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Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumoniae*

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A loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *Mycoplasma pneumoniae* was developed and evaluated. The assay specifically amplified only *M. pneumoniae* sequences, and no cross-reactivity was observed for other *Mycoplasma* species or respiratory bacterial species. The detection limit for this assay was found to be 2×10^2 copies, corresponding to 2–20 colour changing units of *M. pneumoniae* in 1 h, as observed in a real-time turbidimeter and electrophoretic analysis. The accuracy of the LAMP reaction was confirmed by restriction endonuclease analysis as well as direct sequencing of the amplified product. The assay was applied to 95 nasopharyngeal swab samples collected from patients or from healthy individuals, and compared to a real-time PCR assay in-house. A concordance of 100 % was observed between the two assays. The LAMP assay is easy to perform, shows a rapid reaction and is inexpensive. It may therefore be applied in the routine diagnosis of *M. pneumoniae* infection in the clinical laboratory.

INTRODUCTION

Mycoplasma pneumoniae is the causative agent of atypical pneumonia and is also responsible for other respiratory tract infections such as tracheobronchitis, bronchiolitis, croup and less-severe respiratory-tract infections in older children and young adults (Clyde, 1993). Epidemics break out at intervals of 4–7 years (Foy, 1993). Since this organism is not sensitive to β-lactam antibiotics, which are often used for the empirical treatment of lower respiratory tract infections, a rapid diagnosis method is necessary to avoid the use of such ineffective antibiotics (Dorigo-Zetsma *et al.*, 1999).

Currently, three methods are available for the routine diagnosis of infections caused by *M. pneumoniae*: culture, serology and nucleic acid amplification techniques. Culture is time-consuming, taking several weeks to produce results and is relatively insensitive (Dorigo-Zetsma *et al.*, 1999; Harris *et al.*, 1988; Kok *et al.*, 1988). Serological methods are insufficiently sensitive and require paired serum samples from the acute and convalescent phases of the disease, thus only allowing a retrospective diagnosis (Dorigo-Zetsma *et al.*, 1999; Chia *et al.*, 1988; Sillis, 1990; Fedorko *et al.*, 1995). More rapid, higher sensitivity methods were therefore developed, one of them being the PCR for fragments of the P1 gene or the 16S rRNA gene (Dorigo-Zetsma *et al.*, 1999; Tjhie *et al.*, 1994; Ieven *et al.*, 1996). In the last few years, real-time PCR methods for the diagnosis of *M. pneumoniae* have been described (Hardegger *et al.*, 2000; Ursi *et al.*, 2003; Templeton *et al.*, 2003). The advantages of this application, compared to conventional PCR methods, are higher speed and less handling of PCR products such as electrophoretic analysis. However, owing to the expensive systems required, this application is still not very common in hospital laboratories.

Recently, Notomi *et al.* (2000) reported a novel nucleic acid amplification method called loop-mediated isothermal amplification (LAMP), which is capable of amplifying DNA under isothermal conditions with high specificity, efficiency and speed. This method depends on autocycling strand-displacement DNA synthesis performed by the *Bst* DNA polymerase, and the amplification products are stem–loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops (Notomi *et al.*, 2000). The most significant advantage of LAMP is the ability to amplify specific sequences of DNA under isother-

Abbreviation: LAMP, loop-mediated isothermal amplification.

mal conditions between 63 °C and 65 °C, thereby obviating the need for a thermal cycler (Notomi *et al.*, 2000). Moreover, this method can be carried out with simple systems and the LAMP reaction can be monitored in real-time through measurement of turbidity, which is correlated with the production of magnesium pyrophosphate, by means of an inexpensive photometer (Mori *et al.*, 2001).

In the present study, we investigated a real-time quantitative LAMP assay for rapid detection of *M. pneumoniae* in clinical specimens. This is the first research report on the application of this method to the detection of *M. pneumoniae*.

METHODS

Bacterial stains and clinical specimens. For the evaluation of primer specificity, bacterial stains were prepared from 24 reference stains and three clinical isolates representing eight mycoplasmal species and 19 bacterial species (Table 1). Between January and February 2004, nasopharyngeal swab samples were collected from 70 outpatients (age range, 1–74 years; 43 males and 27 females) with acute respiratory symptoms admitted to the University of Tokyo Hospital in Japan, and analysed using the LAMP assay and real-time PCR assay. The diagnosis was based on clinical signs and symptoms (cough, fever, chill, productive sputum, chest pain or abnormal breathing sounds), and radiographic pulmonary abnormalities that were at least segmental and were due to pre-existing or other known causes. As controls, nasopharyngeal swab samples were obtained from 25 healthy volunteers.

Table 1. Bacterial species and strains

Species	Strain*
Mycoplasma pneumoniae PI1428	ATCC 29085
Mycoplasma pneumoniae M129	ATCC 29342
Mycoplasma pneumoniae MAC	ATCC 15492
Mycoplasma orale	ATCC 23714
Mycoplasma salivarium	ATCC 23064
Mycoplasma hominis	ATCC 15056
Mycoplasma fermentans	ATCC 19989
Mycoplasma genitalium	ATCC 33530
Staphylococcus aureus	ATCC 25923
Staphylococcus epidermidis	ATCC 12228
Streptococcus pneumoniae	ATCC 6305
Streptococcus pyogenes	ATCC 19615
Enterococcus faecalis	ATCC 33186
Viridans group *Streptococcus*	Clinical isolate
Moraxella catarrhalis	ATCC 25238
Neisseria lactamica	Clinical isolate
Corynebacterium spp.	Clinical isolate
Haemophilus influenzae	ATCC 35056
Escherichia coli	ATCC 25922
Pseudomonas aeruginosa	ATCC 27853
Proteus vulgaris	ATCC 13315
Enterobacter cloacae	ATCC 23355
Klebsiella pneumoniae	ATCC 13883
Serratia marcescens	ATCC 8100
Stenotrophomonas maltophilia	ATCC 13637
Candida albicans	ATCC 10231
Legionella pneumophila	ATCC 35289

*ATCC, American Type Culture Collection, Rockville, MD, USA.

Design of primers. Oligonucleotide primers used for the LAMP assay of *M. pneumoniae* were designed using the P1 gene sequences (GenBank accession no. M21519). A set of five primers, consisting of two outer (F3 and B3) and two inner (FIP and BIP) primers, and one loop (loop F) primer, capable of recognizing six distinct regions on the target sequence was designed using a LAMP primer design support software program (Net Laboratory). The primer sequences are shown in Fig. 1. FIP consists of a complementary sequence of F1 and a sense sequence of F2. BIP consists of a complementary sequence of B1 and a sense sequence of B2.

DNA preparation. DNA was extracted from bacteria and nasopharyngeal swab samples with a QIAamp DNA mini kit (Qiagen), in accordance with the manufacturer's instructions. The extracted DNA was eluted in a total volume of 30 µl of elution buffer and stored at −80 °C until use.

LAMP. The LAMP assay was conducted on 28 µl of a reaction mixture consisting of a 20 µM concentration of each inner primer (FIP and BIP), a 5 µM concentration of each outer primer (F3 and B3), a 20 µM concentration of the loop primer (loop F), 2× reaction mix (12·5 µl), *Bst* DNA polymerase (1 µl) and 2 µl isolated DNA templates, using the Loopamp DNA amplification kit (Eiken Chemical). For the real-time monitoring of the LAMP assay, the reaction mixture was incubated at 65 °C for 90 min in a Loopamp real-time turbidimeter (LA-200; Teramecs). Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were taken. After making turbidity measurements, LAMP products and those digested with 5 units of the restriction enzyme *Alu*I were electrophoresed in 3 % agarose gels, and then stained with ethidium bromide and visualized on a UV transilluminator at 365 nm. Furthermore, in order to confirm that the LAMP products had the target sequence, direct sequencing was performed using loop F. In addition, direct sequencing of the LAMP products was performed to confirm the accuracy of the LAMP assay. The LAMP products were purified with a PCR purification kit (Qiagen) and sequenced with a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) according to the instructions of the manufacturer. In order to identify inhibition, 5 µl of the purified DNA preparation

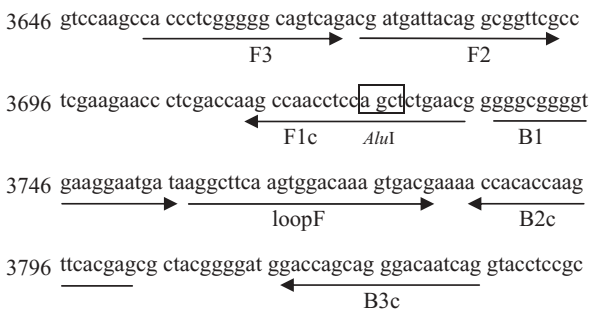

Fig. 1. Oligonucleotide primers used for LAMP assay of *M. pneumoniae* (GenBank accession no. M21519) within P1 gene. F1c, B2c and B3c indicate complementary sequences to F1, B2 and B3, respectively. Arrows and box indicate the position of the target sequences and the site of the restriction enzyme *Alu*I, respectively. F1c, B2c and B3c indicate complementary sequences to F1, B2 and B3, respectively.

from all the LAMP-assay-negative specimens was spiked with 2 µl extract DNA from *M. pneumoniae* M129 at a concentration corresponding to approximately 2×10^2 copies per reaction. All experiments were repeated at least two times.

Real-time PCR. Real-time PCR was performed in a Lightcycler using a Faststart DNA master hybridization probes kit (Roche Diagnostics), by a method described elsewhere (Ursi *et al.*, 2003), except that plasmid DNA (pCRP1) containing the P1 gene of *M. pneumoniae* was used for the standard curve. The primers for amplification were 5′-CACC CTCGGGGGCAGTCAG-3′ (forward) and 5′-CGGGATTCCCCG-CGGAGG-3′ (reverse). The two hybridization probes were 5′-GCCTTATCATTCCTTCACCCCGCCCC-3′FITC and 5′LCRed640-TTCAGAGCTGGAGGTTGGCTTGGTCGAG-3′Ph. The thermal conditions were as follows: initial denaturation step at 95 °C for 10 min and 50 cycles of denaturation at 95 °C for 15 s, hybridization at 65 °C for 10 s, with a touchdown to 60 °C in steps of 1 °C from cycle 20 on and extension at 72 °C for 10 s. The slope was set at 20 °C per second for all steps. The pCRP1 solution was quantified by measuring the optical density at 260 nm, and the copy number was calculated. Serial dilutions of pCRP1 ranging from 2 to 2×10^7 copies per reaction were made in order to prepare standard curves, which were also used for the determination of LAMP detection limits.

RESULTS AND DISCUSSION

The LAMP reaction produced ladder-like patterns on the gel and LAMP products were detected only with the target DNA of *M. pneumoniae* (Fig. 2b).

The LAMP assay specifically amplified DNA from *M. pneumoniae* but not from any of the other *Mycoplasma* species or other respiratory bacterial species listed in Table 1. To establish the detection limit of *M. pneumoniae* by the LAMP assay, serial dilutions of pCRP1 that had been quantified by measuring the optical density at 260 nm were tested and compared with the results for a real-time PCR assay done in-house. The detection limit for the LAMP reaction was found to be 2×10^2 copies for a 60 min reaction in a Loopamp real-time turbidimeter as well as in electrophoretic analysis (Fig. 2a, b). At this point, the turbidity of the reaction tube was discernable by the naked eye. The same result was achieved for the detection limit through serial dilution of a DNA solution of *M. pneumoniae* M129 (data not shown). For the real-time PCR assay, the detection limit was 2×10^1 copies in 60 min (Fig. 2c).

The accuracy of the LAMP reaction was confirmed by digestion with restriction enzyme *Alu*I to ensure that the LAMP products had the corresponding sequence of the P1 gene of *M. pneumoniae*. The sizes of fragments produced by digestion were in good agreement with the sizes predicted theoretically from the expected DNA structures: 66 and 107 bp (Fig. 2b). In addition, the structures of the amplified products were confirmed through direct sequencing, in which the sequences obtained were perfectly matched with the expected DNA sequences (data not shown).

Nasopharyngeal swab samples collected from patients ($n = 70$) or from healthy individuals ($n = 25$) were analysed using the LAMP and real-time PCR assays simultaneously. For both LAMP and real-time PCR assays, six of the 70 patient

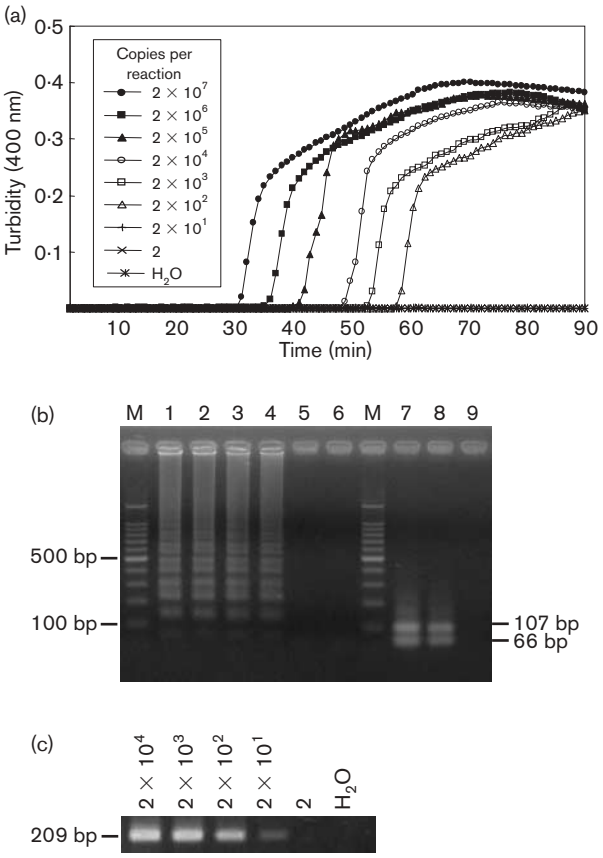

Fig. 2. Comparative sensitivity of LAMP and real-time PCR for detection of *M. pneumoniae* using serial dilutions of pCRP1. (a) Sensitivity of LAMP for *M. pneumoniae* as monitored in a real-time turbidity assay with a Loopamp real-time turbidimeter. (b) Electrophoretic analysis of LAMP products for 60 min reaction. Lane M, 100 bp DNA ladder marker; lanes 1, 2, 3, 4 and 5, 2×10^4, 2×10^3, 2×10^2 and 2×10^1 copies per reaction, respectively; lane 6, no template DNA (H_2O); lanes 7, 8 and 9, LAMP products of lanes 3, 4 and 5, respectively, digested with *Alu*I (bands at 66 and 107 bp). (c) Sensitivity of real-time PCR for the detection of *M. pneumoniae* as observed in electrophoretic analysis.

specimens tested positive and all of the healthy controls tested negative. Thus, the concordance between the two assays was 100 %. No inhibition of the LAMP assay was identified among the LAMP-assay-negative specimens in this study. All of the LAMP-assay-positive specimens were positive with culture using PPLO broth and agar plates (data not shown).

In the present study, we evaluated the LAMP assay, a novel nucleic acid amplification method, for the detection of *M. pneumoniae* in either culture isolates or throat and nasal specimens. This assay is a simple diagnostic tool in which the reaction takes place in a single tube. For this purpose, the buffer, primers and DNA polymerase are mixed together and the mixture is incubated at 65 °C in a regular laboratory water bath or heat block that provides a constant temperature. The specificity of the assay is extremely high because it

uses four primers for the recognition of six distinct regions on the target DNA (Notomi *et al.*, 2000; Iwamoto *et al.*, 2003). The LAMP assay was initially evaluated for detection of hepatitis B virus DNA (Notomi *et al.*, 2000), and has recently been applied to the direct detection of the *Mycobacterium tuberculosis* complex, *Mycobacterium avium* and *Mycobacterium intracellulare* (Iwamoto *et al.*, 2003), and severe acute respiratory syndrome coronavirus (Hong *et al.*, 2004).

In the present study, the assay specifically amplified only *M. pneumoniae* and no cross-reactivity was observed for other *Mycoplasma* species or other respiratory bacterial species. These results demonstrate that it has high specificity for the amplification of *M. pneumoniae* and detected it with high efficiency. We found that the detection limits using pCRP1 for the LAMP and real-time PCR assays in 60 min were 2×10^2 and 2×10^1 copies, respectively, and the detection limit was the same when using serial dilution of a DNA solution of *M. pneumoniae* M129. This indicates that the LAMP assay has almost the same sensitivity as the real-time PCR assay.

We indicated our titres as copies per microlitre. In the literature, the detection limits for *M. pneumoniae* nucleic acid amplification assays are expressed in different units [number of colony-forming units (c.f.u.), number of colour-changing units (c.c.u.), number of cells or quantity of DNA], which makes a straightforward comparison of the sensitivities of assays very difficult. One colour-changing unit corresponds to 10–100 organisms (Loens *et al.*, 2002). For example, Abele-Horn *et al.* (1998) reported that the detection limit of their assay was 3000 genome copies, 30 pg of DNA, 19 c.f.u. or 1900 organisms, and Ursi *et al.* (2003), who used similar a method similar to ours for the real-time PCR assay, noted a detection limit of 2 c.c.u. *M. pneumoniae* per reaction. Taking these findings together, expressed in c.c.u., the detection limit for *M. pneumoniae* by the LAMP assay is estimated to be equivalent to 2–20 c.c.u. of *M. pneumoniae* per reaction.

Another feature of this assay is that if it is used as the standard curve of pCR1, *M. pneumoniae* concentrations in clinical samples may be measured, enabling *M. pneumoniae* infection to be discovered at an early stage and potential source transmitters to be identified. Also, since the concordance between the LAMP and the real-time PCR assay for 95 nasopharyngeal swab samples collected from patients or from healthy individuals was 100 %, it is capable of being applied to the routine diagnosis of *M. pneumoniae* infection in the clinical laboratory, and clinicians will be able to evaluate its clinical utility in comparison with the real-time PCR assay.

Summing up, this assay was highly specific and showed a sensitivity of 2×10^2 copies *M. pneumoniae* for a 60 min reaction in a Loopamp real-time turbidimeter LA-200 as well as in electrophoretic analysis. In turbidity monitoring, the *M. pneumoniae* LAMP assay had almost the same sensitivity as the real-time PCR assay. Considering this together with its ease of operation, rapid reaction and inexpensive system, the LAMP assay is more appropriate than the real-time PCR assay at the genetic point of care in the hospital laboratory.

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