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Development and Evaluation of a Loop-Mediated Isothermal Amplification Method for Rapid Diagnosis of *Bordetella pertussis* Infection

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We developed a loop-mediated isothermal amplification (LAMP) method to detect *Bordetella pertussis* infection. This LAMP assay detected *B. pertussis* with high sensitivity, but not other *Bordetella* species. Among nasopharyngeal swab samples from subjects with suspected pertussis, LAMP results showed a high level of agreement with results of conventional PCR. This method is a rapid, sensitive, and specific method for diagnosis of *B. pertussis* infection even in clinical laboratories with no specific equipment.

Pertussis is a highly contagious disease caused by the bacterial pathogen *Bordetella pertussis*. Pertussis can be diagnosed by culture and serological methods (17, 19). However, they are not practical diagnostic tools. Culture is required for 7 to 10 days to isolate, confirm, or exclude the presence of *B. pertussis*. Similarly, serological diagnosis requires paired sera, acute- and convalescent-phase sera, obtained about 1 month apart. Consequently, results are not available until the patient is recovering. For rapid and sensitive diagnosis, PCR assays have revolutionized the laboratory diagnosis of pertussis infections. Various PCR-based detection methods including nested PCR and real-time PCR have been developed that target different regions of the genome. PCR targeting the pertussis toxin (PT) promoter region is highly specific for *B. pertussis* (10, 12), but its sensitivity is low. In contrast, IS*481*-based PCR is a highly sensitive PCR assay (1, 2, 6, 9, 15, 16); however, IS*481*-based PCR detects not only *B. pertussis* but also *Bordetella holmesii* (4, 20, 25, 26). Thus, there is a need for a specific and more sensitive diagnostic method for pertussis.

Recently, loop-mediated isothermal amplification (LAMP) has been developed as a novel method to amplify DNA with high specificity and simplicity (22). The method consists simply of incubating a mixture of the target gene, four or six different primers, *Bst* DNA polymerase, and substrates. The significant advantages of the LAMP method are (i) high amplification efficiency under isothermal conditions (63 to 65°C) and (ii) visual judgment based on the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube (18). Although it has thus emerged as a powerful tool to facilitate genetic testing for the rapid diagnosis of several viral and bacterial infectious diseases in clinical laboratories (5, 11, 13, 14), the LAMP method has not been evaluated for the diagnosis of *B. pertussis* infection. In the present study, we developed a LAMP method for diagnosis of *B. pertussis* infection and evaluated its sensitivity, specificity, and applicability for clinical specimens. This report, to our knowledge, is the first practical application of the LAMP method for the diagnosis of *B. pertussis* infection.

The bacterial strains used in this study are listed in Table 1. Chromosomal DNAs of *Bordetella* species were extracted

TABLE 1. Strains used in this study and comparison of LAMP and PCR specificity[a]

Strain	Result by[b]:		
	LAMP[c]	IS*481*-PCR	PTp1/p2-PCR
B. pertussis			
Tohama	+	+	+
Yamaguchi	+	+	+
BP256[d]	+	+	+
BP257[d]	+	+	+
BP258[d]	+	+	+
BP259[d]	+	+	+
BP260[d]	+	+	+
BP289[d]	+	+	+
BP290[d]	+	+	+
B. parapertussis			
ATCC 15237	−	−	−
ATCC 15311	−	−	−
BAA-587	−	−	−
BPP01[e]	−	+	−
B. hinzii			
ATCC 51730	−	−	−
B. holmesii			
ATCC 51541	−	+	−
B. avium			
ATCC 35086	−	−	−
B. bronchiseptica			
R05[f]	−	−	−

[a] For PCR detections, 1 ng of DNA was tested. For LAMP assay, 10 fg DNAs of *B. pertussis* strains and 1 ng DNAs of other *Bordetella* species were tested.
[b] +, LAMP or PCR amplification; −, no LAMP or PCR amplification.
[c] Turbidity assay with a 60-min reaction.
[d] Isolated from Japanese patients in the period 2004 to 2005.
[e] Isolated from a Japanese patient in 2002.
[f] Isolated from a rabbit in 1995.

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using a QIAGEN genomic tip (20G) and a genomic buffer set (QIAGEN). For evaluation of this LAMP assay with clinical specimens, 112 nasopharyngeal swabs (NPS) were obtained from 112 Cambodian children with suspected pertussis between January and November 2005. The NPS were obtained with sterilized rayon-tipped swabs (Eiken Kizai Co., Ltd., Tokyo, Japan) (3) and were transported to the National Institute of Infectious Diseases, Japan, in individual sterilized tubes, each containing two or three silica gels under dry conditions. The dried NPS were immersed in 0.5 ml of Casamino Acid solution (1% Difco Casamino Acids, 0.6% NaCl, pH 7.1) and vortexed. Small portions (50 to 100 μl) of the NPS solution were inoculated on a *Bordetella* CFDN or CVA agar plate (Nikken Bio Medical Laboratory, Kyoto, Japan). The inoculated plates were incubated for 5 to 7 days at 36°C. *B. pertussis*-like colonies were subcultured on Bordet-Gengou plates and then identified as *B. pertussis* by agglutination test (Denka Seiken, Co., Ltd., Tokyo, Japan) and/or PCR identification (12). The remaining NPS solutions were used for LAMP and PCRs. The NPS solution (approximately 400 μl) was transferred to a 1.5-ml microcentrifuge tube and then centrifuged at 15,000 rpm for 10 min. Total DNA was extracted from the precipitation using QIAamp DNA microkit (QIAGEN).

For specific detection of *B. pertussis*, six LAMP primers were designed by targeting the PT promoter region of *B. pertussis* strain Tohama (genome position 159549 to 159755; GenBank accession no. BX640422) using Primer Explorer software, version 3 (https://primerexplorer.jp/lamp3.0.0/index.html): two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB). The name and sequence of each primer are shown in Table 2. Before the LAMP reaction, template DNA was denatured at 95°C for 5 min and then cooled on ice. The LAMP reaction was carried out with the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). A 25-μl reaction mixture containing 40 pmol (each) of BP-FIP and BP-BIP primers, 5 pmol (each) of BP-F3 and BP-B3 primers, 20 pmol (each) of BP-LF and BP-LB primers, 2× reaction mixture (12.5 μl), *Bst* DNA polymerase (1 μl), and template DNA (2 μl) was used. The mixture was incubated at 65°C for 60 min (for the initial validation study) or 40 min (for clinical specimens) and then heated at 80°C for 2 min to terminate the reaction. All oligonucleotides (high-performance liquid chromatography purification grade) for the LAMP primers were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). For the initial validation study, the LAMP amplification was confirmed with real-time monitoring of the increase of turbidity using LA-320C (Eiken Chemical Co., Ltd.). For further confirmation, some of the amplified products were analyzed by electrophoresis on 2% agarose gels, followed by ethidium bromide staining and photography. For clinical specimens, the amplified products in the reaction tube were directly detected with the naked eye using Loopamp fluorescent detection reagent (Eiken Chemical Co., Ltd.) according to the manufacturer's instructions. To evaluate the LAMP assay, two conventional single-PCR assays, IS*481*-PCR (4) and PTp1/p2-PCR (12), were also performed.

In a previous report, Nagamine et al. (21) demonstrated that there is no necessity for heat denaturation of the template DNAs for the LAMP assay. However, our LAMP assay showed that LAMP with heat-denatured template DNA was 100 times more sensitive than that with a nondenatured template (data not shown). Therefore, heat-denatured template DNAs were used for all LAMP assays. For analytical sensitivity tests, the LAMP reaction was tested using 10-fold serial dilutions of *B. pertussis* strain Tohama DNA and compared against results from conventional single-PCR assays, IS*481*-PCR and PTp1/p2-PCR. As shown in Fig. 1A, amplification by LAMP was obtained in reaction tubes containing from 1 ng to 10 fg of the DNA template for a 60-min reaction with a turbidity assay. In a gel electrophoresis analysis, the amplified products also showed ladder-like patterns from 1 ng to 10 fg DNA/tube (Fig. 1B). Thus, the detection limit of LAMP was 10 fg DNA/tube for chromosomal DNA in a 60-min reaction. This DNA content corresponds to 2.4 genomic copies, since *B. pertussis* strain Tohama has a genomic size of 4.1 Mbp (24). In contrast, the detection limits for IS*481*-PCR and PTp1/p2-PCR were 1 pg/tube and 100 pg/tube, respectively. This LAMP assay was 100 times more sensitive than the single IS*481*-PCR.

TABLE 2. LAMP primers for *Bordetella pertussis* detection

Primer	Type	Sequence (5′–3′)
BP-F3	F3	CCGCATACGTGTTGGCA
BP-B3	B3c	TGCGTTTTGATGGTGCCT
BP-FIP	F2-F1c	TTGGATTGCAGTAGCGGGATGTGC ATGCGTGCAGATTCGTC
BP-BIP	B1-B2c	CGCAAAGTCGCGCGATGGTAACG GATCACACCATGGCA
BP-LF	LFc	ACGGAAGAATCGAGGGTTTTGTAC
BP-LB	LB	GTCACCGTCCGGACCGTG

Specificity of the LAMP primers was tested using various *Bordetella* species, *B. pertussis*, *B. parapertussis*, *B. hinzii*, *B. holmesii*, *B. avium*, and *B. bronchiseptica*, in a turbidity analysis and compared against the results of conventional single-PCR assays (Table 1). All nine *B. pertussis* strains, two reference strains and seven clinical isolates, showed maximal turbidities above 0.5 at 650 nm with 10 fg of template DNA after incubation for 60 min (data not shown). In contrast, all other *Bordetella* species were negative (<0.01 turbidity) in the assay with 1 ng of template DNAs after incubation for 60 min. The LAMP primer set was as highly specific for *B. pertussis* as PTp1/p2-PCR primers. In contrast, the IS*481*-PCR assay detected not only *B. pertussis* stains but also *B. holmesii* ATCC 51541 and *B. parapertussis* clinical strain BPP01. Although *B. holmesii* reportedly harbors IS*481* elements (25), a *B. parapertussis* strain harboring IS*481*-like sequences has not been reported. Recently, Galadbach et al. (8) demonstrated that 2 of 12 *B. bronchiseptica* isolates tested were PCR positive for IS*481*. Cross-reactivity with *B. bronchiseptica* in IS*481*-based PCR was also observed in the external quality assessment program (20). Therefore, it should be noted that IS*481*-based PCR provided the only evidence for the presence of *Bordetella* species in clinical specimens, as described by Fry et al. (7).

To evaluate the clinical sensitivity of our LAMP method, a total of 112 nasopharyngeal swabs were tested for the presence of *B. pertussis* by LAMP, conventional single PCRs, and culture (Table 3). The overall sensitivities of LAMP and IS*481*-PCR were significantly higher than those of PTp1/p2-PCR and cul-

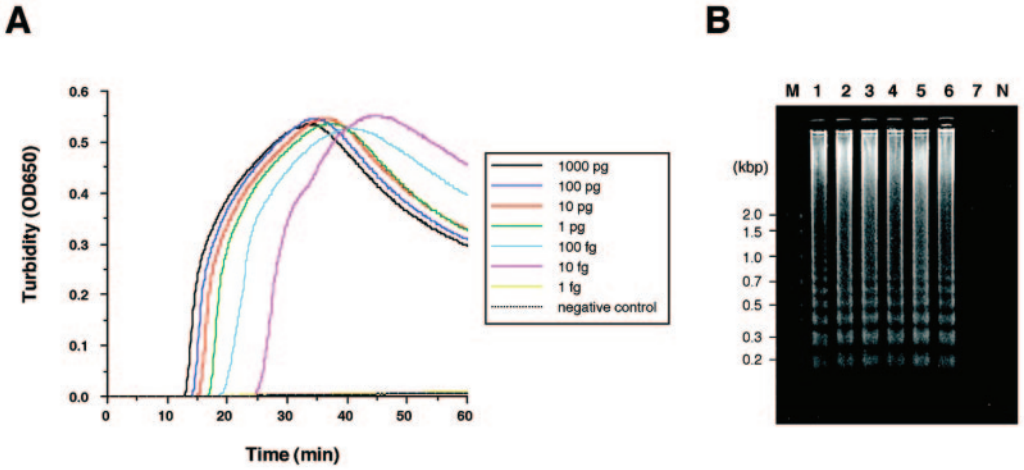

FIG. 1. Analytical sensitivity of LAMP for detection of the *B. pertussis* genome. Total DNA from *B. pertussis* strain Tohama was serially diluted from 1 ng to 1 fg and amplified by LAMP. (A) Real-time turbidity assay with Loopamp real-time turbidimeter. (B) Electrophoretic analysis of LAMP products (2 μl) for a 60-min reaction. Lanes 1 to 7, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg DNA/tube, respectively; lane N, negative control; lane M, molecular size marker. OD650, optical density at 650 nm.

ture. Among the five specimens that were culture positive, all specimens were also positive by LAMP and IS*481*-PCR (100% sensitivity); however, one specimen was negative by PTp1/p2-PCR (80% sensitivity). The specificities of LAMP, IS*481*-PCR, and PTp1/p2-PCR were 82% (88/112), 82% (88/112), and 98% (105/112), respectively, when culture was used as the reference method. LAMP and IS*481*-PCR results showed a high level of agreement (104/112; 93%) with 20/112 positive in both assays and 84/112 negative in both assays (data not shown). Where the results of IS*481*-PCR were considered to be true positive, the sensitivity and specificity of LAMP were 83% (20/24) and 95% (84/88), respectively. The positive and negative predictive values of LAMP were also 83% and 95%, respectively. Although the LAMP assay has greater analytical sensitivity than IS*481*-PCR, no significant difference was observed in its clinical sensitivity. This observation suggests that clinical specimens that contain *B. pertussis* cells around the lower detection limit of IS*481*-PCR would be very rare in practice. In any case, the LAMP assay constructed in the present study would have adequate sensitivity and specificity for the detection of *B. pertussis* cells in clinical specimens.

The LAMP assay targeting the PT promoter region of *B. pertussis* successfully detected *B. pertussis* clinical isolates. However, unfortunately, in the promoter region, polymorphism was found in three *B. pertussis* strains, *B. pertussis* ATCC 9340 and ATCC 9797 (23) and clinical strain CZ (GenBank accession no. AJ006159). The nucleotide variations are present in the LAMP primer annealing regions, and the annealing regions of F2 and LFc have three nucleotide substitutions and one nucleotide substitution, respectively, compared to the sequences of other *B. pertussis* strains. It has been suggested that these nucleotide variations in the LAMP primer annealing regions are a possible source of false-negative LAMP results. Interestingly, however, a high degree of PT promoter sequence homology has been reported among Swedish clinical isolates (23). In fact, the LAMP assay showed that all seven Japanese clinical isolates were LAMP positive. Moreover, five Cambodian clinical specimens that were culture positive were LAMP positive. These results demonstrate that the polymorphism found in *B. pertussis* ATCC 9340, ATCC 9797, and strain CZ is not typical among recently circulating strains, suggesting that the nucleotide variations might not affect the LAMP assay for diagnosis of *B. pertussis* infection. For validation of this test method, a larger-scale clinical evaluation would be necessary.

In conclusion, the LAMP-based assay described here for *B. pertussis* provides rapid and simple diagnosis of pertussis infection. Thanks to its easy operation without the need for a thermal cycler and electrophoresis system, our LAMP assay promises to become a useful and powerful tool for diagnosis of pertussis in clinical laboratories as well as diagnostic laboratories.

TABLE 3. Results of LAMP, IS*481*-PCR, and PTp1/p2-PCR versus results of culture for clinical specimens

Culture result	No. of specimens tested by:								
	LAMP[a]			IS*481*-PCR			PTp1/p2-PCR		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Positive	5	0	5	5	0	5	4	1	5
Negative	19	88	107	19	88	107	2	105	107
Total	24	88	112	24	88	112	6	106	112

[a] Fluorescent-dye-mediated naked-eye visualization with a 40-min reaction.

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