

Detection of *Mycoplasma pneumoniae* by loop-mediated isothermal amplification (LAMP) assay and serology in pediatric community-acquired pneumonia

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Abstract Rapid diagnosis of *Mycoplasma pneumoniae* pneumonia is required for treatment with effective antimicrobial agents without delay; however, this capacity has not yet been established in clinical practice. Recently, a novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) has been used to rapidly diagnose various infectious diseases. In this study, we prospectively evaluated the efficacy of the LAMP assay to rapidly diagnose *M. pneumoniae* pneumonia in clinical practice. Three hundred sixty-eight children (median age, 3.8 years; range, 0.1–14.3 years) admitted to our hospital between April 2009 and March 2010 for community-acquired pneumonia were enrolled in this study. We obtained throat swabs on admission to detect *M. pneumoniae* DNA and paired serum samples on admission and at discharge to assay *M. pneumoniae* antibody titers. *M. pneumoniae* pneumonia was diagnosed by either a positive LAMP assay or a fourfold or greater increase in antibody titer. Overall, 46 children (12.5% of the patients with pneumonia) were diagnosed with *M. pneumoniae* pneumonia; of these, 27 (58.7%) were aged less than 6 years. Of the aforementioned 46 children, 38 (82.6%) and 37 (80.4%) were identified by LAMP and serology, respectively. When the results of serology were taken as the standard, the sensitivity and specificity and positive and negative predictive values of the LAMP assay

were 78.4%, 97.3%, 76.3%, and 97.6%, respectively. We concluded the LAMP assay may be useful for rapid diagnosis of *M. pneumoniae* pneumonia.

Keywords *Mycoplasma pneumoniae* · Pediatric community-acquired pneumonia (CAP) · Rapid diagnosis · Loop-mediated isothermal amplification (LAMP)

Introduction

Mycoplasma pneumoniae is a major cause of community-acquired pneumonia (CAP) in children and young adults, accounting for 10–30% of cases [1–3]. Because of the absence of a cell wall, *M. pneumoniae* is resistant to β -lactams. Minocyclines and fluoroquinolones are not generally recommended for use in pediatric patients because of adverse effects; hence, macrolides are as the first-choice agents for treating *M. pneumoniae* infections. Thus, a rapid and accurate diagnosis is required for appropriate treatment.

No specific clinical, epidemiological, or laboratory findings enable a definite diagnosis of *M. pneumoniae* pneumonia early in the clinical course. Currently, several methods are available for the definitive diagnosis of *M. pneumoniae* infections: culture, serology, and nucleic acid amplification techniques. Isolation of *M. pneumoniae* remains the gold standard diagnostic procedure for this infection. Furthermore, a significant increase in the *M. pneumoniae* antibody titer between acute and convalescent serum samples is diagnostic. However, these two confirmatory methods are extremely slow and too insensitive to be of practical use for diagnosing *M. pneumoniae* infection. The detection of the specific immunoglobulin (Ig) M antibody is useful for rapid diagnosis; however, this

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IgM antibody can persist for years in asymptomatic individuals [4] and is insufficiently sensitive and specific during the acute phase [5]. Polymerase chain reaction (PCR) analysis of nasopharyngeal or throat swabs for *M. pneumoniae* DNA has been reported to be a rapid, sensitive, and specific method by several authors [6–8]. However, because of complicated procedures and the expensive systems required, this application is still uncommon in hospital laboratories. Therefore, development of easier, inexpensive, sensitive, and accurate diagnostic methods has long been desired.

Recently, Notomi et al. [9, 10] reported a novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP), which can amplify DNA under isothermal conditions with high specificity, efficiency, and speed. The LAMP reaction uses a DNA polymerase with strand displacement activity and multiple primers, recognizing six distinct sequences in the target DNA. The most significant advantage of LAMP is its ability to amplify specific DNA sequences at 63°–65°C without thermocycling. Therefore, this technique requires relatively simple and cost-effective equipment, making it amenable for use in hospital laboratories. Because of these advantages, this method has been used to rapidly diagnose various infectious diseases [11–14].

LAMP assay for the rapid detection of *M. pneumoniae* was recently developed and had almost the same sensitivity and specificity as a PCR assay [15, 16]. Meanwhile, the standard laboratory diagnosis of *M. pneumoniae* infection presently relies on serological methods in clinical practice, and no studies have been conducted to compare the diagnostic values of serology and LAMP. Additional work is required until the method can be adopted as a routine procedure in hospital laboratories as well as diagnostic laboratories.

In this study, we prospectively evaluated the efficacy of the LAMP assay for rapidly diagnosing *M. pneumoniae* pneumonia and compared it with that of antibody titer determination in pediatric patients with CAP.

Patients and methods

Patients and specimens

A total of 368 otherwise healthy children, aged 0.1–14.3 years (median, 5.3 years), who had pyrexia, cough, and abnormal chest X-ray findings were diagnosed with pneumonia between April 2009 and March 2010 and were admitted to Konan Kosei Hospital (Aichi, Japan). On admission, we obtained throat swabs from all patients to detect *M. pneumoniae* DNA; furthermore, paired serum samples were obtained on admission and at the

convalescent stage to measure complement fixation titers to *M. pneumoniae*, as previously reported [5, 17]. Throat swabs were obtained only by trained pediatricians and immediately transported to our laboratory; specimens were stored at 4°C until DNA extraction, usually within 72 h [18, 19].

To diagnose other pathogens, we performed bacterial cultures of throat swabs, sputum (if possible), and blood on admission. When a differential diagnosis for influenza virus, adenovirus, or a respiratory syncytial virus (RSV) infection was necessary, we detected each viral antigen from a nasopharyngeal swab using a rapid diagnostic kit, and the antibodies in paired sera were also measured.

The study design and purpose, approved by the Institutional Review Board of Konan Kosei Hospital, were fully explained to all patients and/or guardians, and informed consent was obtained before enrollment.

DNA extraction and the LAMP reaction

Cotton-tipped swabs were placed in 1 ml sterilized water. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) and eluted in 50 µl distilled water. LAMP reactions were conducted as described previously [16]. Briefly, we used a set of six primers (B3, F3, BIP, FIP, BL, and FL) that recognized six distinct sequences. Primers for the LAMP assay were designed based on the SDC1 sequence (DDBJ/EMBL/GenBank accession no. M35024), which resides in the P1 operon [20]. The LAMP assay was conducted in a 25-µl reaction mixture containing 12.5 µl 2× reaction mix, 8 U *Bst* DNA polymerase, the specific primer set (40 µM BIP and FIP, 10 µM B3 and F3, and 20 µM BL and FL), and 5 µl isolated DNA templates using a Loopamp DNA Amplification Kit (Eiken Chemical, Tokyo, Japan). The reaction mixture was incubated at 65°C for 60 min, and LAMP products were detected as turbidity using a Loopamp real-time turbidimeter (RT-160C; Eiken Chemical). LAMP results were available within 1.5 h. The accuracy of the LAMP reaction was confirmed by restriction endonuclease analysis of the amplified product. The lower detection limit for the LAMP reaction was six copies per reaction tube; this was more than or equal to that of the two nested PCRs used as references [16]. The assay specifically amplified only *M. pneumoniae* sequences, and no cross-reactivity was observed for common respiratory bacterial species or other *Mycoplasma* species [16].

Definition of *M. pneumoniae* pneumonia

We diagnosed *M. pneumoniae* as the cause of pneumonia when *M. pneumoniae* DNA was detected using LAMP or when an increase of fourfold or more was observed in the antibody titer.

Statistical analysis

Statistical analyses were conducted using the StatView version 5.0 software (SAS Institute, Cary, NC, USA). An overall difference between the groups was determined by the chi-square test or a one-way analysis of variance (ANOVA). When the one-way ANOVA was significant, differences between individual groups were estimated using the Tukey–Kramer test. *P* values less than 0.05 were considered statistically significant.

Results

During our 1-year prospective study, 46 (12.5%) of the 368 patients with pneumonia were diagnosed with *M. pneumoniae* pneumonia. *M. pneumoniae* caused pneumonia throughout the year, and no seasonal variation was observed in its incidence (data not shown). Figure 1 shows the age distribution of the 46 patients with *M. pneumoniae* pneumonia and the 322 patients with other types of pneumonia. The median age of patients with *M. pneumoniae* pneumonia was 5.4 years (range, 1.4–14.3 years) and included 28 boys and 18 girls; 58.7% (27 of 46) were less than 6 years of age [6 (13.0%) of 46, 1–2 years; 21 (45.7%) of 46, 3–5 years]. The incidence of *M. pneumoniae* pneumonia increased with age [9.6% (27 of 286) among patients aged less than 6 years and 23.1% (19 of 82) among patients aged more than 6 years]. The median intervals between symptom onset (cough, fever) and admission were 5 days (range, 2–9 days) and 6 days (range, 1–11 days), respectively. The median white blood cell (WBC) count and C-reactive protein (CRP) levels were 7,100/ μ l (range, 3,400–14,300/ μ l) and 2.1 mg/dl (range, 0.2–7.6 mg/dl), respectively. Coinfection with other microorganisms was observed in 9 patients (19.6%) (RSV, 3; *Streptococcus pneumoniae*, 2; *Streptococcus pyogenes*, 2; *Haemophilus influenzae*, 1; and influenza virus type A, 1). All 46 patients with *M. pneumoniae* pneumonia were initially treated with macrolides (or plus β -lactams), and 3 were subsequently treated with minocyclines when symptoms did not improve. All patients recovered with no sequelae.

Among the 46 patients with *M. pneumoniae* pneumonia, 38 (82.6%) were positive in the LAMP assay and 37 (80.4%) were diagnosed positive by serology. The results of the LAMP assay and serology correlated well, with corresponding negative and positive results in 322 (87.5%) and 29 patients (7.9%), respectively (Table 1). When serology was considered the standard, the sensitivity and specificity and positive and negative predictive values of the LAMP assay were 78.4% (29/37), 97.3% (322/331), 76.3% (29/38), and 97.6% (322/330), respectively.

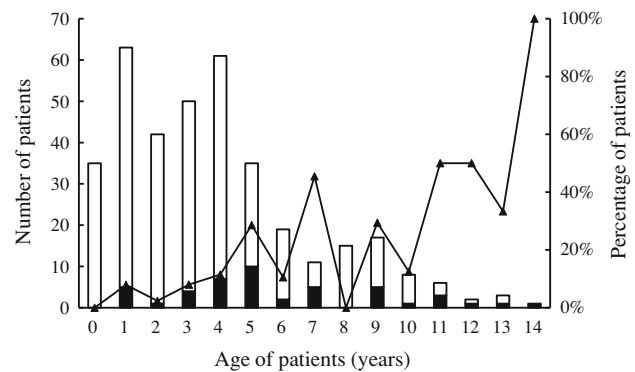


Fig. 1 Number and percentage of patients with *Mycoplasma pneumoniae* pneumonia at each age. Solid bars are number of patients with *M. pneumoniae* pneumonia ($n = 46$); open bars are number of patients with other pneumonia ($n = 322$); triangles are percentage of patients with *M. pneumoniae* pneumonia

Table 1 Correlation between loop-mediated isothermal amplification (LAMP) and *Mycoplasma pneumoniae* pneumonia serology

LAMP	Serology		Total
	Positive	Negative	
Positive	29 (7.9%)	9 (2.4%)	38 (10.3%)
Negative	8 (2.2%)	322 (87.5%)	330 (89.7%)
Total	37 (10.1%)	331 (89.9%)	368 (100%)

Particularly, among 6 patients aged 1–2 years, 4 were positive in the LAMP assay, 5 were diagnosed positive by serology, and 3 were diagnosed by both.

A comparison of the clinical characteristics of patients with *M. pneumoniae* by diagnostic method is shown in Table 2. Patients who received macrolides before sampling for the LAMP assay were less likely to be positive (8/11; 72.7%) than those who did not (30/35; 85.7%). However, the difference was not significant. Mean WBC count and CRP levels on admission were significantly higher in patients diagnosed positive by serology only. No significant differences were observed for other clinical manifestations.

Discussion

Mycoplasma pneumoniae is the main cause of CAP in children and young adults [1–3] and has been reported in 10–30% of pediatric CAP cases [1–3]. Recent studies have shown that pre-school-aged and school-aged children are prone to *M. pneumoniae* infection [21, 22]. In our study, *M. pneumoniae* was the pathogen in 12.6% of patients with pneumonia, and the number of patients with *M. pneumoniae* pneumonia was higher in patients aged less than 6 years than in patients 6 years or more; this finding was similar to those of previous reports.

Table 2 Comparison of clinical characteristics of *Mycoplasma pneumoniae* pneumonia by diagnostic method

	Values of the cases tested			P
	LAMP (+)/serology (+)	LAMP (+)/serology (-)	LAMP (-)/serology (+)	
Number of patients (male:female)	29 (18:11)	9 (7:2)	8 (3:5)	0.24
Age (years) ^a	6.4 ± 2.9	6.9 ± 3.6	5.8 ± 3.9	0.81
Duration of symptoms before LAMP (days) ^a				
Cough	5.6 ± 2.2	5.6 ± 2.3	6.4 ± 2.5	0.61
Fever	5.4 ± 1.6	4.9 ± 1.2	4.5 ± 1.7	0.31
Interval between two serological tests (days) ^a	6.1 ± 1.8	5.7 ± 1.0	5.9 ± 0.7	0.90
Macrolide treatment preceding admission [n (%)]	8 (27.6)	0 (0.0)	3 (37.5)	0.14
Rale on presentation [n (%)]	12 (41.3)	3 (33.3)	4 (50.0)	0.82
White blood cell counts on admission (/μl) ^a	7,000 ± 1,800 [†]	6,500 ± 1,600 [‡]	9,100 ± 3,000 ^{†‡}	0.029
Serum C-reactive protein on admission (mg/dl) ^a	2.0 ± 1.7 [†]	2.3 ± 1.1	3.8 ± 2.1 [†]	0.049
Coinfecting pathogens (n)	HI (3), RSV (3), GAS (2), SP (1), flu (1)	–	SP (1), RSV (1)	–
CF titer to <i>M. pneumoniae</i> on admission ^a	2 ^{1.7} ± 1.2	2 ^{1.4} ± 1.3	2 ^{1.9} ± 1.7	0.80

Bold values are statistically significant

HI, *Haemophilus influenzae*; RSV, respiratory syncytial virus; flu, influenza virus; SP, *Streptococcus pneumoniae*; GAS, *Streptococcus pyogenes*

^a Mean ± SD

[†] $P < 0.05$, between LAMP (+)/serology (+) and LAMP (-)/serology (+) (Tukey–Kramer test)

[‡] $P < 0.05$, between LAMP (+)/serology (-) and LAMP (-)/serology (+) (Tukey–Kramer test)

Several reports have shown that coinfection of *M. pneumoniae* with other pathogens is not rare [2, 3]. Hamano-Hasegawa et al. [3] reported that the proportion of coinfection of *M. pneumoniae* with other pathogens was 17.9%. In our study, 9 of 46 patients with *M. pneumoniae* pneumonia were coinfecting with pathogens other than *M. pneumoniae*. These patients could be appropriately treated with effective antibiotics only after a rapid diagnosis by the LAMP assay.

Macrolide-resistant *M. pneumoniae* has been clinically isolated worldwide in recent years, and a future increase in *M. pneumoniae* pneumonia for which treatment with macrolides will be ineffective is a concern [23–25]. Patient symptoms appear to be prolonged when isolates show macrolide resistance [26]. Furthermore, *M. pneumoniae* pneumonia cannot be distinguished clinically from that caused by other viral or bacterial agents. Thus, a rapid and accurate laboratory diagnosis is required for the treatment of *M. pneumoniae* pneumonia with effective antimicrobial agents without delay.

Detecting *M. pneumoniae* by PCR has been examined by many authors to develop an early diagnosis [6–8, 27]. Several studies have demonstrated that PCR is a good alternative to serological tests for detecting *M. pneumoniae* [6–8, 27]. Martinez et al. [27] showed that the global agreement between PCR and serological results was 343/357 (96.1%). Yoshino et al. [16] established a LAMP

assay, which had almost the same sensitivity and specificity as a PCR assay, for rapidly detecting *M. pneumoniae*. We used a LAMP assay to diagnose *M. pneumoniae* pneumonia in pediatric patients. Moreover, our results showed a good global agreement between the LAMP assay and serological results (351/368; 95.4%).

Among the discordant results in our series, nine patients were LAMP positive but serologically negative. These cases may represent either a mismatch of the serum samples or an *M. pneumoniae* carrier state. We obtained paired serum samples on admission and at discharge to decrease the problem of client visits. However, it is recommended to obtain a second sample after a 2- to 3-week interval to demonstrate a significant rise in antibody titer in cases of a low level of specific IgG [28]. If paired serum samples were obtained at longer intervals, more patients would be serologically diagnosed. Daxboeck et al. [28] stated that transient asymptomatic carrying of *M. pneumoniae* resulted from the persistence of the pathogen after previous disease. The rate of *M. pneumoniae* carriage in asymptomatic healthy children is between 0% and 2.2% [29–32]. It may be helpful to use serological methods simultaneously for a definite diagnosis, but specific IgM antibodies are not produced constantly during the early phase of the disease [5, 28, 33]. Although detecting *M. pneumoniae* DNA in throat swabs is not necessarily indicative of a causative role of the pneumonic pathogen, it is difficult to distinguish the

infectious pathogen from carriage during the acute phase, so it appears to be reasonable to use anti-mycoplasma therapy in LAMP-positive cases.

Eight patients were serologically diagnosed but had negative results in the LAMP assay. These cases probably represented false-negative LAMP results that may have originated either from a low bacterial load resulting from previous antibiotics or inadequate sample dilution. Morozumi et al. [1] reported that PCR positivity was significantly lower among patients receiving antibiotics with susceptibility to *M. pneumoniae* than those who did not, although no significant differences were observed in our study. Furthermore, the specimen type can affect the rate of *M. pneumoniae* DNA detection [18, 34, 35]. We used throat swabs from the oropharynx because of the ease of obtaining other respiratory specimens in pediatric practice. However, some previous reports have shown that sputum is better than other specimens, including throat swabs, for detecting *M. pneumoniae* DNA [18, 36]. It may be more useful to diagnose *M. pneumoniae* pneumonia by the LAMP assay using sputum, if available. Interestingly, for unknown reasons, the WBC count and CRP levels were significantly high in this population. It was suspected that some patients developed bacterial pneumonia after the *M. pneumoniae* infection improved.

In conclusion, we suggest that this LAMP assay will be useful for the rapid diagnosis of *M. pneumoniae* in children. Thus, antimicrobial agent therapy can be initiated without delay. However, the sensitivity of the LAMP method is significantly dependent on procedures of sampling technique, sample storage, and transfer of samples. Appropriate sampling and treatment of samples are essential prerequisites for optimal performance of this method. Thus, additional studies in a larger patient population and in many institutions will be required to confirm our findings.

Conflict of interest The authors did not receive any financial support from any third party

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