academicJournals

Vol. 7(30), pp. 3919-3925, 26 July, 2013
DOI: 10.5897/AJMR12.1508
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Optimisation of reverse transcription loop-mediated isothermal amplification assay for the rapid detection of pandemic (H1N1) 2009 virus

Xin Cai[1],·Zha Lei[2], Wen-liang Fu[1], Zhe-yi Zhu[1], Min-ji Zou[1], Jie Gao[1], Yuan-yuan Wang[1], Min Hong[1], Jia-xi Wang[1], Wu-ju Li[2*], Dong-gang Xu[1]**

[1]Laboratory of Molecular Genetics, Beijing Institute of Basic Medical Sciences, Beijing, P.R.China.
[2]Center of Computational Biology, Beijing Institute of Basic Medical Sciences, Beijing, P.R.China.

Accepted 16 July, 2013

Conventional reverse transcriptase polymerase chain reaction (RT-PCR) and optimized of a closed tube reverse-transcription loop-mediated isothermal amplification (RT-LAMP) were used for detection of pandemic (H1N1) 2009 virus and the optimized of a closed tube RT-LAMP methods were compared with the conventional RT-PCR with respect to specificity and sensitivity. In this study, optimized RT-LAMP detected 2 copies of target RNA by visual detection with modified dye. Reaction time, temperature and quantity of each reagent were optimised for the detection of the virus. The sensitivity of detection limit by optimised RT-LAMP was 100 times as that of conventional RT-PCR. Amplification of DNA can be identified by visualization with modified dye, which reduces the cross-contamination caused by opening tube. The sensitivity of visual detection was equivalent to that of electrophoresis analysis. Additionally, the method was specific as no cross-reaction was observed among samples from human blood, *Escherichia coli* and other related viruses including human seasonal influenza A, subtypes H1N1, H1N2 and H3N2 viruses. These results demonstrate that the optimized RT-LAMP assay for pandemic (H1N1) 2009 virus RNA was a valuable tool with simplicity, rapidity and specificity, as well as its superiority for the screening and surveillance of influenza in developing countries.

Key words: Pandemic (H1N1) 2009 virus, loop-mediated isothermal amplification (LAMP), reverse transcriptase polymerase chain reaction (RT-PCR), HA gene.

INTRODUCTION

A novel subtype of influenza A virus called pandemic (H1N1) 2009 virus was first identified in Mexico and reported to World Health Organisation (WHO) in April 2009 by the Centers for Disease Control and Prevention in Atlanta (CDC, 2009; Fraser et al., 2009; Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009). The virus is strikingly different from previous H1N1 strains as well as the normal seasonal flu virus in that it is a triple-reassortant of swine influenza A (H1N1) virus containing classic swine RNA-segments from the North America lineage hemagglutinin (HA), neuraminidase (NP), non-structural proteins (NS) and avian influenza RNA

*Corresponding authors. E-mail: xudg@bmi.ac.cn; liwj@bmi.ac.cn; Tel.:(86) 10-66931380; Fax: (86) 10-66931380.

Abbreviations: HA, Hemagglutinin; **NP,** neuraminidase; **NS,** non-structural proteins; PB, polymerase basic protein; **PA,** polymerase acidic protein; **RT-PCR,** reverse transcription polymerase chain reaction; **LAMP,** loop-mediated isothermal amplification; **DTT,** dithiothreitol; **PPV,** positive predictive value; **NPV,** negative predictive value.

Table 1. RT-LAMP primers for the detection of pandemic (H1N1) 2009 virus.

Primer name	Sequence (5′ to 3′)	Genome position	Length (bp)
F3	TCAGACAATGGAACGTGTT	303-321	19
B3	CTTTCCCTTTATCATTAATGTAGGA	540-564	25
FIP	GTCTTGGGGAATATCTCAAACCTTT-TTTT-	385-409	50
	ATTATGAGGAGCTAAGAGAGC	340-360	
BIP	CAAAGGTGTAACGGCAGCAT-TTTT-	437-456	47
	GAATTTCCTTTTTTAACTAGCCA	498-520	

segments from the North America lineage polymerase basic protein 2 (PB2), polymerase acidic protein (PA) and polymerase basic protein 1 (PB1) from human seasonal H3N2 viruses. Moreover, the N and M segments were exchanged by N and M from the Eurasian influenza A (H1N1) swine lineage instead of classic North America swine lineage origin (Shinde et al., 2009).

Pandemic (H1N1) 2009 virus transmits easily between humans. More than 213 countries reported confirmed cases including over 16713 deaths within a year of the initial identification of the virus. Therefore, rapid detection and differentiation from seasonal influenza is critical for public health management and disease surveillance. It has been reported that conventional and reverse transcription polymerase chain reaction (RT-PCR) protocols for the detection of this virus (Poon et al., 2009). However, these methods 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. The loop-mediated isothermal amplification (LAMP) method was developed by Notomi et al. (2000). The most significant advantage of LAMP is the ability to amplify specific DNA sequences under isothermal conditions in only 15-60 min (Mori et al., 2006). Thus, it requires only a simple and inexpensive water bath or a heating block which is available in routine laboratories. This technology is cost-effective and widely available which is being used increasingly for rapid detection and typing of emerging viruses (Chen et al., 2009; Suebsing et al., 2012; Venkatesan et al., 2012).

The amplified products of LAMP can be analyzed by agarose gel electrophoresis, which is the most sensitive among other detection assays for LAMP products. However, the disadvantage of electrophoresis assay is that opening tube detection produces aerosol contamination by the very large amount of amplified DNA, which is not beneficial for subsequent experiments. Real-time Turbidimeter and UV detection devices were used to evaluate the reaction results to avoid cap-opening. However, the high price of these detection devices greatly limited its application, especially in the developing countries and the elementary medical organizations. Therefore, in the present study, the RT-LAMP with modified dye and optimized reaction condition for detection of pandemic (H1N1) 2009 virus was developed and evaluated.

MATERIALS AND METHODS

Viral strains and clinical reference samples

Sixteen inactivated seasonal influenza virus strains were provided by Hebei Center for Disease and Prevention (CDC) including five H1N1 subtype strains, five H1N2 subtype strains and six H3N2 subtype strains. Twenty positive clinical specimens from patients infected with pandemic (H1N1) 2009 virus were identified and obtained from Beijing Institute of CDC.

RNA extraction

Total RNA was extracted from inactivated seasonal influenza viruses using a QIAamp Viral RNA mini kit (QIAGEN, US), according to the manufacturer's protocol. After extraction, RNA was eluted in 60 µl of elution buffer and stored at -80°C until further use.

RT-PCR analysis

The first cDNA strand was synthesized as follows: 2 µg of total RNA and 2 µl oligo (dT) were added to a 0.2 ml eppendorf centrifuge tube, then RNase-free water was supplemented to a final volume of 13 µl. After incubation at 70°C for 5 min, 5 µl of 5×buffer, 5 µl of 10 mM dNTPs, 1 µl of RNasin, and 1 µl AMV reverse transcriptase were added, mixed, and incubated at 42°C for 1 h. Subsequently, PCR was performed using the obtained cDNA as the template and the oligonucleotides F3 and B3 (Table 1) as the specific primers. The reaction was carried out with the following procedures: initial denaturation at 95°C for 5 min followed by 30 consecutive cycles of denaturation at 95°Cfor 30 s, annealing for 30 s at 55°C, extension at 72°C for 2 min, and then final extension at 72°C for 7 min. A total of 10 µl of RT-PCR product was then analyzed by 2.5% agarose gel electrophoresis in tris-buffer, and target bands were visualized by staining with ethidium bromide. The amplified products were gel-purified by the gel extraction kit (Omega Biotechnology Co., USA) and confirmed by DNA sequencing.

RT-LAMP primer design

The sequence of pandemic (H1N1) 2009 virus (A/California/04/2009 (H1N1)) (GenBank accession nos.FJ966082) with all available sequences of human H1, H2, H3, B influenza virus, swine H1N1 from the database were compared using a program for multiple sequence alignment - ClustalX. The conserved segment within the HA gene of pandemic (H1N1) 2009 virus was selected as the target region for primer design. A set of four primers, composed of two outer and two inner primers, was designed

using Primer Explorer version 4.0 (Applied Biosystems) and is shown in Table 1. The specificity of designed primer was confirmed by restriction analysis and sequencing amplified PCR product.

In vitro transcription and quantification

To obtain a quantitative RNA standard with which to check the detection limit of the RT-LAMP, a target region (262 bp) of the HA gene for the RT-LAMP assay was initially synthesized (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd, China) according to the target sequence between outer primer (F3) and backward outer primer (B3). Using F3 and B3 primers, the synthesized target DNA was amplified by PCR and the PCR product was purified and inserted into the *Eco*R V site of pMD-20T Vector.

The recombinant plasmid was confirmed by restriction analysis and sequencing. Before transcription in *vitro*, the confirmed recombinant plasmid was linearized by *Bam*HI digestion as the sample was used to perform the transcription reaction. The reaction mixture consists of: 100 mM dithiothreitol (DTT), 0.1% BSA, 10×SP6 RNA polymerase buffer, 10 mM NTP mixture, 40U RNAase inhibitor, 50U SP6 RNA polymerase, and RNase-free water. Reaction was carried out at 37°C for 60 min.

Optimization of dye and RT-LAMP reaction condition

The RT-LAMP reaction mixture contained inner primers, outer primers, dNTPs, AMV reverse transcriptase (Takala Ltd, China) and Bst polymerase (New England Biolabs), together with the supplied buffer, distilled water, betaine (Sigma, German), MgSO4, modified dye including calcein (Beijing Chemical Reagent), manganism chloride ($MnCl_2$) and hydroxy naphthol blue (Beijing Chemical Reagent Factory), and synthesized target fragment RNA or distilled water (blank control) in a final volume of 25 μl.

Optimum quantity of each ingredient to be added was standardised. The following changes were attempted in the reaction to optimise the color change obtained. Optimization of (i) different ratio of calcein, manganism chloride ($MnCl_2$) and Hydroxy naphthol blue concentration viz., 1:50:10, 1:50:50, 1:50:100,1:50:1000 were tried, (ii) different concentration of Mg^{2+} viz., 2, 4, 6, 8, 10 mM were tried, (iii) concentration of betaine — five different concentrations viz., 0.2, 0.4, 0.6, 0.8, 1.0 M were tried, (iv) different concentration of dNTP viz., 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mM were tried, (v) different ratios of outer and inner primers viz., 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10 were tried, (vi) concentration of AMV Reverse transcriptase - three concentration viz., 2.5 U, 5 U, 10 U were attempted. (vii) concentration of Bst DNA polymerase - two concentration viz., 6 and 8 U were attempted. (viii) different temperature of the reaction mix, viz., 58, 60, 63, 65 and 68°C were compared, (ix) different reaction time viz., 15, 30, 45, and 60 min were carried out.

Comparison of different detection methods

Agarose gel analysis

Following amplification, 2 μl of the RT-LAMP assay product was analyzed by 1.2% agarose gel electrophoresis as the positive products should show a typical ladder.

White precipitate analysis

The reaction products can be inspected by naked eye because of the white precipitate produced by positive amplification.

Visualization detection with nucleic acid dye

1 μl of SYBR Green I dye (Invitrogen, USA) was added to the tube after amplification for the evaluation on the results by directly observing the color change as reported (Iwamoto et al., 2003).

Visualization detection with modified dye

1 μl of modified dye was added to the tube prior to amplification. In the case of positive amplification, the original green color of the dye would be changed into purple that can be judged under natural light.

Specificity of RT-LAMP with modified dye Assay

To assess the specificity of RT-LAMP, following RNA samples isolated from seasonal influenza virus including five H1N1 subtype strains, five H1N2 subtype strains and six H3N2 subtype strains, and RNAs extracted from human blood, as well as *Escherichia coli* were detected as the parallel control. RT-LAMP product with modified dye was judged by color change. 2 μl of RT-LAMP product without adding modified dye was then analyzed by 1.2% agarose gel electrophoresis.

Sensitivity of RT-LAMP with modified dye

The sensitivity of RT-LAMP with modified dye assay for the pandemic (H1N1) 2009 virus was determined using the obtained virus RNAs through transcription *in vitro* as positive sample. The RNA fragment was quantitated using UV spectrophotometry at 260 nm (Sigma, German) and its copy was calculated. A 10 × serial dilutions, and 2-2×10^7 copies/tube were used to test the sensitivity of RT-LAMP reaction and compared with RT-PCR by using the same templates at the same concentrations. In addition, 20 samples of RNA extracted from pandemic (H1N1) 2009 virus-infected patients were detected using optimized RT-LAMP visualization assay. The results of RT-LAMP with the modified dye were compared with RT-PCR and determined the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

RESULTS

Optimization of RT-LAMP reaction conditions for pandemic (H1N1) 2009 virus detection

The specificity of amplification of the template was optimum at 63°C by visual observation by the color change with modified dye. An apparent color change was observed when the modified dye with the ratio of 1:50:1000 (Figure 1). RT-LAMP worked in all inner-outer primer ratios tried, that is, 1:2 to 1:10 (figure not shown). But the optimum sensitivity and minimum non-specificity was observed with 1:6. Amplified products were observed as early as 15 min, with a maximal sensitivity reached at 60 min by visualization detection with optimized dye (Table 2). All subsequent experiments included a 60 min reaction time to ensure optimal sensitivity. The addition of

Figure 1. Visual detection of pandemic (H1N1) 2009 virus with modified dye. Lane 1: no template negative control showing purple colour. Lane 2: Reaction positive with template of pandemic (H1N1) 2009 virus RNA showing green colouration.

10 mM $MgSO_4$ to the reaction mixture to obtain a total concentration of 12 mM can achieve an apparent color change. RT-LAMP worked well both with 0.6 and 0.8 M betaine. But the latter concentration, that is, 0.8 M, appeared to be slightly better in terms of a visual color change (data not shown). 2.5 U AMV Reverse transcriptase was found to be sufficient for amplification of DNA by RT-LAMP. There was no significant difference in the visible color change with 6 and 8 U of Bst polymerase. Hence, 6 U was selected for the reaction mix.

Comparison in sensitivity between optimized RT-LAMP and RT-PCR

The sensitivity of optimized RT-LAMP assay for pandemic (H1N1) 2009 virus RNA was determined using 10-fold dilutions of target RNA. Optimized RT-LAMP detected 2 copies of target RNA by visual detection with modified dye, while the RT-PCR detected 200 copies of target RNA (Figure 2). The sensitivity of detection limit by RT-LAMP was 100 times as that of conventional RT-PCR. In addition, a total of twenty identified positive samples and sixteen negative samples were subjected to optimized RT-LAMP and RT-PCR assay. The results of RT-LAMP with modified dye showed that 18 cases out of 20 positive samples displayed positive reaction. In contrast, RT-PCR showed that 15 cases out of 20 positive samples and three cases out of 16 negative samples displayed positive reaction. The results demonstrate that the positive predictive value and negative predictive value of optimized RT-LAMP were higher than that of RT-PCR (Table 2).

Specificity of optimized RT-LAMP detection

The specificity by visual observation with optimized dye demonstrated that only positive target RNA after reaction turned green, while negative samples including human seasonal influenza virus A/H1N1, A/H1N2 and A/H3N2 RNA, human blood total RNA and *E. coli* total after reaction turned purple (Figure 3A). These two colors are separated farther in the chromatic circle. The results of corresponding group without adding dye by agarose gel electrophoresis analysis showed that only positive target RNA can produce the amplified DNA band, and no amplified DNA band was observed with the template of negative samples, which is consistent to that obtained by visual observation (Figure 3B).

A total of 20 identified positive samples and sixteen negative samples were subjected to optimized RT-LAMP and RT-PCR assay. The results demonstrated that RT-LAMP with modified dye assay for pandemic (H1N1) 2009 virus was convincing, which is consistent to that obtained by agarose gel electrophoresis (Figure 4A). The tube containing amplicons turned green, whereas the solution turned into purple indicating no amplicons (Figure 4B). Therefore, the results of the RT-LAMP with modified dye can simply be judged by the naked eye. The positive results of optimized RT-LAMP and RT-PCR assay from twenty identified positive samples and sixteen negative samples were compared. The results demonstrated that the specificity of optimized RT-LAMP was higher than that of RT-PCR (Table 3).

Comparison of different detection methods

Electrophoresis detection showed that 2 copies of the target RNA after amplification had a typical ladder (Figure 5A). The result demonstrated that detection limit per reaction for electrophoresis analysis was no less than 2 copies. Direct detection with the naked eyes showed that 20 copies of target RNA had a slight white turbidity, whereas 2 copies of target RNA without forming white turbidity (Figure 5B). Therefore, the sensitivity of electrophoresis detection is 10 fold higher than that of direct visual observation. Visual identification of amplified product was facilitated by adding the fluorescent nucleic acid stain SYNB Green I . The positive reaction with 20 copies of target RNA turned green after the addition of SYBR Green I, whereas the positive reaction with two copies of target RNA retained the original orange color (Figure 5C). The result demonstrated that the sensitivity

Table 2. The comparison of optimized RT-LAMP with RT-PCR method.

Parameter	Sensitivity	Specificity	Positive predictive value (%)	Negative predictive value (%)
Optimized RT-LAMP	18(20)*	0(16)†	90**	100††
RT-PCR	15(20)*	3(16)†	75**	81††

*Positive case out of 20 positive samples; †positive case out of 16 negative samples; **positive predictive value was calculated by comparing positive reaction cases in 20 positive samples; †† negative predictive value was calculated by comparing negative reaction cases in 16 negative samples.

Table 3. Determination of optimal reaction time for pandemic (H1N1) 2009 virus RT-LAMP.

Nucleic acid	Copies/tube	The results of RT-LAMP reaction for the different time			
		15 min	30 min	45 min	60 min
RNA	20,000	+	+	+	+
	2000	_	+	+	+
	200	_	+	+	+
	20	_	_	+	+
	2	_	_	_	+

The samples were evaluated in triplicate.

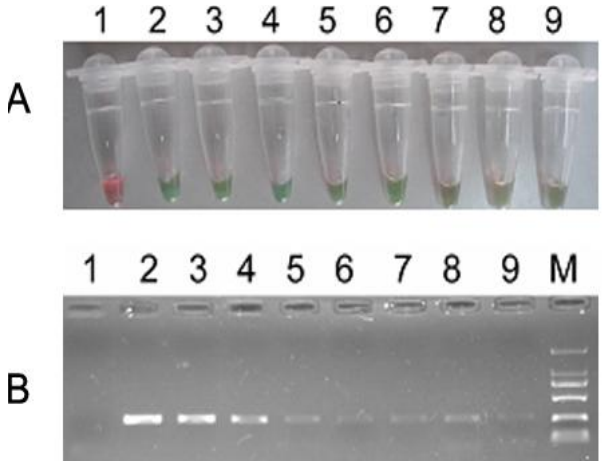

Figure 2. Sensitivity comparison between optimized RT-LAMP and RT-PCR for the detection of pandemic (H1N1) 2009 virus. (A) Sensitivity of optimized RT-LAMP assay by visual inspection. Lane 1: ddH_2O was used as negative control in optimized RT-LAMP assay. Lanes 2-9: Optimized RT-LAMP carried out in the presence of 2-2×10^7 copies/tube target RNAs respectively; (B) Sensitivity of RT-PCR assay. Lane 1-9 is corresponding to template of Fig.2A analysis (A).

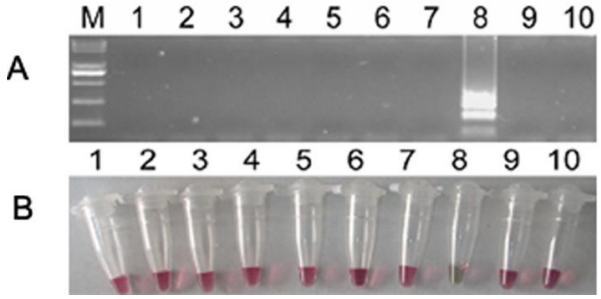

Figure 3. Specificity of pandemic (H1N1) 2009 virus RT-LAMP assay. (A) Electrophoresis analysis of cross-reaction of pandemic (H1N1) 2009 virus RT-LAMP assay. Lane M, DNA Marker DL-2000. Lane 1-6: RT-LAMP carried out in the presence of two H1N1 subtypes, two H1N2 subtypes, two H3N2 subtypes, and human seasonal influenza virus RNAs, respectively; Lane 7: RT-LAMP carried out in the presence of human blood total RNA; Lane 8: RT-LAMP carried out in the presence of target RNA of pandemic (H1N1) 2009 virus; Lane 9-10: ddH_2O and E.coil total RNA were respectively used as negative control in RT-LAMP. (B) Specificity of optimized RT-LAMP assay for pandemic (H1N1) 2009 virus by visual inspection. Lane 1-10 is corresponding to electrophoresis analysis for human seasonal influenza virus RNAs.

of addition with SYBR GREEN I dye was inferior to that of electrophoresis detection. Compared with reported visual detection methods, adding modified dye in reaction mixture prior to reaction showed that 2 copies of target RNA could be observed color change into green (Figure 5D). The sensitivity of modified dye observation is comparable to that of electrophoresis detection.

DISCUSSION

Recently, application of RT-LAMP assay for the detection of the pandemic (H1N1) 2009 virus has been reported (Ma et al., 2010). Although the detection limit reached 10 copies by electrophoresis assay, the disadvantage of the method is that the high sensitivity and very large amount

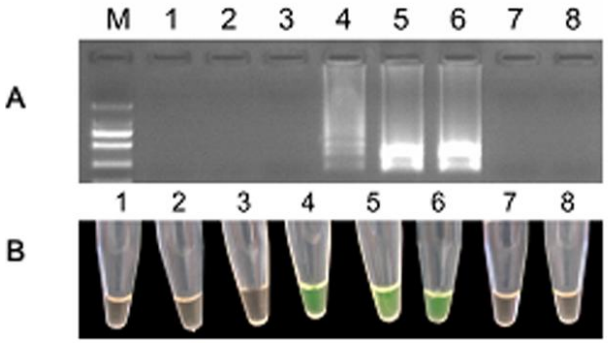

Figure 4. Results are representative of visual inspection and electrophoretic analysis of clinical specimens from patients infected with pandemic (H1N1) 2009 virus by RT-LAMP. (A) Detection of clinical specimens by agarose gel electrophoresis. Lane 1-3: RT-LAMP detection for H1N1, H1N2, H3N2 human seasonal influenza virus RNA, respectively; Lane 4-6: RT-LAMP carried out in the presence of clinical pandemic (H1N1) 2009 virus specimen; Lane 7-8: ddH_2O and total *E.coi*l RNA were respectively used as negative control in carried out RT-LAMP. (B) Visual inspection by modified dye. Lane 1-8 is corresponding to electrophoresis analysis for human seasonal influenza virus RNAs.

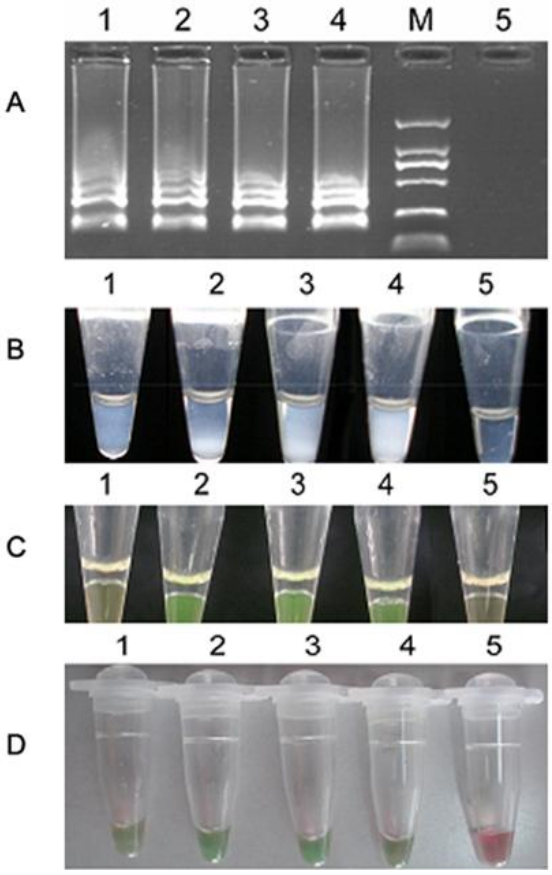

Figure 5. Comparison of different detection methods. (A) Sensitivity of RT-LAMP assay by the electrophoresis analysis. Lanes 1-4: RT-LAMP carried out in the presence of $2\text{-}2\times10^3$ copies/tube target RNAs, respectively; Lane M: DNA Marker DL-2000; Lane 5: ddH_2O was used as negative control in RT-LAMP assay. (B) Visual inspection by observation of magnesium pyrophosphate white precipitate. The solution containing amplicons produced white precipitate, whereas the solution of negative samples remained transparent. (C) Visual inspection by SYNB Green Ⅰ staining. 1 μl of SYBR Green I dye was added to the tube after amplification The negative samples remained the original orange color of SYBR Green I, whereas positive samples turned orange. (D) Visual inspection by modified dye. 1 μl of modified dye was added to the tube prior to amplification. The negative samples changed into purple, whereas positive samples turned green.

of aerosol contamination formed by opening cap assay, which makes it susceptible to false positive reactions because of cross-contamination. Though the addition of HNB dye prior to amplification mediated visual detection of the pandemic (H1N1) 2009 virus can reduce cross contamination (Kubo et al., 2010), the detection limit was 60 copies and inferior to electrophoresis assay because the difference between the negative and the positive color of samples was not obvious. In addition, these detection methods needs corresponding detection devices such as electrophoresis device and UV transilluminator, which did not meet the growing demand for simple and economical molecular tests in the developing countries and the elementary medical organizations.

Therefore, in the present study, we explore closed tube detection methods with low-cost and high sensitivity. First, the dye was improved so as to distinguish negative and positive samples more easily. On the basis of the calcein recipe reported in the literatures, hydroxy naphthol blue was added as adjuvant developer. The obvious color change can be obtained when the ratio of dye was 1:50:1000. After using the modified dye, the negative samples showed purple and that of the positive sample showed green. These two colors were separated farther in the chromatic circle, thus can be distinguished more easily by naked eye. Therefore, the improved closed tube dye system can be applied to other LAMP detection kits. Additionally, we estimated that the cost of the modified dye was less than $0.04 dollars per 1000 RT-LAMP reactions which will be helpful to advance the technology.

In addition, in order to increase the sensitivity of the closed tube system, the RT-LAMP reaction with modified dye for the detection of pandemic (H1N1) 2009 virus was optimized. The RT-LAMP reaction works well at 63 and 65°C. However, since reverse transcriptase is used in the RT-LAMP assay, and considering the convenience of use of the LAMP method for detection of the pandemic (H1N1) 2009 virus, all further reactions were carried out at 63°C. Although RT-LAMP worked in all inner-outer

primer ratios tried, obvious color contrast was observed in 1:6. This observation suggests that primer ratio influences the color change of reaction. Experiments proved that the concentration of $MgSO_4$, betaine, reverse transcriptase enzyme and Bst DNA polymerase play a role in the visible detection with modified dye. Through optimizing the reagents of the RT-LAMP, the maximum sensitivity of RT-LAMP assay with modified dye was observed with two copies per reaction within 60 min, which showed a sensitivity higher than that of reported RT-LAMP detecting pandemic (H1N1) 2009 virus (Kubo et al., 2010; Ma et al., 2010).

Furthermore, the present studies with modified dye showed that amplification begins to occur in 15 min. Although earlier studies had demonstrated the time for visible detection including observing white precipitate and color change at 60 min (Iwamoto et al., 2003; Pillai et al., 2006). The time to obtain a color change has been reduced with modified dye by the optimization of reagent concentrations on the reaction kinetics and production of color change in the reaction mixture.

In conclusion, application of the optimized dye not only enhanced sensitivity of the visual detection of pandemic (H1N1) 2009 virus but also eliminated the risk of cross-contamination with the open tube analysis. These characteristics render this method promising for the rapid screening of pandemic influenza A H1N1 virus in less well equipped laboratories in developing countries and in mobile laboratories during outbreaks in remote areas.

ACKNOWEDGEMENT

This work was supported by the Institute of Basic Medical Sciences.

REFERENCES

Centers for Disease Control and Prevention (2009). Outbreak of swine-origin influenza A (H1N1) virus infection—Mexico, March-April 2009. MMWR Morb. Mortal. Wkly. Rep. 58:467-470.

Chen HT, Zhang J, Ma LN, Ma YP, Ding YZ, Liu XT, Chen L, Ma LQ, Zhang YG, Liu YS (2009). Rapid pre-clinical detection of classical swine fever by reverse transcription loop-mediated isothermal amplification. Mol. Cell. Probes 23:71-74.

Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van K, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F, Ghani AC, Ferguson NM, Rambaut A, Pybus OG, Lopez-Gatell H, Pluche-Aranda CM, Chapela IB, Zavala EP, Guevara DM, Checchi F, Garcia E, Hugonnet S, Roth C (2009). Pandemic potential of a strain of influenza A (H1N1): early findings. Science 324:1557-1561.

Iwamoto T, Sonobe T, Hayashi K (2003). Loop-Mediated Isothermal Amplification for Direct Detection of Mycobacterium tuberculosis Complex, M. avium, and M. intracellulare in Sputum Samples. J. Clin. Microbiol. 41:2616-2622.

Kubo T, Agoh M, Mai le Q, Fukushima K, Nishimura H, Yamaguchi A, Hirano M, Yoshikawa A, Hasebe F, Kohno S, Morita K (2010). Development of a Reverse Transcription-Loop-Mediated Isothermal Amplification Assay for Detection of Pandemic (H1N1) 2009 Virus as a Novel Molecular Method for Diagnosis of Pandemic Influenza in Resource-Limited Settings. J. Clin. Microbiol. 48:728-735.

Ma XJ, Shu YL, Nie K, Qin M, Wang DY, Gao RB, Wang M, Wen LY, Han F, Zhou SM, Zhao X, Cheng YH, Li DX, Dong XP (2010). Visual detection of pandemic influenza A H1N1 Virus 2009 by reverse-transcription loop-mediated isothermal amplification with hydroxynaphthol blue dye. J. Virol. Methods 167:214-217.

Mori Y, Hirano T, Notomi T (2006). Sequence specific visual detection of LAMP reactions by addition of cationic polymers. BMC Biotechnol. 6:3.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000). Loop mediated isothermal amplification of DNA. Nucleic Acids Res. 28:E63.

Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM (2009). Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N. Engl. J. Med. 360: 2605-2615.

Pillai D, Bonami JR, Widada JS (2006). Rapid detection of Macrobrachium rosenbergii nodavirus (MrNV) and extra small virus (XSV), the pathogenic agent of white tail disease of Macrobrachium rosenbergii (De Man), by loop mediated isothermal amplification. J. Fish Dis. 29:275-283.

Poon LL, Chan KH, Smith GJ, Leung CS, Guan Y, Yuen KY, Peiris JS (2009). Molecular detection of a novel human influenza (H1N1) of pandemic potential by conventional and real-time quantitative RT-PCR assays. Clin. Chem. 55:1555-1558.

Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, Lindstrom S, Gubareva LV, Deyde V, Garten RJ, Harris M, Gerber S, Vagoski S, Smith F, Pascoe N, Martin K, DuYcy D, Ritger K, Conover C, Quinlisk P, Klimov A, Bresee JS, Finelli L (2009). Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009. N. Engl. J. Med. 360:2616-2625.

Suebsing R, Oh MJ, Kim JH (2012). Development of a reverse transcription loop-mediated isothermal amplification assay for detecting nervous necrosis virus in olive flounder Paralichthys olivaceus. J. Microbiol. Biotechnol. 22:1021-1028.

Venkatesan G, Bhanuprakash V, Balamurugan V, Singh RK, Pandey AB (2012). Development of loop-mediated isothermal amplification assay for specific and rapid detection of camelpox virus in clinical samples. J. Virol. Methods 183:34-39.