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Short communication

Visual detection of pandemic influenza A H1N1 Virus 2009 by reverse-transcription loop-mediated isothermal amplification with hydroxynaphthol blue dye☆

Xue-jun Ma[1], Yue-long Shu[1], Kai Nie[1], Meng Qin[1], Da-yan Wang, Rong-bao Gao, Miao Wang, Le-ying Wen, Feng Han, Shu-mei Zhou, Xiang Zhao, Yan-hui Cheng, De-xin Li, Xiao-ping Dong*

National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Ying-Xin Road 100, Xuan Wu District, Beijing, 100052, China

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ABSTRACT

A sensitive reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for rapid visual detection of pandemic influenza A H1N1 virus infection. The reaction was performed in one step in a single tube at 65 °C for 60 min with the addition of hydroxynaphthol blue (HNB) dye prior to amplification. The detection limit of the RT-LAMP assay was approximately 60 copies, and no cross-detection was observed. The assay was evaluated further with 50 clinical specimens diagnosed clinically with seasonal influenza or pandemic influenza A H1N1 virus infection. RT-LAMP with HNB dye was demonstrated to be a sensitive and easy assay for rapid detection of pandemic influenza A H1N1 virus.

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The currently circulating novel influenza A H1N1 virus (referred to as swine influenza previously) is a new influenza virus of swine origin that was first detected in Mexico and the United States in April 2009. The virus infects human beings and spreads from person to person much in the same manner as seasonal influenza viruses. The outbreak has expanded rapidly and an increasing number of cases are being reported internationally. As of March 7, 2010 more than 213 countries have reported laboratory-confirmed cases of pandemic influenza A H1N1 infection, including at least 16,713 deaths.

To control the pandemic influenza A H1N1 infection, early detection with a rapid, cost-effective and efficient assay is needed, particularly in developing countries such as China with large populations and relatively insufficient and uneven health resources. Currently, the US Center for Disease Control and Prevention (CDC) has developed a qRT-PCR diagnostic kit for the detection of pandemic H1N1 virus which is distributed internationally. However, this method might not be suitable in primary clinical settings in developing countries or for field use, because of the sophisticated instrumentation required, elaborate and complicated assay procedures and expensive reagents. There is therefore a growing demand for simple and economical molecular tests.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method developed by Notomi et al. (2000), and has emerged as a powerful gene amplification tool due to its simplicity, speed, specificity and cost-effectiveness. This technique is being used increasingly for rapid detection and typing of emerging viruses (Hong et al., 2004; Dukes et al., 2006; Toriniwa and Komiya, 2006; Yoneyama et al., 2007; Parida et al., 2007).

In the present study, a simple RT-LAMP and visual detection assay is described, in which the reaction is carried out in a single tube by mixing primers, reverse-transcriptase and DNA polymerase together with the tested samples at 65 °C for 60 min. The high sensitivity and specificity of the RT-LAMP reaction are due to continuous amplification under isothermal conditions. This assay employs six primers that recognize eight distinct regions of the HA gene from pandemic influenza A H1N1 virus. One of the most attractive features of this RT-LAMP assay is that the results can be observed and determined by hydroxynaphthol blue (HNB) dye-mediated visualization using the naked eye and without opening the tubes after amplification.

Field isolates of human seasonal influenza A viruses including H1N1, H3N2, influenza B virus and 16 swine influenza virus mixtures (H1N1) from the China National Influenza Cen-

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* Corresponding author at: State Key Laboratory for Infectious Disease Control and Prevention, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Ying-Xin Road 100, Xuan Wu District, Beijing, 100052, China. Tel.: +86 10 83534616; fax: +86 10 83534616.
E-mail addresses: dongxp238@sina.com, maxj2004@yahoo.com.cn (X.-p. Dong).
[1] The first four authors contributed equally to this study.

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Table 1
RT-LAMP primers designed for the detection of the HA gene sequence of pandemic influenza A H1N1.

Primer name	Genome position	Sequence (5′-3′)
F3	757–777	CCGGGAGACAAAATAACATTC
B3	919-937	GTATATTCTGAAATGGGAGGC
FIP (F1c + F2)	(818–841) + (778–798)	CAGATCCAGCATTTCTTTCCATTGGAAGCAACTGGAAATCTAGTG
BIP (B1 + B2c)	(846–870) + (897–916)	TATCATTTCAGATACACCAGTCCACTGGTGTTTATAGCACCCTTG
Loop-1	799–817	CGAATGCATATCTCGGTAC
Loop-2	878–896	ATACAACTTGTCAAACACC

ter were used as control viruses to evaluate the specificity of the RT-LAMP assay. All isolates had been verified previously by RT-PCR and sequencing. A/California/07/2009 (H1N1; circulating virus of this outbreak) from the US CDC was used as the reference virus. Throat swab specimens collected from 24 cases confirmed seasonal influenza viruses infections and 26 cases diagnosed definitively with pandemic influenza A H1N1 infection in China were included in this study, for which the presence of pandemic influenza A H1N1 virus-associated sequences had been confirmed previously by qRT-PCR (US CDC test kit), RT-PCR (China National Influenza Center test kit) and later by virus isolation.

The primers were designed based on sequence information obtained from the Influenza Sequence Database (http://www.flu.lanl.gov). All available sequences of human H1, H2, H3, B influenza virus, swine H1N1 and A/California/07/2009 (H1N1) viruses from the database were compared (data not shown). The most conserved segment within the HA gene (nt 757–937) of A/California/07/2009 (H1N1) was selected as the target. All primers were designed by a software program for LAMP primer design (Eiken Chemical Co., Ltd., Tokyo, Japan) and HPLC purified as shown in Table 1.

To test the specificity of the established RT-LAMP assay, total RNA was extracted from 140 μl of various stock control viruses and the reference virus (HA titers:1:32–1:1024) with the commercial QIAampViral RNA Mini Kit (Qiagen, Hilden, Germany). The extracts were centrifuged at 6000 × *g* for 1 min and pellets suspended in 50 μl distilled water. One microliter of the resuspended pellet was brought up to a total volume of 25 μl in RT-LAMP reaction mixture containing 1 μl Bst DNA polymerase (8 U/μl, New England Biolabs, Ipswitch, MA, USA), 1 μl avian myeloblastosis virus reverse-transcriptase (10 U/μl, Invitrogen, Carlsbad, CA), 2.5 μl dNTPs (10 mM), 8 μl Betaine (250 mM), 1 μl $MgSO_4$ (150 mM), 1 μl HNB (3 mM, Lemongreen, Shanghai, China) and 1 μl of each primer (F3 and B3: 5 pmol/μl; BIP and FIP:40 pmol/μl; Loop-1 and Loop-2: 20 pmol/μl). The RT-LAMP reaction was incubated in a conventional water bath at 65 °C for 60 min, followed by heating at 85 °C for 2 min to terminate the reaction. A positive amplification was indicated by a color change from violet to sky blue, as shown in Fig. 1A, and verified by agarose gel electrophoresis (data not shown). A

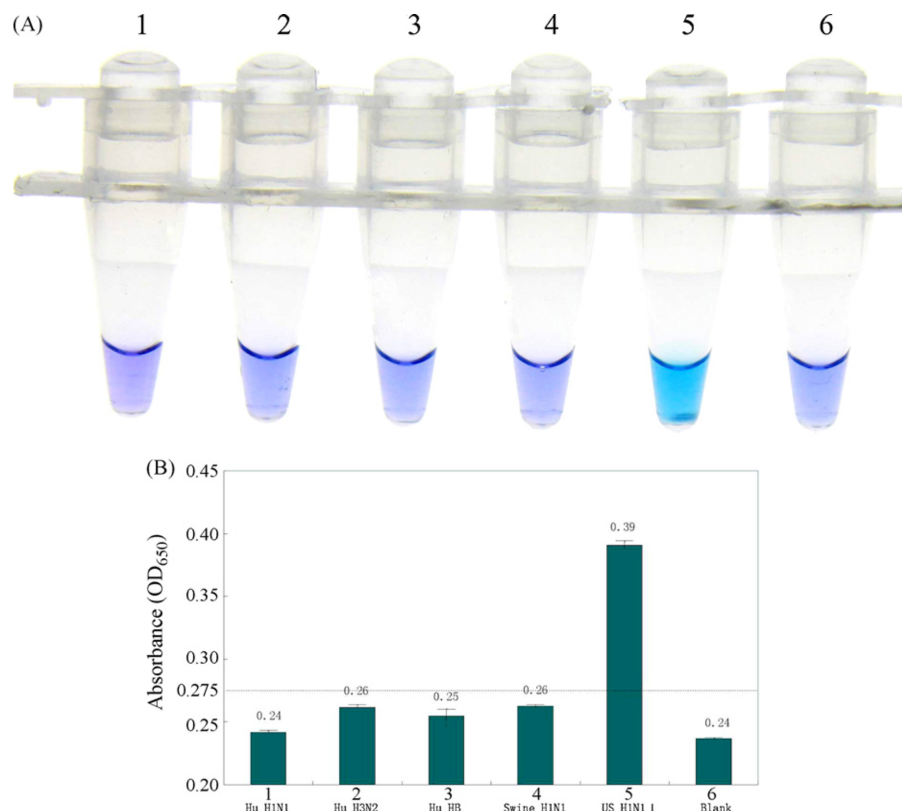

Fig. 1. Specificity analyses of colorimetric RT-LAMP for the pandemic influenza A H1N1 HA gene. (A) Colorimetric detection of amplified viral RNA. Tube (1) human seasonal influenza A H1N1 virus; (2) human seasonal influenza A H3N2 virus; (3) human influenza B virus; (4) mixture of 16 swine H1N1 isolates; (5) human pandemic influenza A H1N1 virus kindly supplied by the US CDC; (6) blank control of PBS. (B) The OD values measured at 650 nm. Column (1) human seasonal influenza A H1N1 virus; (2) human seasonal influenza A H3N2 virus; (3) human influenza B virus; (4) mixture of 16 swine H1N1 isolates; (5) human pandemic influenza A H1N1 virus kindly supplied by the US CDC; (6) blank control. The average result for each concentration of RNA was evaluated from three independent tests and represented as the mean ± S.D. The dashed horizontal line indicates the OD_{650} cut-off value (0.275) that was calculated by adding two standard deviations to the mean of the negative reaction.

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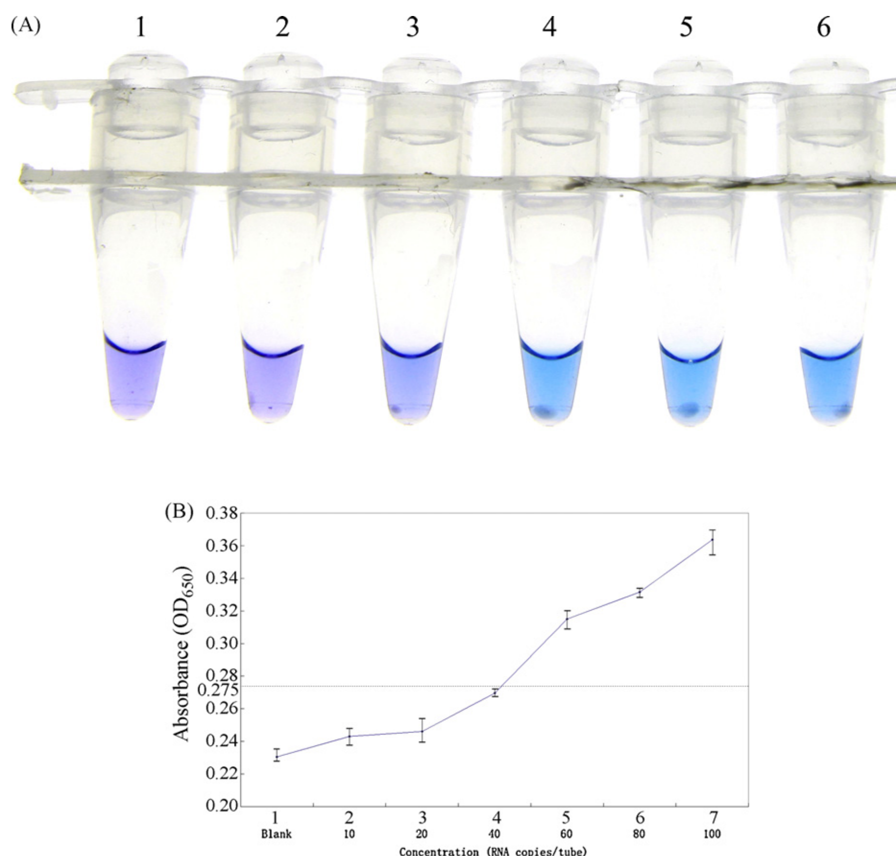

Fig. 2. Sensitivity analyses of colorimetric RT-LAMP for the pandemic influenza A H1N1 HA gene. (A) Colorimetric detection of amplified viral RNA. RT-LAMP was carried out using different concentrations of RNA transcripts per reaction transcribed *in vitro* from the recombinant plasmid pMD20 containing the full HA gene of human pandemic influenza A H1N1. Tube (1) 10 copies of RNA; (2) 20 copies of RNA; (3) 40 copies of RNA; (4) 60 copies of RNA; (5) 80 copies of RNA; (6) 100 copies of RNA. (B) The OD values measured at 650 nm. Sample (1) blank control; (2) 10 copies of RNA; (3) 20 copies of RNA; (4) 40 copies of RNA; (5) 60 copies of RNA; (6) 80 copies of RNA; (7) 100 copies of RNA. The average result for each concentration of RNA was evaluated from three independent tests and represented as the mean ± S.D. The dashed horizontal line indicates the OD_{650} cut-off value (0.275) that was calculated previously as in Fig. 1B.

positive color (sky blue) was only observed in the preparation of the reference virus, whereas none of the control viruses showed a color change. The corresponding absorbance values of 20 μl of reaction solution at 650 nm were measured further to distinguish the positive (OD_{650} absorbance of 0.39) from negative (OD_{650} absorbance of ≤0.26) reactions. An OD_{650} absorbance cut-off set at 0.275 was calculated based on the mean of the negative reactions plus two standard deviations (Fig. 1B). Similar to previous publications using LAMP detection (Hong et al., 2004; Dukes et al., 2006; Toriniwa and Komiya, 2006; Yoneyama et al., 2007; Parida et al., 2007), no internal control was included in this study.

To determine the detection limit of the LAMP assay, quantitative RNA transcripts were synthesized *in vitro* from the recombinant plasmid pMD20 containing the full HA gene of the reference virus with a commercial Riboprobe combination system-SP6/T7 kit (Promega, Madison, WI). A panel of RNAs with concentrations ranging from 10 to 100 copies per assay was prepared. As shown in Fig. 2A, the detection limit of the RT-LAMP assay was approximately 60 copies per reaction, which is comparable to that of the conventional RT-PCR method supplied by the China National Influenza Center using the same RNA panel as the template (data not shown). The reaction at each template concentration was repeated three times and similar results were obtained. The corresponding absorbance values of each reaction solution at 650 nm indicated that the detection of 40 copies of RNA was just below the predetermined cut-off value (Fig. 2B).

In parallel, the RT-LAMP products were detected turbidometrically using LA-320C (Eiken Chemical Co., Ltd., Tokyo, Japan), and the sensitivity and specificity of the RT-LAMP assay was also confirmed (data not shown). Finally, the assay was evaluated with 50 throat swab specimens (China National Influenza Center) including 26 cases diagnosed definitively with pandemic influenza A H1N1 virus infection in China, 10 cases with seasonal influenza H1N1, 10 cases with seasonal influenza H3N2 and four cases with seasonal Influenza B virus (HB). Clear positive amplifications were detected from all 26 samples of pandemic influenza A H1N1 virus infection, but not from any of the 24 samples of seasonal influenza H1N1, H3N2 or influenza B (data not shown).

Thus, RT-LAMP with HNB dye was shown to be a sensitive and easy assay for detection of pandemic influenza A H1N1 virus. The HNB dye-based assay has a remarkable advantage compared with other color-based assays (Goto et al., 2009; Parida et al., 2005; Hill et al., 2008; Curtis et al., 2008) in that HNB is mixed prior to amplification. A need to open the assayed samples to add the dye is thereby omitted, thus reducing the risk of cross-contamination. Additional studies, including improvements in sensitivity (Li et al., 2006, 2008), validation of visual testing with a larger number of clinical samples and proficiency testing, are necessary before this method can be applied widely for routine laboratory testing. The simplicity, ease of use and cost-effectiveness of this method makes it an attractive assay for the rapid screening of pandemic influenza A H1N1 virus.

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