Jpn. J. Infect. Dis., 60, 55-58, 2007

Short Communication

Detection of *Haemophilus influenzae* by Loop-Mediated Isothermal Amplification (LAMP) of the Outer Membrane Protein *P6* Gene

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(Received July 21, 2006. Accepted October 27, 2006)

SUMMARY: It is difficult and time-consuming to distinguish *Haemophilus influenzae* from the genotypically similar *Haemophilus parainfluenzae*, which is a commensal of the human oral cavity. The novel nucleic acid amplification technique of loop-mediated isothermal amplification (LAMP), which amplifies DNA under isothermal conditions (63°C) with high specificity, efficiency, and rapidity, was evaluated for *H. influenzae* detection. A *H. influenzae*-specific LAMP primer set was designed for the outer membrane protein *P6* gene. Primer set specificity was validated using 4 *Haemophilus* spp. and 13 other species. Within 60 min, LAMP detected 100 or more copies of purified DNA with a sensitivity that was 10-fold higher than that of conventional PCR. This method can be used to differentiate *H. influenzae* from *H. parainfluenzae* strains. Thus, LAMP may represent a sensitive and reliable means of diagnosing *H. influenzae* infection.

Haemophilus influenzae is a common major pathogen of humans and is associated with community-acquired pneumonia, bacteremia, meningitis, and otitis media. While traditional antimicrobial therapy has proven efficacious, the isolation of antimicrobial-resistant *H. influenzae* strains has increased rapidly, especially in Japan (1). Procedures for isolating and identifying *H. influenzae* strains are complicated and time-consuming, owing to the difficulty of distinguishing *H. influenzae* from closely related *Haemophilus* spp., such as *H. parainfluenzae*, which are commensals of the human oral cavity. Detection using classical techniques, which depend on growth-based assays, colonial morphology, tests for V (NAD) and X (haemin) growth factor requirements, porphyrin tests, and serological determination, is time-consuming and complicated. Despite these problems, most diagnostic laboratories continue to use these conventional identification techniques. Sensitive and specific assays that can be completed promptly in the clinical laboratory are essential for early diagnosis and effective antibiotic therapy (2). Molecular assays are inherently valuable because detection can be achieved with enhanced sensitivity and specificity and they are applicable to nonviable microorganisms.

H. influenzae detection has been achieved with varying degrees of success using PCR-based assays with primers specific for rRNA-encoding genes (3,4). However, since the rRNA sequences of *H. influenzae* and *H. parainfluenzae* show approximately 95% homology, these genes are not ideal targets for the unequivocal identification of *H. influenzae* in clinical and epidemiological studies. The genes for the capsulation-associated protein Bex A and the outer membrane protein (OMP) P6 represent specific diagnostic targets (5). The primer set for the Bex A protein gene reacts only with capsulated strains of *H. influenzae*, but does not detect nontypeable *H. influenzae* strains, which are important pathogens of *H. influenzae* infection. OMP P6 is highly conserved among most strains of *H. influenzae* (6,7), and has been shown to be a potential vaccine component for the prevention of infections caused by *H. influenzae* (8). The *H. influenzae*-specific OMP *P6* gene is well characterized (9), and attempts have been made to use this gene to detect *H. influenzae* (5,10,11). However, these studies also detected other *Haemophilus* spp., such as *H. parainfluenzae* (5,10,11).

Ueyama et al. (11) reported that the sequences of the *H. parainfluenzae* and *H. influenzae* OMP *P6* genes differ in 30 of 274 bases, which corresponds to eight amino acid substitutions in the *H. parainfluenzae* OMP, as compared to the *H. influenzae* sequence. Therefore, using *H. influenzae*-specific sequences, we are able to develop highly specific and reliable molecular diagnostics for detecting *H. influenzae* infection. A novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), amplifies DNA with high specificity, efficiency, and speed under isothermal conditions (12). The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of four specially designed "inner" and "outer" primers that recognize six distinct sequences on the target DNA (12). This reaction can be accelerated using loop primers (13). The method requires simple, cost-effective equipment that is available in hospital laboratories. LAMP exhibits extremely high amplification efficiency due in part to its isothermal nature; no time is lost as a result of changes in temperature, and the reaction can be conducted at the optimal temperature for enzymatic activity. Therefore, LAMP does not require skilled personnel or the use of a thermal cycler. Consequently, LAMP may be potentially useful for the rapid diagnosis of infectious diseases caused by *H. influenzae* in commercial and hospital laboratories.

The goal of this study is to establish a sensitive, species-specific LAMP-based *H. influenzae* DNA amplification method by examining the reliability of the method for distinguishing between *H. influenzae* and *H. parainfluenzae*.

Genomic DNAs used to evaluate the primers were pre-

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Table 1. *Haemophilus* strains used in this study

Strain	note	Amplification	
		PCR[1)]	LAMP[2)]
Control *Haemophilus* strains			
H. parahaemolyticus GTC1529[3)]		+	−
H. parainfluenzae IID991[4)]		+	−
H. haemolyticus HK45[5)]		+	−
H. influenzae IID983[4)]	serotype a	+	+
H. influenzae IID984[4)]	serotype b	+	+
H. influenzae IID985[4)]	serotype c	+	+
H. influenzae IID986[4)]	serotype d	+	+
H. influenzae IID987[4)]	serotype e	+	+
H. influenzae IID988[4)]	serotype f	+	+
H. influenzae IID989[4)]	nontypeable	+	+
H. influenzae IID993[4)]	biotype aegyptius	+	+
Clinical strains			
H. parainfluenzae HK79[5)]		+	−
H. parainfluenzae HK82[5)]		+	−
H. parainfluenzae HK146[5)]		+	−
H. parainfluenzae HK155[5)]		+	−
H. parainfluenzae HK330[5)]		+	−
H. parainfluenzae HK758[5)]		+	−
H. parainfluenzae HK759[5)]		+	−
H. parainfluenzae HK760[5)]		+	−
H. parainfluenzae HK825[5)]		+	−
H. parainfluenzae NUSD1		+	−
H. parainfluenzae NUSD2		−	−
H. parainfluenzae NUSD3		+	−
H. parainfluenzae NUSD4		+	−
H. influenzae HK390[5)]	serotype a	+	+
H. influenzae HK176[5)]	serotype b	+	+
H. influenzae HK177[5)]	serotype b	+	+
H. influenzae HK179[5)]	serotype b	+	+
H. influenzae HK180[5)]	serotype b	+	+
H. influenzae HK195[5)]	serotype b	+	+
H. influenzae HK196[5)]	serotype b	+	+
H. influenzae HK827[5)]	serotype b	+	+
H. influenzae HK838[5)]	serotype b	+	+
H. influenzae HK635[5)]	serotype c	+	+
H. influenzae HK638[5)]	serotype f	+	+
H. influenzae HK2109[5)]	serotype f	+	+
H. influenzae HK864[5)]	biotype aegyptius	+	+
H. influenzae HK870[5)]	biotype aegyptius	+	+
H. influenzae HK882[5)]	biotype aegyptius	+	+
H. influenzae HK856[5)]	nontypeable	+	+
H. influenzae HK2112[5)]	nontypeable	+	+
H. influenzae HK2115[5)]	nontypeable	+	+
H. influenzae HK2117[5)]	nontypeable	+	+
H. influenzae HK2119[5)]	nontypeable	+	+
H. influenzae HK2121[5)]	nontypeable	+	+
H. influenzae NUSD5	nontypeable	+	+
H. influenzae NUSD6	nontypeable	+	+
H. influenzae NUSD7	nontypeable	+	+

[1)]: +, amplification detected; −, amplification not detected.
[2)]: +, amplification was seen after a 35-min incubation; −, amplification was not seen after a 60-min incubation.
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pared from 66 strains, representing 4 *Haemophilus* spp. (Table 1) and 13 non-*Haemophilus* spp.: *Streptococcus mitis* (ATCC9811), *S. oralis* (ATCC10557), *S. gordonii* (ATCC10558), *S. mutans* (XC47), *S. sanguis* (ATCC10556), *S. salivarius* (HHT), *S. pneumoniae* (ATCC6305, R6, GTC261, IID553, IID554), *Escherichia coli* (DH5α), *Actinobacillus actinomycetemcomitans* (Y-4), *Porphyromonas gingivalis* (381, ATCC49417), *Actinomyces naeslundii* (WVU627), *Prevotella intermedia* (ATCC25611), and *P. nigrescens* (ATCC25261). Genomic DNA was extracted and purified using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, Calif., USA) in accordance with the manufacturer's instructions. For the sensitivity study, genomic DNA purified from *H. influenzae* IID984 was used. The DNA concentration was quantified using an Ultrospec 3300 Pro spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK). The number of genomic copies for the LAMP mixture was calculated by assuming a molecular size of 1.9 Mbp.

Based on a comparison between the OMP *P6* (*pal*) gene sequences of the *H. influenzae* strain Rd (GenBank accession no. L42023 and GeneID 949485) and the *H. parainfluenzae* gene for the OMP P6 protein homologue (DDBJ accession no. D28887), a LAMP primer set targeting *H. influenzae* composed of five primers was designed using the LAMP primer support software program (Net Laboratory, Kanagawa, Japan). This primer set comprised the outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and a loop primer forward (LF) (Table 2).

Twenty-five microliters of the reaction mixture contained 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 0.4 μM of LF, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs Inc., Beverly, Mass., USA), 1.4 mM deoxynucleoside triphosphate, 0.8 M betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, and template DNA (2 μl). The mixture was incubated at 63°C for 35 or 60 min and then heated at 80°C for 2 min to terminate the reaction.

The LAMP reaction causes turbidity in the reaction tube proportional to the amount of DNA amplified. Therefore, we were able to observe the turbidity in the reaction tube with the naked eye. To confirm the sensitivity, a Loopamp real-time turbidimeter (LA-200; Teramecs, Kyoto, Japan) was used. For further confirmation, the amplified products were detected using electrophoresis in 3% agarose gels, followed by ethidium bromide staining. To confirm the structures of the amplified products, some of the amplified products were digested with the restriction enzyme *Tas*I (Fermentas Inc., Hanover, Md., USA) and their sizes were analyzed by electrophoresis in 3% agarose gels, followed by staining with ethidium bromide. The LAMP assay with the proper template DNA at 63°C for 35 or 60 min successfully amplified the 205-bp target sequence of the *H. influenzae* OMP *P6* gene (Tables 1 and 3). This product was observed by agarose gel electrophoresis and showed a ladderlike pattern on the gel; this is characteristic of the LAMP reaction and indicates the production of stem–loop DNA with inverted repeats of the target sequence (data not shown).

To ascertain the detection limit of the LAMP assay for *H. influenzae*, serial 10-fold dilutions of genomic DNA were amplified, and the results were compared to those obtained using conventional PCR with the following primers targeting the OMP *P6* gene: primer F1, 5′-AACTTTTGGCGGTTACTCTG-3′ and primer R1, 5′-CTAACACTGCACGACGGTTT-3′ (11). The sensitivity of the LAMP assay was the same using the Loopamp real-time turbidimeter as it was using naked eye assessment (Table 3) or electrophoresis. Using chromosomal DNA, the LAMP primer set without the LF had a detection limit of 10^4 copies/tube for a 60-min reaction (Table 3). In contrast, the LAMP primer set with the LF

Table 2. Oligonucleotide primers for LAMP

Primer	Sequence
Hi-F3	5′-TAG AAG GTA ACA CTG ATG AAC G-3′
Hi-B3	5′-TAC GCT AAC ACT GCA CGA-3′
Hi-FIP	5′-ACA CCT TTA CCA GCT AAA TAA CCT TTG GTA CAC CAG AAT ACA ACA TC-3′
Hi-BIP	5′-AGG CAC AGT ATC TTA CGG TGA ATA TGC AGC TTC ATC ATG ACC-3′
Hi-LF	5′-TGC ACG ACG TTG GCC TAA-3′

Table 3. Sensitivities of the LAMP and PCR assays for *Haemophilus influenzae* IID984

Assay	Detection of genomic DNA (copies/tube) with[1]:							
	10^6	10^5	10^4	10^3	10^2	10	1	0
PCR[2]	+	+	+	+	−	−	−	−
LAMP without LF, 35 min[3]	+	−	−	−	−	−	−	−
LAMP with LF, 35 min[3]	+	+	+	+	−	−	−	−
LAMP without LF, 60 min[3]	+	+	+	−	−	−	−	−
LAMP with LF, 60 min [3]	+	+	+	+	+	−	−	−

[1]: +, amplification detected; −, amplification not detected.
[2]: The results were obtained by electrophoretic analysis.
[3]: The results were determined with the naked eye.

was faster and more sensitive; the detection limits were 10^3 copies/tube for chromosomal DNA in a 35-min reaction and 10^2 copies/tube for chromosomal DNA in a 60-min reaction (Table 3). The detection limits for the PCR were 10^3 genomic DNA copies; therefore, the sensitivity of the LAMP assay was ten times greater than that of conventional PCR (Table 3). No amplification was apparent when the sample tube lacked the target DNA.

To evaluate the species specificity of the LAMP primers, we tested 4 *Haemophilus* spp. and 13 non-*Haemophilus* spp. (Table 1). Significant amplification of *H. influenzae* DNA was observed after the 35-min incubation. In contrast, the genomic DNAs of the other strains were not amplified after a 60-min incubation. The specificity of the amplification was also confirmed by *Tas*I digestion to ensure that the amplification product contained sequences that corresponded to the selected target. The products of *Tas*I digestion were 90 and 125 bp in size, and thus were in good agreement with the predicted sizes (data not shown). The amplified products were confirmed by sequencing, and the derived sequences were identical to the expected nucleotide sequences (data not shown).

To confirm the efficacy of LAMP for evaluating strains, we used the conventional PCR assay (11) to screen four *Haemophilus* spp. (Table 1). The genomic DNA concentration of each strain was 10^6 copies per assay mixture, allowing a direct comparison of the results of the PCR and LAMP assays. LAMP detected only *H. influenzae* strains, while PCR produced 15 false-positive results. These findings concurred with those of previous reports that have described the presence of the OMP *P6* gene in other *Haemophilus* spp. (5,10,11). To differentiate *H. influenzae* from *H. parainfluenzae*, Ueyama et al. (11) added a hybridization step using the PCR products as a template in order to eliminate false products. In contrast, in our study, LAMP successfully distinguished *H. influenzae* from *H. parainfluenzae* without having to perform an extra step (Table 1).

The OMP *P6* nucleotide sequence of *H. parainfluenzae* differs in several places from that of *H. influenzae*, as described above (11). Using this variation, we designed *H. influenzae*-specific sequence primers for LAMP in order to improve the specificity and reliability of the molecular diagnosis of *H. influenzae* infection. The LAMP reaction is more discriminating than PCR, as it uses five primers that recognize seven distinct regions on the target DNA (12,13). The reliability and sensitivity of this LAMP method are in full agreement with our previous finding for a *S. pneumoniae* assay (14). In addition, the simple operation, rapid reaction, and detection ease of the LAMP assay are advantages in a clinical microbiological laboratory where experience with PCR or molecular testing is lacking. One of the most likely applications of the LAMP technique in clinical laboratories is the identification of suspected *H. influenzae* colonies without the need for V (NAD) and X (haemin) growth factor requirement testing. In this case, identification using the LAMP method proposed here is less troublesome because *H. influenzae*-specific DNA amplification can be achieved using LAMP with genomic DNA prepared by boiling cells from a tiny colony on an agar plate (data not shown). Establishing a distinct method that enables us to distinguish *H. influenzae* from similar non-virulent bacterial species is important in clinical diagnosis.

ACKNOWLEDGMENTS

This investigation was supported in part by the Sato Fund, Nihon University School of Dentistry, the Nihon University Research Grant for Assistants and Young Researchers (2003), the Grant-in-Aid for Scientific Research (C) (No. 16592097) and a Grant to promote multidisciplinary research projects from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study was also supported by special research grants for the development of distinctive education from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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