Clinical utility of loop-mediated isothermal amplification for rapid diagnosis of *Mycoplasma pneumoniae* in children

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Loop-mediated isothermal amplification (LAMP), which rapidly amplifies DNA with high specificity and efficiency under isothermal conditions, has almost the same sensitivity as PCR for *Mycoplasma pneumoniae* detection (Saito et al., 2005; Yoshino et al., 2008) and is simpler (Mori et al., 2001) and less expensive than PCR. A previous study showed that the LAMP assay and serology are strongly correlated in the diagnosis of MP pneumonia among children (Gotoh et al., 2012). However, the clinical utility of this assay for MP infection has not been reported. We therefore investigated if use of the LAMP assay had an effect on diagnostic utility.

**INTRODUCTION**

*Mycoplasma pneumoniae* (MP) is a major cause of community-acquired pneumonia (CAP) among school-age children (Korppi et al., 2004). MP infections occur worldwide and throughout the year but are slightly more frequent during late summer and early autumn (Ferwerda et al., 2001). The clinical picture is related to age and pre-exposure immunity (Kliegman, 2011). MP infection is usually mild and may be asymptomatic, particularly among adults with a previous history of MP infection (Waites, 2003). High seroprevalence is observed in healthy individuals because antibody titres remain elevated for years after infection (Nir-Paz et al., 2006). Early diagnosis of MP is important for the appropriate use of antimicrobials, because β-lactams are ineffective (Long et al., 2012) and macrolides are the first-line therapy for this pathogen (Kuroki et al., 2004). There are presently three methods to identify MP infection: culture, serological tests, and nucleic acid amplification techniques (Daxboeck et al., 2003). Serological tests such as particle agglutination require paired serum specimens collected 2–3 weeks apart to confirm a fourfold or greater rise in antibody, which is the criterion for a reliable diagnosis (Long et al., 2012). However, it is difficult to apply such testing results to actual treatment decisions. Nucleic acid amplification technologies such as PCR are fast, sensitive (Kuroki et al., 2004), strongly correlated with serology (Blanco et al., 2011) and provide useful information for selecting antimicrobials; however, they can be costly and require complicated and expensive instrumentation.

Notomi et al. (2000) developed loop-mediated isothermal amplification (LAMP), which rapidly amplifies DNA with high specificity and efficiency under isothermal conditions. The LAMP assay has almost the same sensitivity as PCR in detecting MP (Saito et al., 2005; Yoshino et al., 2008) and is simpler (Mori et al., 2001) and less expensive than PCR. A previous study showed a good correlation between the LAMP assay and serology in the diagnosis of MP pneumonia among children (Gotoh et al., 2012). However, the clinical utility of this assay for MP infection has not been reported. We therefore investigated if use of the LAMP assay had an effect on diagnostic utility.

clinical interventions in treating MP pneumonia among children.

METHODS

Patients and sample collection. During the period from April 2012 through September 2012, 111 children (median age, 4 years; range, 2 months to 15 years) were diagnosed with CAP at Niigata Prefectural Shibata Hospital, a tertiary community hospital in Shibata City (approximate population, 100,000) in Niigata Prefecture, Japan. This group included both inpatients and outpatients presenting with respiratory symptoms and fever but without other symptoms or underlying diseases. Chest radiographs showed abnormal findings indicative of MP pneumonia, in the context of the patients’ ages and contact with other sick individuals. Paediatricians obtained throat swabs by using sterile cotton swabs, and a commercial kit (Serodia-Myco II, Fujirebio) was used to collect paired serum samples (at admission and during convalescence) to measure antibody titres to MP by particle agglutination. MP pneumonia was diagnosed among patients who had a fourfold or greater rise in MP antibodies. When respiratory syncytial virus or adenovirus was suspected, rapid antigen tests were performed using a commercially available kit. This study was approved by the ethics committee of Niigata Prefectural Shibata Hospital.

DNA extraction and LAMP assay. DNA was extracted from throat swabs using the QIAamp DNA Mini kit (Qiagen) in accordance with the manufacturer’s instructions. For the LAMP assay, six primers – inner primers (Forward Inner Primer and Backward Inner Primer), outer primers (F3 and B3) and loop primers (LoopF and LoopB) – were designed by targeting the SDC1 sequence specific to MP, as previously described (Yoshino et al., 2008). Using the commercially available Loopamp Mycoplasma P Detection kit (Eiken Chemical), 5 μl of the extracted DNA sample was added to 20 μl of master mix composed of 20 μl reaction mix and 1 μl of Bst DNA polymerase, which resulted in a final concentration of 1.6 μM of each inner primer, 0.4 μM of each outer primer, 0.8 μM of each loop primer, 1.4 mM dNTPs, 8 mM magnesium sulfate, and 12 units Bst DNA polymerase. The mixture was incubated at 65 °C for 60 min in a Loopamp EXIA turbidity meter (Eiken Chemical), which qualitatively monitors the turbidity of magnesium pyrophosphate as a LAMP product. The total time required for the LAMP assay, including the DNA extraction step, was approximately 2.5 h.

The accuracy of the LAMP assay was evaluated by real-time PCR analysis of the same extracted DNA samples, as previously described (Morozumi et al., 2006).

Statistical analysis. We compared the LAMP assay and paired sera with respect to the time required for MP diagnosis. For the comparison, the Wilcoxon rank-sum test was used because the data were distributed in a non-parametric manner. The analysis was performed using the JMP 10 software package (SAS Institute). A P-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Patient characteristics

In total, MP pneumonia was diagnosed in 45/111 (41 %) patients, among whom 43 patients (96 %) were positive by the LAMP assay; 34 (79 %) of those 43 patients were inpatients and 9 (21 %) were outpatients. Twenty-four patients (56 %) were male, and their median age was 9 years (range, 1–15 years). The number of patients aged 1–4, 5–9 and 10–15 years were 8 (19 %), 14 (32 %) and 21 (49 %), respectively. The remaining 66 patients, who had no significant rise in MP titres, had negative results by the LAMP assay. No patient had a diagnosis of coinfection with respiratory syncytial virus or adenovirus.

Concordance between the LAMP assay and serum titres

The rate of concordance between the LAMP assay and a substantial increase in titre was 98 % (Table 1). The sensitivity, specificity, positive predictive value, and negative predictive value of the LAMP assay were 96 %, 100 %, 100 %, and 97 %, respectively.

Positivity with the LAMP assay

The interval from fever onset to collection of throat swabs ranged from 4 to 10 days (median, 7 days). Twenty-five of 43 (58 %) patients had already been administered antibiotics such as clarithromycin (n=14), azithromycin (n=5), rokitamycin (n=1), tosufloxacin (n=9), and minocycline (n=2), which are considered effective against MP. MP titres of 12 patients (28 %) were less than 1:40, and 16 patients (37 %) had titres greater than 1:160 when a positive result was obtained on the LAMP assay. Among the 43 patients, 33 samples were available for confirmatory real-time PCR analysis, which showed 100 % concordance.

Difference between the LAMP assay and serum titres in the interval before diagnosis of MP pneumonia

We compared the LAMP assay and paired serum samples with respect to the time required for a definitive diagnosis of MP pneumonia (Table 2). The median duration from onset of symptoms to a positive test result for MP pneumonia by the LAMP assay (i.e. diagnosis) was 7 days, which was significantly shorter than the 13 days required for paired sera (P<0.0001). The median difference between

| Table 1. Comparison of LAMP assay results and MP particle agglutination titres |
|-------------------------------------|------------------|------------------|------------------|
| Positive [n (%)] | Negative [n (%)] | Total [n (%)] |
| Positive | 43 (96) | 0 (0) | 43 (39)|
| Negative | 2 (4)* | 66 (100) | 68 (61) |
| Total | 45 (41) | 66 (59) | 111 (100) |

*A subsequent LAMP assay yielded positive results for both patients.
the LAMP assay and serology in the time required to diagnose MP pneumonia was 6 days (range, 1–12 days).

**DISCUSSION**

This study demonstrated the clinical utility of the LAMP assay in diagnosing MP among children. The LAMP assay required less time than serology for diagnosis and enabled appropriate management of children with CAP.

The LAMP assay is an acknowledged alternative to PCR (Sidoti et al., 2013). It has almost the same sensitivity as PCR in detecting MP (Saito et al., 2005; Yoshino et al., 2008), and the time required to obtain results is the same as real-time PCR – approximately 1 h (Blanco et al., 2011). The LAMP assay has a few advantages over real-time PCR assay. First, the device for the LAMP assay is approximately 2–3 times less expensive than the instrumentation platform for real-time PCR (including a thermal cycler), which enables institutions to incorporate the LAMP assay into their routine clinical practice, especially in areas where medical resources are limited. Second, LAMP is an isothermal assay, and therefore amplification can be performed simply with a heating block. Device maintenance is minimal; usually no regular maintenance is required during the first 5 years of use. Furthermore, the LAMP assay does not require complicated fluorescence detection settings. LAMP amplification yields a by-product, magnesium pyrophosphate, that appears as white precipitate (Mori et al., 2001). Because the turbidity of the reaction mixture increases with the production of precipitate, it correlates with the amount of DNA synthesized, and real-time monitoring of the LAMP reaction can be achieved by measuring turbidity with an inexpensive turbidimeter. The LAMP assay has been applied to many other organisms such as *Mycobacterium tuberculosis* (Aryan et al., 2013) and *Bordetella pertussis* (Kamachi et al., 2006), with the above advantages.

Because it is easily transmitted by droplets, MP can cause outbreaks in schools and communities (Kliegman, 2011). Thus, it is crucial to diagnose MP early enough to start appropriate antimicrobial therapy to prevent further transmission of MP infection. Therapy is likely to be more effective when started within 3–4 days after disease onset (Long et al., 2012). In many cases, this organism remains contagious during and after antimicrobial therapy (Long et al., 2012); however, antimicrobial treatment helps reduce the duration of symptoms and signs of MP pneumonia (Ferwerda et al., 2001). Currently, serological assay is the most common method for diagnosing MP infection (Waites, 2003). However, it is not practical in clinical settings because of the time required to obtain paired serum samples (an interval between samples of more than 2 weeks is ideal) (Long et al., 2012). Although a fourfold or greater increase in titre is required for a diagnosis of MP infection, a titre over 1:160 by particle agglutination with only a single serum sample might be a reliable criterion of diagnosis (Daxboeck et al., 2003). In contrast, single serum serology is considered unsuitable for diagnosis of MP infection because of the high seroprevalence in healthy individuals (Nir-Paz et al., 2006). Thus, caution is warranted when only serology is used to diagnose MP infection at an early stage. An assay using genetic materials is suitable for rapid and accurate diagnosis of MP infection, although the interpretation of results requires caution because a positive result does not mean that patients have acute infection; it could instead represent remnant genetic materials after acute infection. Nucleic acid amplification techniques should be limited to patients with a clinical picture suggestive of MP infection, as there are more than a few asymptomatic carriers, with and without positive serological results (Waites, 2003).

β-Lactam antibiotics are the drugs of choice for paediatric CAP and target mainly *Streptococcus pneumoniae*, but they have no activity against MP because it lacks a cell wall (Long et al., 2012). For this reason, macrolides are the first choice for MP infection in children (Ferwerda et al., 2001). Recently, the number and incidence of macrolide-resistant MP strains have increased in Japan (Morozumi et al., 2010), which has led to the use of antimicrobials other than macrolides, including the fluoroquinolones and tetracyclines (for patients ≥8 years of age), when MP infection is suspected or initial macrolide treatment fails. These antimicrobials must be used cautiously because of the possible development of drug-resistant strains and the risk of adverse effects. Furthermore, although it is difficult to diagnose MP infection in children younger than 5 years of age because they tend to have mild illness and are less likely to have overt illness (Kliegman, 2011), a study in Finland found that MP was the cause of CAP in 9% of patients 0–4 years of age (Korppi et al., 2004). In the current study, the LAMP assay was helpful in diagnosing MP infection in young children, accounting for 19% of all MP cases among children 1–4 years of age.

Swab samples for the LAMP assay were obtained from the oropharynx because a previous report highlighted the usefulness of throat swabs in detecting MP using PCR (Michelew et al., 2004). The LAMP assay yielded a positive result as soon as 4 days after fever onset, and the results were not affected by use of antibiotics known to be effective.

**Table 2.** Numbers of patients with a diagnosis of MP pneumonia, by days from fever onset, as determined by LAMP assay and analysis of paired sera

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<th>Days from fever onset</th>
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The median interval required for diagnosis of MP pneumonia was 7 days for the LAMP assay and 13 days for paired sera (P<0.0001).
against MP. The concordance between the LAMP assay and real-time PCR was 100%. Our findings indicate that, as compared with serum titres, MP pneumonia could be diagnosed 6 days earlier with the LAMP assay. The current study clearly demonstrated the rapidity of the LAMP assay in diagnosing MP pneumonia in a clinical setting.

Two patients had negative results on the LAMP assay and a significant increase in antibody titres. MP pneumonia was suspected clinically despite a negative result on the LAMP assay at admission. In both patients, a subsequent LAMP assay obtained 1–3 days after the first test was positive. These two cases can be considered false negatives for the LAMP assay if serology is regarded as the standard method of diagnosing MP infection. These false negatives may have resulted from technical difficulties in obtaining samples or in the care of samples. On the basis of our limited experience with these two patients, a repeat LAMP assay is recommended for patients strongly suspected of having MP pneumonia.

Our study had a few limitations. First, we were unable to evaluate the early stage of MP pneumonia because our centre is a tertiary hospital and thus most patients are referred from their primary physicians. Second, our study was performed in one centre, and the findings might not reflect the characteristics of MP pneumonia in other areas. Third, not all test results were obtained on the day the samples were collected. Although the LAMP assay was performed within 72 h of laboratory receipt of samples, the laboratory was not always able to process them immediately. Finally, not all positive LAMP samples could be subjected to confirmatory real-time PCR analysis, because the samples were retrospectively reviewed and not all samples were available for confirmation.

In conclusion, the LAMP assay was clinically useful in diagnosing MP pneumonia. The LAMP assay has a few advantages over PCR and can be implemented as an alternative method for rapidly diagnosing MP pneumonia in an actual clinical setting.

REFERENCES


