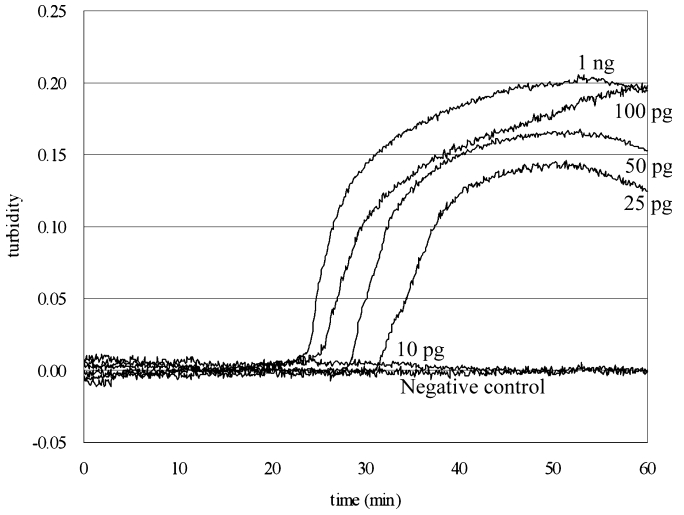

FIG. 1—*An increase in turbidity results from performance of the loop-mediated isothermal amplification procedure with various concentrations of purified* S. salivarius *DNA using the Loopamp Real-time Turbidimeter.*

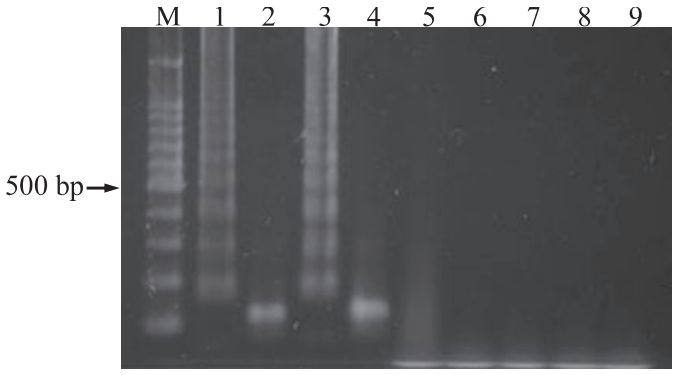

FIG. 2—*Analysis of loop-mediated isothermal amplification (LAMP) product and its digestive product. The ladder-like product (lanes 1 and 3) was digested with* CelI*I to confirm amplification of the intended product LAMP product (lanes 2 and 4). And the other samples were not amplified by LAMP method (lanes 5–8). Lane M, 100-bp molecular mass marker; lane 1, LAMP product of purified* S. salivarius *DNA; lane 2,* CelI*I digests of the LAMP product of* S. salivarius*; lane 3, LAMP product of a saliva sample; lane 4,* CelI*I digests of the LAMP product of a saliva sample; lanes 5–8, LAMP product of semen, urine, vaginal fluid, and a sample taken from the surface of skin, respectively. All the products were analyzed using 2.0% agarose gel electrophoresis with SYBR Green I staining.*

skin. The results of the LAMP assay obtained using various dilutions of saliva are shown in Table 2. We could detect *S. salivarius* from saliva collected after cleaning of teeth and after drinking coffee; moreover, the presence of a decayed tooth or pyorrhea alveolaris did not prevent the detection of *S. salivarius* (data not shown).

As shown in Table 3, we detected *S. salivarius* in all mock forensic samples (cigarette butts, used chewing gum, cotton swab wiped against licked skin or water bottles, and the saliva stains stored for 6 years). This method also detected *S. salivarius* in mixtures of saliva with semen or urine. However, *S. salivarius* was not detected in saliva samples from three dogs or one cat.

In tests of the carrier alone, cigarette paper, cotton gauze, filter paper, and cotton swabs did not give false positive results in the absence of added saliva.

Discussion

Our previous method for demonstrating the presence of saliva by PCR and gel electrophoresis (8) was, as we noted, relatively complicated, time-consuming, and difficult to use with extremely degraded saliva samples. The results of the present study confirm that the LAMP method can overcome the limitations of our previous method. Use of the LAMP method will greatly facilitate the identification of saliva in forensic samples. In the LAMP method, if there is a minimum amount of template DNA to amplify, the white precipitate or fluorescence will be sufficiently observed. Thus, the assessment based on the turbidity or fluorescence can be easily performed, and the presence of saliva is easily determined. In addition, the time required for sample analysis has been significantly reduced; completion of all steps of our previously developed method, including DNA extraction, took 6 h (8), but the current method can be completed in only 2.5 h. LAMP makes this speed possible by shortening the amplification procedure and eliminating the necessity for a separate detection process. Moreover, we consider that because the LAMP reaction system is resistant to inhibitors (16), the crude sample can be added directly to the LAMP reaction mix without first extracting the DNA, which also shortens the time required for the assay.

S. salivarius was successfully detected in all of the aged saliva stains and all of our mock forensic samples using the LAMP method. Therefore, we propose that this method will be useful in demonstrating the presence of saliva in various forensic specimens. It will be particularly valuable for the analysis of saliva mixtures

TABLE 3—*The LAMP results for* S. salivarius *identification from various mock forensic samples.*

Samples	*n*	Detected	Not Detected
Used cigarette butt	5	5	0
Cotton swab wiped against licked skin	5	5	0
Used chewing gum	5	5	0
Cotton swab wiped against the mouths of used water bottles	5	5	0
Saliva stain stored for 6 years	7	7	0
Stained with saliva mixed with a 10-fold excess (v/v) of semen	1	1	0
Stained with saliva mixed with a 10-fold excess (v/v) of urine	1	1	0
Dog saliva	3	0	3
Cat saliva	1	0	1

LAMP, loop-mediated isothermal amplification.

TABLE 2—*The number of positive reactions of the LAMP in diluted saliva collected from 10 donors.*

Dilution Rate	×1	×2	×4	×10	×20	×40	×100	×200	×400	×1000
Net Volume	2 μL	1 μL	0.5 μL	0.2 μL	0.1 μL	0.05 μL	0.02 μL	0.01 μL	0.005 μL	0.002 μL
Number of Positive Reactions	10	10	10	9	7	4	2	1	1	0

LAMP, loop-mediated isothermal amplification.

contaminated with semen or vaginal fluid at the site of a sexual crime. The presence of saliva can be positively demonstrated using the LAMP method because, unlike α-amylase, *S. salivarius* is not present in other body fluids. In this study, we did not examine fecal material and any mixtures of fecal material and other body fluids. It has been reported (17) that *S. salivarius* is present in large quantities in the fecal matter of adults. This LAMP method, therefore, could not differentiate between saliva and fecal matter. And, as a result, a positive result with the LAMP method in a forensic specimen that could contain fecal material could not be interpreted to mean that saliva was present in that specimen.

On average, a total of ~750 million oral bacterial cells are found per milliliter of saliva. *S. salivarius* accounts for an average of 4.6% of cultivable organisms in the adult oral cavity and is the most common species of oral bacteria (18). All humans are infected with *S. salivarius* by the age of 1 year (18). Therefore, it is assumed that most people have a considerable number of *S. salivarius* in their oral cavity. The number of *S. salivarius* might be reduced because of the effects of mouthwash, disease, etc. However, we detected *S. salivarius* in samples from donors with a decayed tooth or with pyorrhea alveolaris, and *S. salivarius* was even detected in samples taken after drinking coffee or cleaning teeth. Although the present study might be insufficient, the results suggest that the oral environment and oral diseases may have little effect on detection of *S. salivarius*.

This study showed that the LAMP method is an easy technique to perform, has a short assay time and can give a clear result, and is useful for the identification of saliva in forensic practice. Therefore, we consider that this method is a novel approach for saliva identification with the advantages of both simplicity and speed.

Conflict of interest: The authors have no relevant conflicts of interest to declare.

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Additional information and reprint requests:
Hiroaki Nakanishi, Ph.D.
Forensic Science Laboratory of Yamanashi Prefectural Police H.Q.
312-4 Kubonakajima
Isawa
Fuefuki
Yamanashi 406-0036
Japan
E-mail: nakanishi@384.jp