Development of a loop-mediated isothermal amplification method for diagnosing *Pneumocystis pneumonia*

Natsu Uemura,^{1,2} Koichi Makimura,¹ Masanobu Onozaki,³ Yoshihito Otsuka,⁴ Yasuhiro Shibuya,⁵ Hirohisa Yazaki,⁶ Yoshimi Kikuchi,⁶ Shigeru Abe¹ and Shoji Kudoh²

Correspondence
Natsu Uemura
s3016@nms.ac.jp

¹Teikyo University Institute of Medical Mycology, 539 Otsuka, Hachioji, Tokyo 192-0395, Japan

²Department of Pulmonary Medicine/Infection and Oncology, Nippon Medical School, Tokyo, Japan

³Kanto Chemical Co. Inc., Tokyo, Japan

⁴Social Insurance Central General Hospital, Tokyo, Japan

⁵Tokyo Metropolitan Hiroo General Hospital, Tokyo, Japan

⁶AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan

Loop-mediated isothermal amplification (LAMP) is a novel, rapid nucleic acid amplification method with high specificity and sensitivity under isothermal conditions. In this study a LAMP assay for diagnosing *Pneumocystis pneumonia* (PCP) was developed. Oligonucleotide primers specific for *Pneumocystis* species were designed corresponding to 18S rRNA gene sequences. The assay, performed for 30 min at 61 °C, was capable of detecting 50 copies per tube (2×10^3 copies ml^{-1}) in 30 min and did not show cross-reactivity to other species of fungi, including the genera *Candida*, *Aspergillus* and *Cryptococcus*. A total of 21 of 24 clinical specimens (sputum and bronchoalveolar lavage fluid) from patients with suspected PCP tested positive using the LAMP assay by real-time fluorescence detection. The results of the LAMP reaction were also observed by real-time turbidity detection and end-point visual turbidity or fluorescence detection. With real-time fluorescence detection, melting curves of the products were effective at distinguishing specific amplification from non-specific amplification or self-amplification. Visual detection was also possible as a rapid and easy assay using only a heat block and a black light.

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INTRODUCTION

Pneumocystis is a fungus belonging to the class Archiascomycetes, phylum Ascomycota. The human pathogen was known as *Pneumocystis carinii* f. sp. *hominis*, but has been renamed *Pneumocystis jirovecii* based on the results of molecular biological studies (Frenkel, 1999; Stringer *et al.*, 2002). This opportunistic fungus causes *Pneumocystis pneumonia* (PCP) in patients infected with human immunodeficiency virus (HIV), as well as other immunocompromised patients. Recently, human-to-human transmission (Helweg-Larsen *et al.*, 1998; Hocker *et al.*, 2005) and colonization by *P. jirovecii* have been reported in the general population (Medrano *et al.*, 2005). Although the standard method for diagnosis of PCP is

based on microscopic examination of clinical specimens [bronchoalveolar lavage fluid (BALF), sputum or lung biopsy], the sensitivity of this method is poor except in the hands of highly skilled examiners (Flori *et al.*, 2004). Therefore, PCR-based methods are widely used in Japan for the diagnosis of *Pneumocystis* infection. However, conventional PCR methods require a thermal cycler and electrophoresis for detection, whilst real-time PCR allows simultaneous amplification and detection, but requires expensive equipment and therefore is not available in general clinical laboratories.

Recently, a new specific DNA amplification technique called loop-mediated isothermal amplification (LAMP) was developed (Nagamine *et al.*, 2002; Notomi *et al.*, 2000), and has already been applied to the detection of pathogenic viruses (Poon *et al.*, 2005), bacteria (Iwamoto *et al.*, 2003), parasites (Poon *et al.*, 2006) and fungi (Endo *et al.*, 2004). LAMP, which does not involve the use of PCR, allows the rapid amplification of DNA with high specificity under isothermal conditions using DNA polymerase with

Abbreviations: BALF, bronchoalveolar lavage fluid; BLF, bronchial lavage fluid; HIV, human immunodeficiency virus; LAMP, loop-mediated isothermal amplification; PCP, *Pneumocystis pneumonia*.

The GenBank/EMBL/DBJ accession number for the 18S rRNA gene sequence of *Pneumocystis jirovecii* reported in this paper is AB266392.

strand-displacement activity. Specific nucleotide extension is achieved with four primers recognizing six distinct regions on the target. The amplification time can be further shortened by using an additional two primers, termed the loop primers (Nagamine *et al.*, 2002). Moreover, LAMP products can be detected not only using specialized equipment but also by visual observation of turbidity or fluorescence (Mori *et al.*, 2001). Here, we report the development of a *Pneumocystis*-specific LAMP assay, which has been applied successfully to clinical specimens for diagnosis of PCP.

METHODS

Fungal strains. A total of 69 strains from 51 species of fungi were used as controls in the present study (Table 1).

Preparation of DNA from fungal cells. With the exception of *P. jirovecii*, all fungal strains were grown on Sabouraud dextrose agar [1% (w/v) peptone; 1% (w/v) glucose; 1.5% (w/v) agar] at 27 or 37 °C for 1–5 days. Rapid preparation of DNA from the fungi was performed by a modification (Makimura *et al.*, 1994) of a method described by Ceniz (1992). Briefly, small samples of mycelia grown on Sabouraud dextrose agar were placed in lysis buffer [200 mM Tris/HCl (pH 8.0), 0.5% (w/v) SDS, 250 mM NaCl, 25 mM EDTA] and crushed with a conical grinder. These samples were incubated at 100 °C for 15 min, mixed with 150 µl 3.0 M sodium acetate, kept at –20 °C for 10 min and then centrifuged at 10 000 g for 5 min. The supernatants were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and once with chloroform. DNA was precipitated with an equal volume of 2-propanol at –20 °C for 10 min, washed with 0.5 ml 99% ethanol, dried and resuspended in 50 µl ultrapure water (MilliQ Synthesis A10; Millipore). Aliquots of 1 µl of the resulting solutions were used as templates for PCR.

DNA from yeasts was prepared by a modification of the method described by Makimura *et al.* (1994). Briefly, small samples of individual yeast colonies were suspended in 100 µl lysis buffer. The samples were mixed using a vortex for 5 s and incubated at 100 °C for 15 min. Next, 100 µl 3.0 M sodium acetate was added and the preparations were mixed. After incubation at –20 °C for 10 min, the samples were centrifuged at 10 000 g for 5 min, after which the supernatants were transferred to fresh tubes. DNA was precipitated with an equal volume of 2-propanol, washed with 0.5 ml 99% ethanol, dried and resuspended in 100 µl ultrapure water.

Clinical specimens. A total of 38 clinical specimens (24 from PCP and 14 from non-PCP patients) were examined. Twenty-four specimens, comprising eight sputum samples and sixteen BALF samples, were collected from twenty-one patients diagnosed with PCP and admitted to three hospitals in Tokyo (Social Insurance Central General Hospital, Tokyo Metropolitan Hiroo Hospital or the International Medical Center of Japan) between January 1998 and July 2006. In the PCP group (Table 2), PCP was diagnosed by microscopic examination, or symptoms were improved by PCP therapy (co-trimoxazole, i.e. sulfamethoxazole/trimethoprim and/or pentamidine isethionate). In addition, 1 BALF sample and 13 bronchial lavage fluid (BLF) samples were collected from the 14 non-PCP patients (Table 3). The subjects in the non-PCP group, which comprised 1 patient with idiopathic pulmonary fibrosis and 13 with lung cancer, did not have fever or inflammatory signs. The PCP group included six patients with collagen disease (rheumatoid arthritis) treated with steroids or immunosuppressive agents, and one lung cancer patient treated with chemotherapy. The patients in

the non-PCP group received no treatment with anti-*Pneumocystis* agents.

DNA preparation from clinical specimens. Clinical specimens were stored at –80 °C before DNA extraction using a NucliSENS kit manually with MiniMAG (bioMérieux) in accordance with the manufacturer's instructions. Samples of 200–1000 µl sputum, BALF or BLF were used for extraction and sample DNA was stocked at –80 °C before use.

Primer design. Oligonucleotide primers specific for the genus *Pneumocystis* were designed corresponding to regions of the 18S rRNA genes of *P. jirovecii* reported by Shah *et al.* (1996) and to genomic sequences determined in our laboratory. The following *Archiascomycetes*, *Hemiascomycetes* and human sequences were also used as references: *P. carinii* (host, ferret; GenBank accession no. S83267), *P. carinii* (host, rat; L27658), *Pneumocystis murina* (AY532651), *P. carinii* (X12708), *Taphrina carnea* (AB000948), *Taphrina virginica* (AB000960), *Taphrina robinsoniana* (AB000958), *Taphrina nana* (AB000955), *Taphrina wiesneri* (D12531), *Taphrina communis* (AB000949), *Taphrina ulmi* (AB000959), *Schizosaccharomyces pombe* (X58056), *Candida albicans* (E15168), *Saccharomyces cerevisiae* (Z75578) and *Homo sapiens* (M10098). Sequences were analysed using GENETYX software (Genetyx).

The sequences of our newly designed oligonucleotide PCR primers were as follows: 5'-TTCGGGGCTTACTTTGGTC-3' (Pjf9), 5'-GTAGTTAGTCTTCAATAAATCT-3' (Pjr4), 5'-AGGCCTACCATG-GTTTCG-3' (Pjf8) and 5'-CTTCGAGGACCGGGCCGT-3' (Pjr8). In addition to these primers, the 18S rRNA gene-based universal fungal primers NS1, NS3, NS5, NS4, NS6 and NS8 (White *et al.*, 1990) were also used for nested PCR and sequencing as shown in Fig. 1.

As LAMP primers, a set of six oligonucleotides comprising two outer (F3 and B3), two inner (FIP and BIP) and two loop primers (FL and BL) recognizing eight distinct regions on the target sequence were designed using LAMP primer design support software (Primer Explorer version 3.0; Eiken Genome; <http://primerexplorer.jp/>). The FIP primer consisted of F2 and the complementary strand of F1 (F1c). The BIP primer consisted of B2 and the complementary strand of B1 (B1c). The sequences and positions of these primers are shown in Table 4 and Fig. 1. All oligonucleotides were synthesized by Sigma-Aldrich.

PCR and plasmid preparation. Nested PCR was performed to amplify part of the 18S rRNA gene using primers NS1, NS8, Pjf9 and Pjr4. Each PCR mixture contained 10 µl 10 × reaction buffer (GE Healthcare Bio-Sciences), 200 µM each dATP, dCTP, dGTP and dTTP (Toyobo), 2.5 U *Taq* polymerase (GE Healthcare Bio-Sciences), 30 pmol each primer and 1 µl DNA template solution. Ultrapure water was added to a final volume of 100 µl. First-round PCR was performed with primers NS1 and NS8 under the following conditions: 94 °C for 4 min; 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1.5 min; and 72 °C for 10 min. Second-round PCR was performed with primers Pjf9 and Pjr4 under the following conditions: 94 °C for 4 min; 25 cycles of 94 °C for 1 min, 60 °C for 15 s and 72 °C for 15 s; and 72 °C for 10 min. PCR products were separated by electrophoresis on 1.2% agarose gels, stained with ethidium bromide and visualized by UV irradiation.

The PCR product amplified with the Pjf9 and Pjr4 primers was ligated into the TA cloning vector pCR 2.1 (Original TA cloning kit; Invitrogen). The ligated product was introduced into *Escherichia coli* DH5α (Nippon Gene) by transformation. After propagation and purification of the plasmid, the concentration (copies µl⁻¹) was calculated from the measurement of A₂₆₀ and the molecular mass of the plasmid.

Table 1. Fungal species and strains used in the LAMP assay to determine specificity

Species	Strain
<i>Candida albicans</i>	ATCC 10231, TIMM 1768, TIMM 1308
<i>Candida glabrata</i>	ATCC 90030, CBS 138
<i>Candida krusei</i>	TIMM 3404, ATCC 6258
<i>Candida dubliniensis</i>	CBS 7987, CBS8500
<i>Candida tropicalis</i>	ATCC 750, Lucy colony S 9/1
<i>Candida parapsilosis</i>	ATCC 90018, ATCC 22019
<i>Candida guilliermondii</i>	TIMM 0257, TIMM 3400
<i>Clavispora lusitanae</i>	JCM 1610, TIMM 4124
<i>Saccharomyces cerevisiae</i>	ATCC 9763
<i>Schizosaccharomyces pombe</i>	L972h-
<i>Geotrichum candidum</i>	TIMM 0693
<i>Cryptococcus gattii</i>	CN 03010907, CN 03010906
<i>Cryptococcus neoformans</i>	ATCC 90113, CN 03010904
<i>Pichia anomala</i>	TIMM 3420, TIMM 3826
<i>Trichosporon asahii</i>	CBS 2497, 1/26 No.43
<i>Aspergillus fumigatus</i>	TIMM 0108, TIMM 1776
<i>Aspergillus fumigatus</i> var. <i>fumigatus</i>	JCM 10253
<i>Aspergillus niger</i>	JCM 10254, TIMM 0114, TIMM 0115
<i>Aspergillus flavus</i>	JCM 2061, TIMM 0057, TIMM 0059
<i>Aspergillus conicus</i>	JCM 1725
<i>Aspergillus restrictus</i>	JCM 1727
<i>Aspergillus ustus</i>	JCM 1928
<i>Aspergillus sclerotiorum</i>	JCM 1962
<i>Aspergillus unguis</i>	JCM 2256
<i>Aspergillus tamarii</i>	JCM 2259
<i>Aspergillus avenaceus</i>	JCM 2719
<i>Aspergillus sydowii</i>	JCM 2722
<i>Aspergillus nidulans</i> var. <i>nidulans</i>	JCM 2728
<i>Aspergillus varians</i>	JCM 2760
<i>Aspergillus clavatus</i>	JCM 10080
<i>Aspergillus oryzae</i>	JCM 10114
<i>Aspergillus clavatonanicus</i>	JCM 10183
<i>Aspergillus candidus</i>	JCM 10250
<i>Aspergillus ochraceus</i>	JCM 10255
<i>Aspergillus penicillioides</i>	JCM 10256
<i>Aspergillus terreus</i> var. <i>terreus</i>	JCM 10257
<i>Aspergillus versicolor</i>	JCM 10258
<i>Eurotium amstelodami</i>	JCM 1565
<i>Eurotium chevalieri</i>	JCM 1568
<i>Eurotium herbariorum</i>	JCM 1575
<i>Neosartorya fischeri</i> var. <i>fischeri</i>	JCM 1740
<i>Neosartorya quadricincta</i>	JCM 1855
<i>Petromyces alliaceus</i>	JCM 1948
<i>Emmericella rugulosa</i>	JCM 2729
<i>Fennellia nivea</i>	JCM 2731
<i>Penicillium marneffei</i>	TIMM 4090
<i>Penicillium expansum</i>	TIMM 1293
<i>Pseudallescheria boydii</i>	TIMM 0886
<i>Rhizopus oryzae</i>	TIMM 0921
<i>Mucor circinelloides</i>	TIMM 3177
<i>Fusarium moniliforme</i>	TIMM 1294

For nested PCR to detect *P. jirovecii* from clinical specimens, we used the *Pneumocystis*-specific primers PjF9 and PjR4 for first-round PCR and PjF8 and PjR8 for second-round PCR. The other PCR components were as described above. Both first- and second-round PCRs were performed under the following conditions: 94 °C for 4 min; 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1.5 min; and 72 °C for 10 min.

18S rRNA gene sequencing. The 18S rRNA gene DNA extracted from clinical sample number 1 (PCP group) was sequenced. Fig. 1 shows the location of the primers on the 18S rRNA gene. Both strands of the primary PCR product (generated with primers NS1 and NS8) were sequenced directly using an ABI PRISM BigDye terminator v1.1 cycle sequencing kit; Applied Biosystems) using primers NS1, NS3, NS4, NS5, NS6 and NS8 as reported by White *et al.* (1990) and an automated sequencer (ABI PRISM 310 genetic analyser; Applied Biosystems), in accordance with the manufacturer's instructions.

LAMP assay. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical) in reaction mixtures composed of 40 pmol each of primers FIP and BIP, 5 pmol each of primers F3 and B3, 20 pmol each of primers FL and BL, 12.5 µl 2 × reaction mixture, 1 µl *Bst* DNA polymerase, 2 µl DNA sample and distilled water up to a final volume of 25 µl. The mixtures were incubated at 61 °C for 30 min (Realoop-30; Eiken Chemical) or 60 min (LightCycler V3; Roche Diagnostics), and then heated at 80 °C for 2 min to terminate the reaction. When using the LightCycler, 20 mU *Tth* pyrophosphatase ml⁻¹ (thermostable; Roche Diagnostics) and 0.25 µg YO-PRO1 ml⁻¹ (a propidium dye; Molecular Probes) were added to the reaction mixtures. The temperature transition rate was 0.2 °C s⁻¹, increasing from 60 to 98 °C, and the cooling temperature was 30 °C for 1 min for melting curve analysis. For visual fluorescence detection, 1 µl fluorescent detection reagent (Eiken Chemical) was added to the reaction mixture. Control samples of plasmid solution (10², 10⁴ and 10⁶ copies per tube, i.e. 4 × 10³, 4 × 10⁵ and 4 × 10⁷ copies ml⁻¹, respectively) were checked each time.

Detection of LAMP products. LAMP products were detected by real-time fluorescence detection with a LightCycler, real-time turbidity detection with a Realloop-30, and end-point turbidity or visual fluorescence detection, the latter being judged under UV irradiation. When using the LightCycler, melting curves of the products were analysed and specific reactions were identified.

Sensitivity and specificity of the LAMP assay. The sensitivity of the LAMP assay was examined using serial dilutions of plasmid solution (1, 10, 10², 10³, 10⁴ and 2 × 10⁶ copies per tube, i.e. 40, 4 × 10², 4 × 10³, 4 × 10⁴, 4 × 10⁵ and 8 × 10⁷ copies ml⁻¹, respectively) with triplicate samples. Specificity was examined for 51 fungal species (69 strains), as shown in Table 1.

RESULTS AND DISCUSSION

Specificity

A plasmid construct containing the nucleotide sequence of the 18S rRNA gene of *P. jirovecii* (GenBank accession no. AB266392) was amplified by the LAMP method. The experiment using LAMP primers for *P. jirovecii* did not show cross-reactivity with pathogenic or environmental fungi. DNA samples extracted from 69 strains of 51 species of fungi shown in Table 1, including the genera *Candida*,

Table 2. Clinical information on specimens collected from PCP patients

Sample no.	Case no.	Clinical specimen	Underlying disease	Result				
				Microscopic examination	LAMP			PCR
					Real-time fluorescence detection	Copy no.*	Visual detection†	
1	C1	BALF	HIV carrier	+	+	<10 ⁶ ‡	ND	+
2	C2	BALF	RA	+	+	<10 ⁶ ‡	ND	+
3	C3	Sputum	RA	—	+	10 ² –10 ⁴	ND	—
4	C4	BALF	WG	+	+	10 ⁶	ND	+
5	C5	Sputum	HIV carrier	+	+	10 ⁶	ND	+
6	C6	BALF	HIV carrier	+	+	10 ⁴	ND	+
7	C6	Sputum	HIV carrier	+	+	10 ² –10 ⁴	ND	+
8	C7	BALF	RA	+	+	10 ²	ND	—
9	C8	Sputum	RA	+	+	<10 ²	ND	—
10	C9	BALF	RA	—	—	—	ND	—
11	C9 (after Tx)§	Sputum	RA	—	—	—	ND	—
12	C10	Sputum	LC (chemo)	+	+	<10 ²	ND	+
13	C10 (after Tx)§	Sputum	LC (chemo)	—	—	—	ND	—
14	C11	BALF	IPF	—	+	<10 ²	ND	—
15	C12	BALF	HIV carrier	+	+	10 ⁴	ND	+
16	C13	Sputum	HIV carrier	+	+	10 ²	ND	+
17	C14	BALF	HIV carrier	+	+	10 ⁴	+	+
18	C15	BALF	HIV carrier	+	+	10 ² –10 ⁴	+	+
19	C16	BALF	HIV carrier	+	+	10 ² –10 ⁴	+	+
20	C17	BALF	HIV carrier	+	+	10 ² –10 ⁴	+	+
21	C18	BALF	HIV carrier	+	+	10 ² –10 ⁴	+	+
22	C19	BALF	HIV carrier	+	+	10 ² –10 ⁴	+	+
23	C20	BALF	HIV carrier	+	+	10 ² –10 ⁴	+	+
24	C21	BALF	HIV carrier	+	+	10 ⁴ –10 ⁶	+	+

IPF, Idiopathic pulmonary fibrosis; LC, lung cancer; ND, not determined; RA, rheumatic arthritis; WG, Wegener's granulomatosis.

*When clinical specimens were examined by LAMP assay with the LightCycler, 10⁶, 10⁴ and 10² DNA copies of *P. jirovecii* plasmid were included together. The estimate of copy number for each sample is based on these amplification curves.

†Visual detection was by end-point visual turbidity and fluorescence detection. Both methods gave identical results. ND indicates that experiments to check visual detection could not be done as the amount of clinical specimen was too small.

‡As the amount of clinical specimens was small, experiments to check quantitative capability could not be performed.

§After PCP therapy.

||Undergoing chemotherapy.

Aspergillus and *Cryptococcus*, were not detected by the LAMP method.

Sensitivity

With a LAMP assay specific for *Pneumocystis*, it was possible specifically to detect *Pneumocystis* DNA in 30 min, with lower limits of detection of 50 or 100 copies (2 × 10³ or 4 × 10³ copies ml⁻¹) of template DNA using a LightCycler or by visual detection, respectively.

Fig. 2 shows the results of real-time fluorescence detection using a LightCycler. DNA amplification is indicated by the rising curve of fluorescence in Fig. 2(a). With the LAMP assay, it was possible to detect 100 copies per tube (4 × 10³

copies ml⁻¹), with the curve beginning to rise at 12–13 min and reaching a plateau at 22–23 min. Detection of 50 copies per tube (2 × 10³ copies ml⁻¹) could be achieved in 30 min. In reactions of 10 copies per tube (4 × 10² copies ml⁻¹), products were detected in two out of three tubes, with no product detected in the remaining tube.

On visual detection, the results were judged at a reaction time of 30 min. By both visually determined turbidity and fluorescence detection, the assays could detect 100 copies of the target sequence per tube (4 × 10³ copies ml⁻¹). However, the results were unstable at concentrations below 50 copies per tube (2 × 10³ copies ml⁻¹). Fig. 3 shows the results of visual fluorescence detection (Fig. 3a) and turbidity detection (Fig. 3b).

Table 3. Clinical information on specimens collected from non-PCP patients

Sample no.	Case no.	Clinical specimen	Underlying disease	Result				
				Microscopic examination	LAMP			PCR
					Real-time fluorescence detection	Copy no.	Visual detection	
25	C22	BALF	IPF	—	+	<10 ²	ND	—
26	C23	BLF	LC	—	—	—	ND	—
27	C24	BLF	LC	—	—	—	ND	—
28	C25	BLF	LC	—	—	—	ND	—
29	C26	BLF	LC	—	—	—	ND	—
30	C27	BLF	LC	—	—	—	ND	—
31	C28	BLF	LC	—	—	—	ND	—
32	C29	BLF	LC	—	—	—	ND	—
33	C30	BLF	LC	—	—	—	ND	—
34	C31	BLF	LC	—	—	—	ND	—
35	C32	BLF	LC	—	—	—	ND	—
36	C33	BLF	LC	—	—	—	ND	—
37	C34	BLF	LC	—	—	—	ND	—
38	C35	BLF	LC	—	—	—	ND	—

IPF, Idiopathic pulmonary fibrosis; LC, lung cancer; ND, not determined.

Quantitative capability

The times to when fluorescence started rising and when it reached a plateau were short when there were high gene copy numbers in the tube. The graphs for each of the triplicate samples showed good accordance between *P. jirovecii* plasmid copy numbers of 2×10^6 to 100 per tube (8×10^7 to 4×10^3 copies ml⁻¹) (Fig. 2a). However, there was no quantitative capability at template DNA copy numbers of less than 100 per tube (4×10^3 copies ml⁻¹).

Melting-curve analysis

As shown in Fig. 2(b), the melting curves of each sample were almost identical. This assay did not show a non-specific reaction within 30 min; however, the reaction of some templates present at less than 10 copies per tube (400 copies ml⁻¹) for more than 30 min yielded melting-curve peaks that were different from those seen in the specific reactions. Non-specific reactions were observed using

clinical specimens when the assay was continued for 1 h. However, the melting-curve peaks of non-specific reactions could be distinguished from those of specific reactions (Fig. 4). Fig. 4 shows examples of the results of LAMP assays of PCP patients using a LightCycler. The melting curve of sample 12 gave a similar result to the positive control. One of the two distilled water samples and sample 13 showed non-specific reactions, and the peaks of their melting curves were different from those of the positive control.

In this study, we used melting-curve analysis rather than electrophoresis to distinguish specific LAMP products, as the process of agarose gel electrophoresis tends to cause severe contamination. There have been several recent reports of melting-curve analysis of PCR products (Bu *et al.*, 2005; Haverstick *et al.*, 2004; Shrestha *et al.*, 2003). PCR products can be characterized by melting-curve analysis because each double-stranded DNA molecule has a specific melting temperature (T_m) at which 50% of the DNA becomes single-stranded. The T_m value is dependent on

Table 4. Sequences of the LAMP primers used in this study

Primer	Sequence (5'→3')
F3	AGCGTATATTAAAGTTGCTGC
B3	GCTTTGAACACTCTAATTTCTCA
FIP	CCAGTGACACACTTCGGAGTTAAAAAGCTCGTAGTTGAACCTA
BIP	GCGATCCTTCTCTGGATTACAAGTAAAAAGGCCCTGGTTA
FL	GACCGGGCCGTCAAC
BL	GTATGCCCTTCATTGGGTGTAT

Fig. 1. Map showing the location of primers on the 18S rRNA gene of *P. jirovecii*. ITS, Internal transcribed spacer.

both base sequence and GC content. Amplification of primer-dimers or non-specific products will be observed as peaks with different T_m values. Bu *et al.* (2005) reported identification of PCR products amplified from *Candida* spp. and *Aspergillus* spp. using melting-curve analysis, and specific probes have been applied to melting-curve analysis typing of hepatitis virus (Haverstick *et al.*, 2004) and differentiation of *Mycobacterium* species (Shrestha *et al.*, 2003). This is believed to be the first report of melting-curve analysis of LAMP products. Identification of LAMP products may theoretically be determined by probes. However, as a relatively large number of oligonucleotides are already used, this would add to the complexity of this assay.

Clinical samples

A total of 22 samples (21 from PCP patients and 1 from a non-PCP subject) tested positive in the LAMP assay using the LightCycler (Tables 2 and 3). The diagnostic sensitivity of this assay was 87.5 % (21/24 PCP cases). In comparison, *P. jirovecii* DNA was detected in only 17 samples from PCP patients by nested PCR. The nested PCR used in the present study could detect 100 copies of plasmid per tube (10^3 copies ml^{-1}) (data not shown) and the sensitivity of this method was 20 times lower than a quantitative real-time PCR reported by Brancart *et al.* (2005). However, this nested PCR did not detect target DNA in any specimens from non-PCP cases or LAMP reaction-negative specimens. With regard to quantitative capability, we speculated that the target DNA copy number in sample 12 in Fig. 4(a) was equivalent to less than 100 copies per tube (4×10^3 copies ml^{-1}). Similarly, clinical samples were examined with control plasmid (10^2 , 10^4 and 10^6 copies per tube, i.e. 4×10^3 , 4×10^5 and 4×10^7 copies ml^{-1} , respectively) each time, and we estimated the equivalent DNA copy numbers of clinical specimens, as shown in Table 2.

Only one patient (case 22) in the present study showed colonization with *P. jirovecii*. There have been a number of recent reports of *P. jirovecii* colonization in both the general population and in patients with respiratory disease (Maskell *et al.*, 2003; Medrano *et al.*, 2005; Vidal *et al.*, 2006), with colonization rates of about 20 % in healthy subjects (Medrano *et al.*, 2005) and about 30 % in patients with interstitial lung disease (Vidal *et al.*, 2006). Further evaluation of this LAMP method with larger numbers of clinical specimens is currently under way in our laboratory.

Fig. 2. Sensitivity of the LAMP assay. Triplicate samples of serial dilutions of *P. jirovecii* plasmid solution were used as DNA template. LAMP products were amplified and analysed using a LightCycler. (a) Amplification analysis is shown, where the rising curves of fluorescence indicate DNA amplification. (b) In the melting-curve analysis, the melting temperature of each sample is shown as a peak. Number of *P. jirovecii* plasmid copies: $\circ$, 2×10^6 copies (8×10^7 copies ml^{-1}); $\bullet$, 10^4 copies (4×10^5 copies ml^{-1}); $\square$, 10^3 copies (4×10^4 copies ml^{-1}); $\blacksquare$, 10^2 copies (4×10^3 copies ml^{-1}); $\triangle$, 50 copies (2×10^3 copies ml^{-1}); $\blacktriangle$, 10 copies (4×10^2 copies ml^{-1}); $\square$, 1 copy (40 copies ml^{-1}); $\blacklozenge$, distilled water.

Fig. 3. Visual detection of LAMP products. (a) For fluorescence detection, the upper panel shows DNA templates used at $1-10^6$ copies ($40-4 \times 10^7$ copies ml^{-1}) of *P. jirovecii* plasmid, and the lower panel shows clinical samples. (b) Examples of positive (P) and negative (N) controls for turbidity detection are shown.

Cost of the LAMP reaction

The LAMP reaction cost about €2.70 per test. Inclusion of the fluorescence detection reagent increased the cost to €3.20 per test. The costs were about the same as those of the PCR method. The LAMP method will be cheaper than the PCR method if visual detection without special equipment is used.

DNA extraction

In this study, we used a NucliSENS kit (bioMérieux) for extraction of *P. jirovecii* DNA, as described by Fischer *et al.* (2001) and Larsen *et al.* (2002). This was completed in 1–2 h, generally with satisfactory results. However, simpler and easier methods of DNA extraction are necessary for clinical on-site application of this LAMP system. DNA extraction using this kit cost €4.50 per sample.

A method for extraction of *P. jirovecii* DNA using an FTA card (Whatman) has been reported (Jaijakul *et al.*, 2005). We attempted the LAMP assay with FTA cards for some specimens, but the results were not consistent (data not shown).

Advantage of the LAMP method

Visual detection is possible with the LAMP method (Fig. 3a). A sensitive real-time PCR method capable of detecting 50 copies of target sequence ml^{-1} has been developed (Brancart *et al.*, 2005), although this system requires the use of specialized equipment. In contrast, amplification and visual detection of the LAMP reaction with a conventional incubator is a simple identification

Fig. 4. Examples of clinical samples (PCP group) in the LAMP assay using a LightCycler: (a) amplification analysis, (b) melting-curve analysis. The arrow in (b) indicates the specific peak. -, Sample 11 (negative reaction); +, sample 12 (positive reaction); $\times$, sample 13 (negative with non-specific reaction). Positive controls: $\circ$, *P. jirovecii* plasmid DNA 10^6 copies (4×10^7 copies ml^{-1}); $\bullet$, 10^4 copies (4×10^5 copies ml^{-1}); $\blacksquare$, 10^2 copies (4×10^3 copies ml^{-1}). Negative control: $\blacklozenge$, distilled water.

method, although its sensitivity is slightly lower than that of the real-time PCR-based method. In patients with suspected PCP, this LAMP method is competent to diagnose PCP. Therefore, visual detection of the LAMP reaction is faster and easier than the PCR-based method and may be applied in general clinical laboratories.

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