Journal of Virological Methods 196 (2014) 133–138

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Identification of human metapneumovirus genotypes A and B from clinical specimens by reverse transcription loop-mediated isothermal amplification

Qinwei Song, Runan Zhu*, Yu Sun, Linqing Zhao, Fang Wang, Jie Deng, Yuan Qian

Laboratory of Virology, Capital Institute of Pediatrics, Beijing 100020, China

Article history:
Received 20 May 2013
Received in revised form 29 October 2013
Accepted 30 October 2013
Available online 20 November 2013

Keywords:
Human metapneumovirus
Reverse transcription loop-mediated isothermal amplification
Clinical specimen

A B S T R A C T

Human metapneumovirus (hMPV) has been recognized as an important pathogen for acute respiratory infections in children worldwide and classified into genotypes A and B. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay is a rapid diagnostic method for detecting nucleic acids with a single step under isothermal conditions in less than 1 h. RT-LAMP targeting the M gene of hMPV was developed for detecting and identifying hMPV genotypes A and B. The detection limit of the genotype-specific hMPV RT-LAMP assay was 10 times greater than that of conventional reverse transcription polymerase chain reaction (RT-PCR). No cross-reactivity was found with respiratory syncytial virus, parainfluenza virus 1–3, adenovirus, human bocavirus, human rhinovirus, influenza virus A and B, human coronaviruses and enteroviruses. One hundred and fifteen clinical specimens were detected for hMPV genotypes A and B with RT-LAMP, RT-PCR and real-time SYBR PCR. Kappa coefficients showed that there was a good agreement among these three methods. Compared with RT-PCR and real-time SYBR PCR, the genotype-specific RT-LAMP showed better specificity, sensitivity and is more convenient to perform with reduced turn-around time.

1. Introduction

Human metapneumovirus (hMPV) has been recognized as an important virus associated with respiratory tract diseases in infants and children. It is the first member of the Metapneumovirus genus in Pneumoviriae, *Paramyxoviridae*, associated with human diseases (van den Hoogen et al., 2001). Currently, sequence analysis for hMPV isolates indicates that there are two major genetic lineages (genotypes A and B) (Bastien et al., 2003; Biacchesi et al., 2003). As an important viral pathogen causing acute lower respiratory infections in children, hMPV is the second viral pathogen of bronchiolitis during infancy and early childhood next to respiratory syncytial virus (RSV) (Skiadopoulos et al., 2006). HMPV associated acute respiratory infections in infants and young children was reported by the same research group of this study in 2003 (Zhu et al., 2003), which was the first report about hMPV infection in China. Since then, surveillance of hMPV infections in children in Beijing has been conducted by antigen test, virus isolation and reverse transcription-polymerase chain reaction (RT-PCR) (Zhu et al., 2007; Wang et al., 2010).

Due to the difficulty of virus isolation and the shortage of specific antibody for antigen test, the technique currently used for rapid detecting hMPV is RT-PCR (Lopez-Huertas et al., 2005; Zhu et al., 2007). However, RT-PCR has some limitations, such as time-consuming, contamination and need for expensive equipment, especially for application in clinical diagnostic laboratories in hospitals.

With the development of molecular techniques, the methods for virus detection are constantly updated and improved. Loop-mediated isothermal amplification (LAMP) was developed originally as a rapid diagnostic method for the detection of nucleic acids under isothermal conditions (Notomi et al., 2000; Mori and Notomi, 2009). The LAMP assay utilizes four primers, which are specific for six gene sequences on the targeted DNA. The products can be observed as a white precipitant of magnesium pyrophosphate. LAMP and RT-LAMP have been used for detecting various DNA and RNA viruses (Mori et al., 2004; Yoneyama et al., 2007; Curtis et al., 2009; Kubo et al., 2010; Luo et al., 2011). To obtain a rapid and reliable assay to detect hMPV from clinical specimens collected from pediatric patients with acute respiratory infections, a RT-LAMP assay was developed for identifying hMPV A and hMPV B from clinical specimens under isothermal conditions at 63 °C within

* Corresponding author at: Capital Institute of Pediatrics, 2 Yabao Road, Chaoyang District, Beijing 100020, China. Tel.: +86 10 85695576; fax: +86 10 85610550.
E-mail addresses: songtsinwei@163.com (Q. Song), runanzhu@163.com, runan@sina.com (R. Zhu), sunyu780312@163.com (Y. Sun), linqingzhao@gmail.com (L. Zhao), fangwang1128@sina.com (F. Wang), jie_deng@sina.com (J. Deng), yqianbjc@263.net (Y. Qian).

0166-0934/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2013.10.037

Table 1
Primers for the genotype-specific hMPV RT-LAMP assay.

Genotype	Primer[a]	Sequence (5′–3′)	Genome position[b]
A	A-F3	TTCAGGCCAATACACCAC	2287–2304
	A-B3	GTCAAGTGCTACAGTCGC	2445–2462
	A-FIP	ACTTTGTGATGCAGCATACAGA-TTTTCAGTTCTGCTTGATCAGCT	(2348–2369)+TTTT+(2308–2326)
	A-BIP	ACTAAAAGTGAATGCATCAGCYC-TTTTACTTCAAACYTTTTGGGAAG	(2378–2400)+TTTT+(2421–2440)
B	B-F3	GTGTCAAAATTTGTGAGTTCAG	2545–2566
	B-B3	GGCYTCRCTGCTTATTGC	2707–2724
	B-FIP	TTCTCTAGGTCCATGAAGTCACA-TTTTCAAATCAGTTGGCAAAAAGACA	(2608–2630)+TTTT+(2568–2589)
	B-BIP	TACCTGTGACAATACCAGCATTC-TTTTCAGTRGCTGACTCACTCTCT	(2636–2658)+TTTT+(2679–2698)

[a] F: forward outer primer; B: backward outer primer; FIP: forward inner primer; BIP: backward inner primer.
[b] Positions for genotype A are defined according to the sequence of the BJ1887 strain from China (GenBank accession no. DQ843659) and positions for genotype B are defined according to the sequence of the BJ1816 strain from China (GenBank accession no. DQ843658).

60 min and the results can be judged by naked eyes. This assay could be used as a diagnostic method in laboratories, especially in those hospitals or clinics with limited equipment.

2. Materials and methods

2.1. Primer designing

The sequences of M genes from hMPV genotypes A and B were retrieved from GenBank and aligned by DNAStar software. The highly conserved regions of the M genes of hMPV genotypes A (GenBank accession no. DQ843659) and B (GenBank accession no. DQ843658) were chosen as the targets for primer designing. Oligonucleotide genotype-specific primers for hMPV were designed with the online LAMP primer design software PrimerExplorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html) and synthesized by Invitrogen (Shanghai, China). The locations and sequences for the set of four primers including outer primers (F3 and B3), a forward inner primer (FIP) and a backward inner primer (BIP) are shown in Table 1.

2.2. Clinical specimens and RNA extraction

Clinical specimens including throat swabs from outpatients and nasopharyngeal aspirates from the hospitalized were collected from infants and young children with acute respiratory infections when they visited the Affiliated Children's Hospital of Capital Institute of Pediatrics, Beijing, China. RNA was extracted from the specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was stored at −80 °C until further processing.

This project was approved by the Institutional Review Board of Capital Institute of Pediatrics (IRB number: SHERLL2011039). This project did not involve personal data of the patients, such as name, age, and gender.

2.3. Genotype-specific RT-LAMP for hMPV

Two RT-LAMP reactions were assembled, including hMPV genotype A specific RT-LAMP and genotype B specific RT-LAMP. Each sample was detected for hMPV genotype A and B by genotype-specific RT-LAMP simultaneously. The RT-LAMP was performed in a one-step reaction in a 25 μL mixture containing 5 μL RNA template, 18 μL reaction solution of Loopamp RNA Amplification Kit (RT-LAMP) (Eiken Chemical, Tokyo, Japan) [12.5 μL 2 × Reaction Mix, 1 μL Enzyme Mix, 4.5 μL distilled water] and 0.5 μL of each of the primers (F3 and B3, 10 μM; FIP and BIP, 80 μM). The template RNA was replaced by distilled water as a negative control. The reaction mixture was incubated at 63 °C for 1.5 h and heated at 80 °C for 5 min to terminate the reaction.

A Loopamp® real-time LA-320C turbidimeter (Eiken Chemical, Tokyo, Japan) was used to monitor the accumulation of magnesium pyrophosphate spectrophotometrically at 650 nm. The amplification of the targeted RNA was indicated by the turbidity real-timely, and the turbidity was shown after the automatically correction of the turbidity of amplification. Each graphic shown by the turbidimeter only includes the results of eight reactions. In the mean while, the result of the reaction could be observed by naked eyes under the natural light. When a white precipitate of magnesium pyrophosphate appeared, the reaction was considered as positive.

In order to make the results easier observed under the natural light or UV light, the Fluorescent Detection Reagent (FDR) (Eiken Chemical, Tokyo, Japan) (1 μL) was added to the reaction tube before amplification started. FDR (calcein) binds with manganese ions and remains quenched. When the LAMP amplifying reaction occurs, a by-product, pyrophosphate, deprives calcein of the manganese ion, which results in the emission of fluorescence. When the free calcein binds to the magnesium ions in the reaction mixture, the fluorescence emission becomes stronger. After amplification, the positive reaction will be indicated by a color change from orange to light green under the natural light or by a green fluorescent change from light to brighter under UV light.

2.4. Sensitivity of genotype-specific RT-LAMP assays for hMPV

The recombinant plasmids with inserted M gene fragments of hMPV genotypes A (pUCm-T-A) or B (pUCm-T-B) amplified from isolated strains A (GenBank accession no. DQ843659) and B (GenBank accession no. DQ843658) were generated previously (Zhu et al., 2005). The recombinant plasmids pUCm-T-A and pUCm-T-B were linearized by vector-specific restriction enzyme *Sal I* (Promega, Madison, USA) and then transcribed in vitro to obtain RNA using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, USA) following the manufacturer's instructions. The RNA was purified and quantified by a Qubit® nucleic acid protein quantitative instrument (Invitrogen, Oregon, USA). The RNA copy numbers of hMPV A and B were 4.33×10^{10} copies/μL and 4.53×10^{10} copies/μL, respectively. The 10-fold serial dilutions of RNA ranging from 10^{-6} to 10^{-13} were applied to determine the minimum limit of hMPV genotype-specific RT-LAMP assay. In addition, the RNA of hMPV A and B was mixed by the same concentration (the concentrations of A and B were both 1×10^9 copies/μL), then made a 10-fold serial dilution, and tested by the genotype-specific RT-LAMP assay, respectively.

All the negative controls presented the turbidities below 0.05 within 60 min in the preliminary experiments. The threshold value of amplification was recommended by the manufacturer's instructions at 0.1 or 0.2 of the turbidity of judgment. According to the results of genotype-specific RT-LAMP using serial diluted transcribed RNA from recombinant plasmids (pUCm-T-A