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Combination of multiplex reverse-transcription loop-mediated isothermal amplification with an immunochromatographic strip for subtyping influenza A virus



Jae Hwan Jung^a, Seung Jun Oh^a, Yong Tae Kim^a, So Yeon Kim^b, Won-Jung Kim^b, Jaean Jung^b, Tae Seok Seo^{a,*}

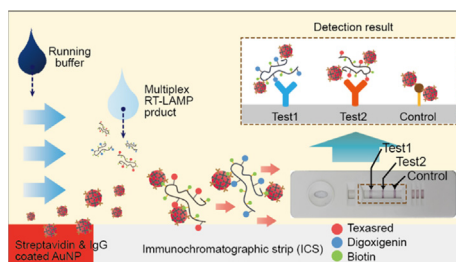
^a Department of Chemical and Biomolecular Engineering (BK21+ Program) and Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea

^b Medisensor GH, Inc., 155-1 Nongso-ri, Juchon-myeon, Gimhae-si, Gyeongsangnam-do 621-841, Republic of Korea

HIGHLIGHTS

- Multiplex RT-LAMP for HA and M genes was developed for subtyping influenza A virus.
- Multiplex RT-LAMP amplicons were simply analyzed by the colorimetric ICS detection.
- Multiplex RT-LAMP (40 min) and ICS detection (15 min) could be complete in 55 min.
- Detection sensitivity for the multiplex RT-LAMP and ICS was 10 copies of viral RNA.
- Our methodology provides simple, rapid genetic analysis platform for viral detection.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 12 August 2014

Received in revised form 30 September 2014

Accepted 13 October 2014

Available online 16 October 2014

Keywords:

Immunochromatographic strip
Influenza A virus
Multiplex reverse transcription loop-mediated isothermal amplification
Subtyping

ABSTRACT

Considering the fatal human victims and economic loss by the annual epidemic influenza virus, the development of a rapid and convenient genetic analysis methodology is demanding for timely on-site pathogen detection. In this study, we utilized reverse-transcription loop-mediated isothermal amplification (RT-LAMP) for multiplex target gene amplification, and the resultant amplicons were analyzed on the immunochromatographic strip (ICS) for subtyping influenza A virus. Through the optimized primer design, reaction temperature and time, and concentration of enzymes (*Bst* DNA polymerase and AMV reverse transcriptase) and dNTP, the HA (H1, H3, and H5 gene) and conserved M gene were amplified. The ICS contains two test lines in addition to a control line in order to detect the presence of the HA and M gene, thereby informing us of influenza virus A type as well as its subtype (H1N1, H3N2, and H5N1). The combination of the multiplex RT-LAMP with the ICS could be complete in 40 min and the pathotyping and subtyping of influenza A virus were performed even with 10 copies of viral RNA templates. Moreover, the subtyping of clinical samples, which were obtained from patients infected by influenza A virus was successfully confirmed using the multiplex RT-LAMP and ICS techniques, showing great feasibility of our methodology for real sample analysis with high speed, simplicity and sensitivity.

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* Corresponding author. Tel.: +82 42 350 3933; fax: +82 42 350 3910.

E-mail addresses: jjahwan@kaist.ac.kr (J.H. Jung), osj237@kaist.ac.kr (S.J. Oh), surpass7@kaist.ac.kr (Y.T. Kim), sykim@medi-sensor.com (S.Y. Kim), wjkim@medi-sensor.com (W.-J. Kim), jj@medi-sensor.com (J. Jung), seots@kaist.ac.kr (T.S. Seo).

1. Introduction

Pandemic influenza A H1N1 virus swept across the globe from 2009 to 2010, resulting in 18,000 of human victims over 214 nations according to the World Health Organization (WHO). Annual occurrence of influenza A virus causes significant casualties and economic loss [1,2]. Considering the detrimental effect on human being and livestock even with low quantity of virus, early detection of the influenza A virus and its subtype is paramount of importance to prevent social damages [3,4]. Among the diagnostic tools, the genetic analysis of epidemics caused by influenza A viruses, severe acute respiratory syndrome (SARS), and foot-and-mouth-disease virus (FMDV) is regarded as most sensitive, accurate, and multiplex [5,6]. In contrast to the conventional polymerase chain reaction (PCR) based target gene detection which requires a long PCR time due to the thermal cycling and slow ramping rate, and a bulky and expensive instrumentation, recent researches have focused on the development of the isothermal amplification methods for simple and user-friendly genetic analysis including loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), recombinase polymerase amplification (RPA), and helicase-dependent amplification (HDA). In particular, LAMP is rapid, sensitive, and specific for boosting up the target genes [7]. Generally, LAMP uses six primers spanning eight distinct sequence on the target gene and a DNA polymerase for strand displacement reaction at 60~65 °C in 1 h, leading to the ladder-patterned multiple amplicon products [8,9]. Besides targeting DNA templates, LAMP can be used for RNA amplification by simply adding a reverse transcriptase enzyme under the identical reaction conditions as LAMP, so-called reverse transcriptase loop-mediated isothermal amplification (RT-LAMP). Thus, a variety of pathogens can be identified through the RT-LAMP reaction using their viral RNA as a template with one-step (without a separate reverse transcription step), high speed, specificity, and sensitivity [10–12]. Ge et al. reported a reverse transcription loop-mediated isothermal amplification combined with a lateral flow device to detect influenza A H7N9 virus targeting hemagglutinin (HA) and neuraminidase (NA) gene [13]. The detection sensitivity was 10 copies of RNA, and 100% specificity was observed. Fang et al. demonstrated an RT-LAMP microdevice to detect multiplex influenza A viruses with an optical detector [14]. A PDMS-glass hybrid microdevice could simultaneously identify 8 samples of influenza A viruses with 8 copies of a limit of detection. Since the RT-LAMP employs only one reaction temperature, the temperature controller system can be simplified, enabling us to construct a miniaturized genetic analysis system for on-site RT-LAMP based pathogen detection [15,16]. Regarding the detection methods for LAMP amplicons, gel electrophoresis, real-time turbidity monitoring which is derived by phosphate precipitates, and calcein mediated colorimetric detection have been proposed [17,18]. However, these detection techniques require complicated, bulky and specialized instrumentations, which diminish the point-of-care testing capability of RT-LAMP. To fully take advantages of the RT-LAMP, the detection methodology should be simple, rapid and cost-effective [19]. In this sense, the colorimetric detection would be ideal due to its user-friendliness, simplicity, and ease to interpret the result. To this end, an immunochromatographic strip (ICS) has been widely utilized, and commercialized for pathogen diagnostic tools [11,13,20–22]. However, the existing ICSs are designed only to identify one influenza A virus type, not for subtyping influenza A virus whose information is important for medical prognosis and treatment. In addition, despite the usefulness of the multiplex gene amplification in a single reaction tube for accurate and reliable pathogen detection with low cost [23,24], multiplex RT-LAMP has been rarely investigated. The differentiation of the ladder-like RT-LAMP amplicons derived from multiple target genes is still challengeable [14,15].

In this study, we developed multiplex RT-LAMP targeting HA gene and conserved matrix (M) gene for pathotyping and subtyping influenza A virus among influenza A H1N1, A H3N2, and A H5N1 virus strains, and the multiplex RT-LAMP amplicons could be clearly analyzed on the multiple test lines of the ICS. The combination of the advanced multiplex RT-LAMP and ICS provides us a simple, rapid, cheap, sensitive, and user-friendly diagnostic tool for identification of influenza A virus.

2. Experimental

2.1. Materials

Viral lysates and purified viral RNAs of influenza A H1N1, A H3N2, and A H5N1 viruses were supplied from College of Medicine in Chungbuk National University. Viral RNAs were extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Germany). Target specific primers for HA gene and universal M gene were ordered from Neoprobe (Daejeon, Korea). To prepare the RT-LAMP reaction mixture, 10× ThermoPol Reaction buffer and 100 mM MgSO₄ were purchased from New England Biolabs (MA, USA). 5 M betaine solution was provided from Sigma–Aldrich (MO, USA), and 5 mM of biotin-18-dUTP and 100 mM of dATP, dCTP, dGTP, dTTP solution were purchased from Jena Bioscience (Germany). *Bst* DNA polymerase (large fragment) and AMV reverse transcriptase were obtained from New England Biolabs (MA, USA) and iNTRON Biotechnology (Korea), respectively.

2.2. Design of the ICS

The packaged ICS in a polystyrene case and the structure of an ICS are illustrated in Fig. 1. The ICS consists of four parts: a buffer loading pad, a conjugate pad, a detection region with test and control lines, and an absorbent pad. Streptavidin and mouse IgG coated gold nanoparticles (AuNPs) were concentrated in the conjugate pad to capture the biotin labeled RT-LAMP products. In the detection region, two test lines and one control line were patterned. The test line 1 and line 2 were coated with Digoxigenin monoclonal antibodies (Medisensor Inc., Korea), and Texas Red monoclonal antibodies (Medisensor Inc., Korea) respectively, and the control line was immobilized with goat anti-mouse IgG.

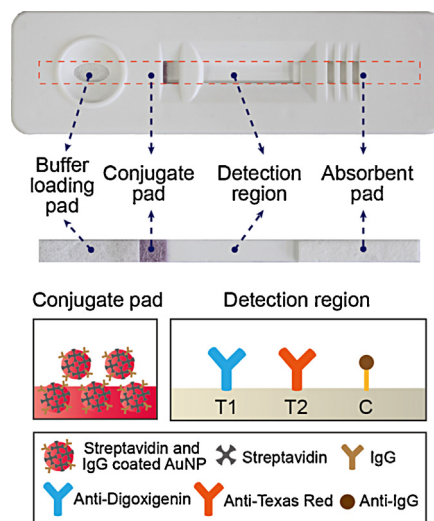


Fig. 1. Digital image of the packaged ICS and its structure. Streptavidin and IgG coated gold nanoparticles are immobilized in the conjugate pad. In the detection region, anti-Digoxigenin, anti-Texas Red and anti-IgG are immobilized on the test line 1, test line 2, and control line, respectively.

2.3. Design of primers

The full sequence of the influenza A viruses were obtained from Genbank. The strains of influenza A virus were A/Korea/CJ01/2009 (H1N1), A/reassortant/NYMC X-175C (Uruguay/716/2007 × Puerto Rico/8/1934)(H3N2), and A/environment/Korea/W149/2006 (H5N1). Hemagglutinin (HA) genes (accession number: [HM189479.1](#) for H1 gene, [CY058503.1](#) for H3 gene, [EU233731.1](#) for H5 gene), and conserved matrix (M) gene from the three viral strains (accession number: [HM189449.1](#), [CY058506.1](#), [EU233732.1](#)) were targeted. Target HA gene specific primers were designed using a Primer Explorer V4 program on the Eiken website (<http://primerexplorer.jp/e/>). Six primers including outer primers (F3/B3), inner primers (FIP/BIP), and loop primers (LF/LB) for targeting HA genes, and five primers including F3/B3, FIP/BIP, and LF for targeting conserved M gene were designed as shown in Fig. 2 [25,26]. The sequence information is shown in Table S1. For subtyping influenza A virus, H1, H3, and H5 were targeted, and LF and LB primers were labeled with Texas Red hapten (Fig. 2a–c). To ensure the influenza A virus, the conserved region of M gene was amplified, and the LF primer was labeled with Digoxigenin (Fig. 2d). Universal M gene expression in the three viral strains was confirmed by revealing the same pattern of the RT-LAMP products in the gel electropherogram (Fig. S1).

2.4. Multiplex RT-LAMP reaction

Multiplex RT-LAMP reaction was performed in 25 µL of an RT-LAMP cocktail containing 0.5 µL of each outer primer (F3 and B3, 20 uM), 0.5 µL of each inner primer (FIP and BIP, 200 uM), 0.5 µL of each loop primer (LF and LB, 100 uM), 12.5 µL of the reaction mixture (1× ThermoPol buffer, 0.8 M betaine, 8.0 mM

MgSO₄, 1.6 mM dATP, dGTP, dCTP, 1.0 mM dTTP, and 0.06 mM biotin-dUTP), 1 µL of *Bst* DNA polymerase (8 U), 0.2 µL of AMV reverse transcriptase (2 U), 3.3 µL of deionized water and 2 µL of a purified viral RNA solution of influenza A H1N1, A H3N2, and A H5N1 viruses. The concentration of viral RNAs was controlled from 10⁶ copies to 10 copies. The RT-LAMP cocktail was incubated at 66 °C for 40 min and deactivated at 80 °C for 5 min.

2.5. Colorimetric detection of the RT-LAMP amplicons on the ICS

The RT-LAMP amplicons of HA genes were labeled with Texas Red haptens and biotin moieties due to the incorporation of Texas Red-labeled loop primers and biotin-dUTPs, while those of M gene were labeled with Digoxigenin haptens and biotin moieties by using Digoxigenin-labeled primers and biotin-dUTPs (Fig. 3a). After the RT-LAMP reaction, 1 µL of the amplicon solution was loaded in the conjugation pad window and 45 µL of a running buffer (25 mM sodium carbonate buffer (pH 9.6), 1% casein, 0.4 mM EDTA, 0.2% alpha-cyclodextrin, 0.2% Triton-100, 0.4 M urea, 0.1% sodium azide) were introduced to the buffer loading reservoir on the ICS (Fig. 3b and c). After 15 min, multiplex RT-LAMP products were captured and visualized by the hapten (Texas Red and Digoxigenin) of the resultant amplicons and anti-hapten interaction on the test lines (Fig. 3d).

2.6. Clinical sample analysis

To investigate the feasibility of our methodology for real sample analysis, we performed the subtyping of influenza A virus from clinical samples, which were obtained from patients infected by influenza A virus. First, human DNAs in the clinical samples were removed by treatment with an RNase-Free DNase Kit (Qiagen,

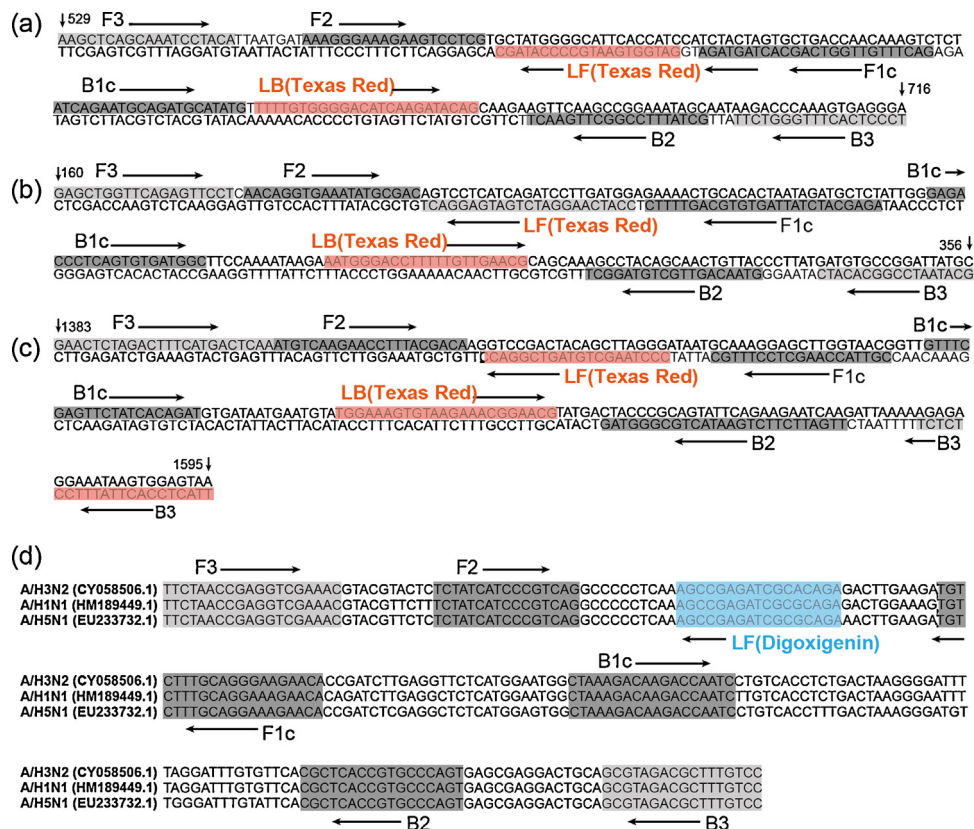


Fig. 2. Primer design for multiplex RT-LAMP. (a) H1 gene of influenza A H1N1. (b) H3 gene of H3N2. (c) H5 gene of H5N1. LF and LB loop primers for targeting HA genes were labeled with Texas Red haptens. (d) Primer design for targeting conserved M gene of influenza A viruses (H1N1, H3N2, and H5N1). An LF loop primer for M gene was labeled with a Digoxigenin hapten.

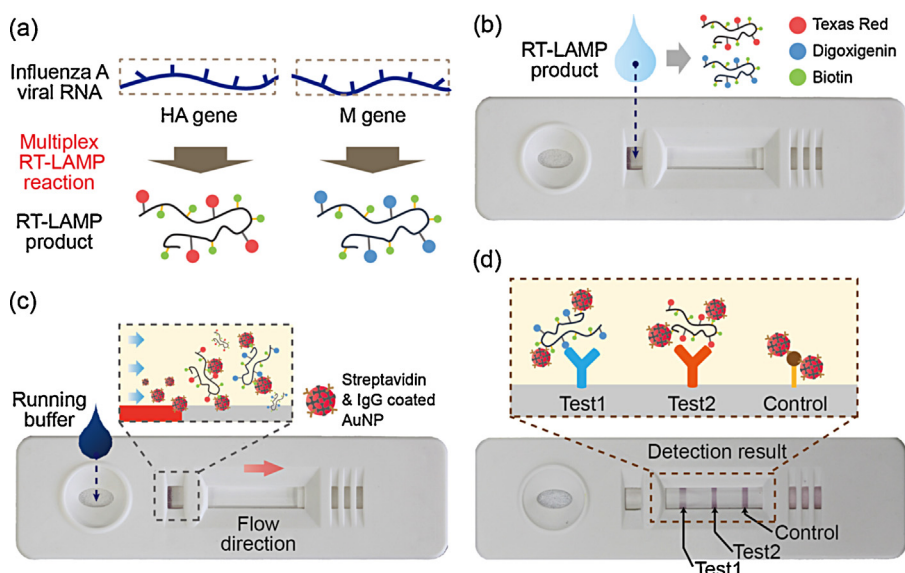


Fig. 3. Analysis of multiplex RT-LAMP amplicons on the ICS for subtyping influenza A virus. (a) Multiplex RT-LAMP reaction to amplify HA and M genes. (b) Loading of RT-LAMP amplicons. (c) Loading of a running buffer. (d) Colorimetric detection of the gene expression on the ICS.

Germany). 140 μL of a nasopharyngeal swab solution in a transport buffer were incubated for 10 min with 20 μL of a Buffer RDD, 5 μL of a DNase I stock, and 35 μL of RNase-Free water. Then, viral RNAs were extracted using a QIAamp[®] Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Purified viral RNAs were used as templates for multiplex RT-LAMP to subtype influenza A virus. To confirm the results, the conventional real-time RT-PCR was also performed by using a One-Step SYBR Amplification Kit (Takara Korea, Daejeon).

3. Results and discussion

3.1. Optimization of the multiplex RT-LAMP conditions

To optimize the multiplex RT-LAMP conditions, we controlled the reaction temperature, the amount of the used enzymes, and the concentration of dNTPs. The reaction temperature was varied from 62 to 66 °C as shown in Fig. S2a. H1 and M genes of influenza A virus H1N1 were isothermally amplified, and the resultant RT-LAMP products were analyzed by gel electrophoresis as well as on the ICS. Gel electropherogram shows that an additional product band of 125 bp appeared as the reaction temperature increased more than 64 °C (Fig. S2a(i)). The intensities of the gel bands became strongest at 66 °C. The analysis of multiplex RT-LAMP amplicons on the ICS are shown in Fig. S2a(ii). Violet color on the test line 1 was displayed in all the reaction temperatures, indicating the expression of H1 gene. However, the violet color on the test line 2, which means the M gene amplicon, was turned up from 65 °C. Thus, we chose 66 °C of the reaction temperature to clearly reveal the HA and M gene expression on the ICS.

We also optimized the concentrations of dNTPs and biotin-dUTP in the multiplex RT-LAMP reaction. The incorporated biotin-dUTPs in the amplicons serve as binding moieties with the streptavidin coated gold nanoparticles in the conjugate pad. So, the high amount of biotin-dUTP can increase the detection sensitivity in the ICS, but can simultaneously decrease the isothermal amplification efficiency. Thus, the concentration ratio of dTTP and biotin-dUTP was fixed to 50:3 based on our previous report [27], and the concentration of dNTPs was tuned with 0.8, 1.2, 1.6, 2.0, and 2.4 mM in the multiplex RT-LAMP reaction. At the concentration of 0.8 and 1.2 mM dNTPs (lane 1 and 2 in Fig. S2b(i)),

only H1 gene was dominant. At the concentration of 1.6 mM dNTPs, both H1 and M genes were amplified, showing the additional band around 125 bp (lane 3 in Fig. S2b(i)). Interestingly, the band pattern at the concentration of 2.0 mM dNTPs (lane 4) looks similar to that of lane 3, but the strong red color at the bottom suggested that the Texas Red-labeled primers which were added to amplify H1 gene were not successfully incorporated. At high 2.4 mM concentration (lane 5), there was also a red band in the gel electrophoresis, and both H1 and M genes were not produced, probably due to inhibition to the enzyme activity [28]. We analyzed the same multiplex RT-LAMP products on the ICS (Fig. S2b(ii)). The amplicon products at the concentration of 0.8 and 1.2 mM dNTPs showed only H1 gene expression (#1 and #2 in Fig. S2b(ii)), which was matched with the gel pattern of lane 1 and 2 in Fig. S2b(i). #3 on the ICS shows both H1 and M gene expression, which was correspondent to the lane 3 in the electropherogram. However, in case of #4 on the ICS, only M gene test line was visualized, although the gel band patterns of lane 3 and 4 in Fig. S2b(i) were similar. These results imply that the ICS could obviously distinguish the multiplex RT-LAMP products, which could not be discernible in the gel electropherogram. Although it is not clear at this moment why the dNTP concentration affected the gene amplification efficiency in the multiplex RT-LAMP reaction, it is likely that the control of dNTP concentration is critical to produce the comparable amount of multiple target genes in the multiplex RT-LAMP.

The amount of *Bst* DNA polymerase was also controlled with 8, 12, 16, 20, and 24 unit, and AMV reverse transcriptase was also tuned with 1, 2, 3, 4, and 5 unit. The concentrations of betaine and MgSO_4 were fixed to 0.8 M and 8.0 mM, respectively. The low amount of *Bst* DNA polymerase (8 units) was enough to produce the equivalent gel patterns with similar band intensities in the multiplex RT-LAMP reaction as shown in Fig. S2c(i). In case of AMV reverse transcriptase, although 1 unit of AMV reverse transcriptase could produce all the amplicon bands, the band intensities became saturated from 2 units as shown in Fig. S2c(ii). Thus, 8 units of *Bst* DNA polymerase and 2 units of AMV reverse transcriptase were employed in the multiplex RT-LAMP reaction.

3.2. Monoplex and multiplex detection of influenza A viruses

Under the optimized conditions as described above, we performed the monoplex of HA and M gene as well as multiplex

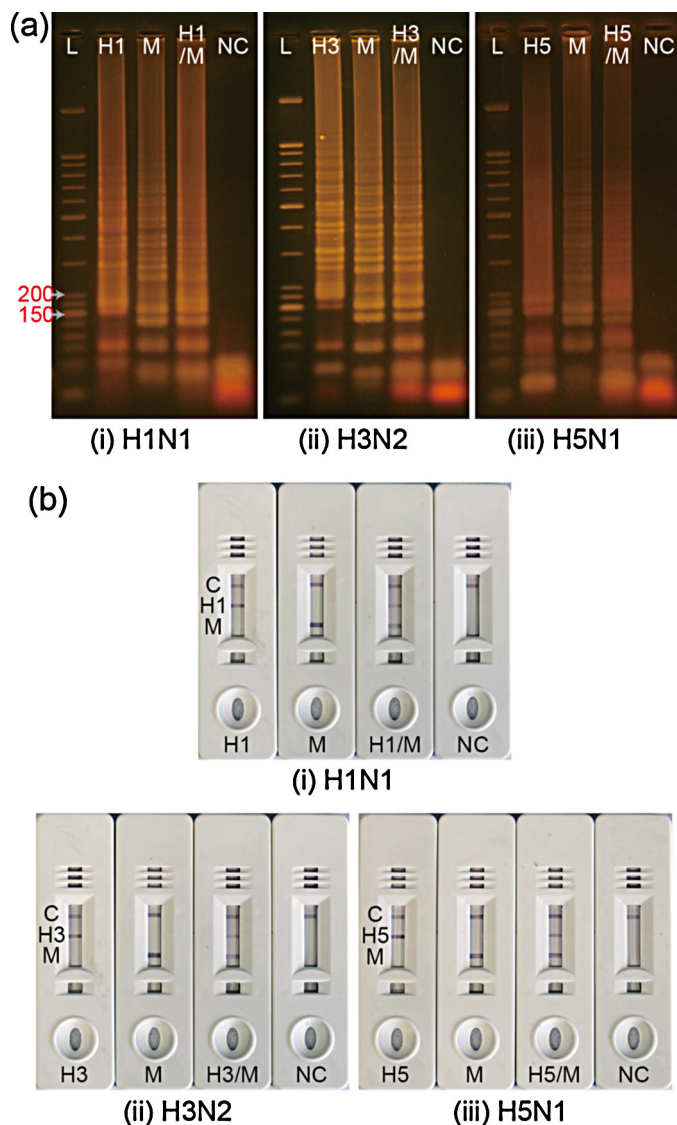


Fig. 4. Monoplex and multiplex RT-LAMP amplicons of HA and M genes by (a) gel electrophoresis and (b) ICS.

isothermal amplification of both genes using H1N1, H3N2, and H5N1 strains at 66 °C for 60 min with 10^6 copies of viral RNAs. Triplicate experiments were performed in all cases. For monoplex amplification, either HA gene-targeting primers or M gene-targeting primers were added into the RT-LAMP cocktail, while, in the multiplex reaction, both the HA gene and M gene-targeting primers were added. Fig. 4a shows the gel electropherograms for the monoplex and multiplex RT-LAMP results. Band patterns of monoplex HA gene and conserved M gene were distinguishable. Reddish ladder-like gel patterns of the HA gene amplicons is due to the Texas Red labeling tag. Multiplex RT-LAMP amplicons for HA and M genes were successfully produced from three viral strains, which could be confirmed by observing the combination of the gel patterns of monoplex HA and M gene. The negative control which omitted the RNA templates in the RT-LAMP cocktail did not produce any gel bands with strong red band at the bottom.

Although the multiplex RT-LAMP amplicons could be verified by carefully comparing with the gel band patterns of the monoplex RT-LAMP amplicons, it could be ambiguous and misjudged if the HA gene and M gene pattern are similar. To solve this problem, Iseki et al. suggested a digestion process by using restriction enzymes

for the multiplex LAMP products to distinguish multiple targets, but the proposed method is time-consuming, high-priced, and inadequate for on-site detection [29]. On the contrary, our ICS can clearly show the monoplex and multiplex gene expression. Fig. 4b displays the monoplex and multiplex HA and M gene amplicons of three influenza A viral strains on the ICS. In addition to the control line (the top line), the HA gene (H1, H3, and H5) expression was observed in the middle test line, and the M gene expression was monitored from the bottom test line. All the data were matched with those of gel electropherograms with higher confidence. Influenza A H1N1 virus was confirmed by detecting both H1 and M genes, influenza A H3N2 virus was verified by observing both H3 and M gene expression, and influenza A H5N1 virus was identified due to the expression of both H5 and M gene. Thus, the subtyping of three influenza A viruses was achieved simply and rapidly by combining the multiplex RT-LAMP with ICS.

3.3. Time control of multiplex RT-LAMP for colorimetric detection on the ICS

We controlled the reaction time from 60 min to 20 min with 10 min interval to determine the minimum time for efficient

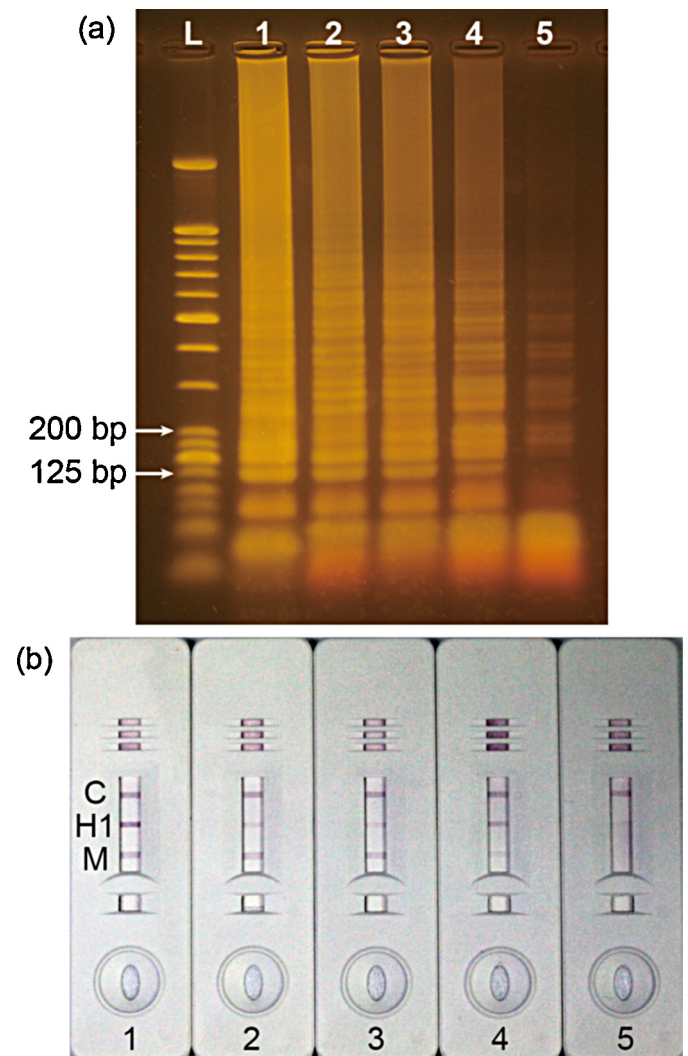


Fig. 5. Multiplex RT-LAMP for influenza A H1N1 virus with variation of RT-LAMP time from 60 min to 20 min. Both H1 and M genes were targeted and the resultant amplicons were analyzed by (a) gel electropherogram and (b) ICS. (L: DNA ladder, 1: 60 min, 2: 50 min, 3: 40 min, 4: 30 min, 5: 20 min).

multiplex RT-LAMP at 66 °C. Influenza A H1N1 viral RNA was used with 10^6 copies to amplify H1 and M genes, and the experiments were performed at least three times in all cases. The band intensities were gradually reduced as the reaction time decreased (Fig. 5a). Thirty minutes of the multiplex RT-LAMP were enough to produce the amplicon ladder patterns on the gel as well as the three lines on the ICS (Fig. 5b). At 20 min of the reaction time, the product ladder patterns became faint on the gel and only test line 1 indicating H1 gene amplification was positive, suggesting that H1 gene was more efficient than M gene during the multiplex RT-LAMP reaction. We chose 40 min as an optimized reaction time considering the multiplex RT-LAMP with low copy of viral RNAs.

3.4. Sensitivity test for multiplex RT-LAMP amplicons on an ICS

To determine the limit of detection of our methodology, influenza A H1N1 viral RNAs were serially diluted ranging from 10^6 to 10 copies and were isothermally amplified for 40 min at 66 °C. Both the H1 and M genes were successfully amplified and detected even with 10 copies by presenting three lines on the ICS as shown in Fig. 6. Previously, our group reported an integrated RT-PCR and ICS microdevice to detect H1 gene of influenza A H1N1 virus with 1.41 pg (about 10^5 copies) LOD [27]. In this study, we improved the detection sensitivity (10 copies) and multiplexity using multiplex RT-LAMP and ICS technology [15], and will incorporate them in the advanced microdevice for on-site viral detection.

3.5. Clinical sample analysis by the combined multiplex RT-LAMP and ICS

Three clinical nasopharyngeal swab samples obtained from the patients infected by influenza A virus were analyzed using the RT-LAMP and ICS. After RNAs were extracted, the purified RNAs were isothermally amplified for 40 min at 66 °C with the multiplex RT-LAMP primer sets for targeting H1 and M genes, H3 and M genes, or H5 and M genes. Sample 1 revealed three lines only with the H1 and M gene targeting primer sets, indicating the subtype of influenza A H1N1 virus. Samples 2 and 3 showed three lines with the H3 and M gene targeting primer sets, suggesting that the clinical sample was infected by influenza A H3N2 virus (Fig. 7). To confirm the result accuracy of the combined multiplex RT-LAMP and ICS, the clinical samples were also analyzed by a conventional real-time RT-PCR (Fig. S3) [30]. While sample 1 produced positive results only for H1 and M gene amplification, samples 2 and 3 showed positive results for H3 and M gene amplification. Thus, the results obtained from the RT-LAMP and ICS were perfectly matched with those of the real-time RT-PCR profile with reduced time. These results demonstrated that the multiplex RT-LAMP and ICS techniques could be applied for real clinical samples to pathotype and subtype influenza A virus with high speed,



Fig. 6. LOD test of the multiplex RT-LAMP reaction targeting H1 and M genes by using influenza A H1N1 viral RNA templates ranging from 10^6 to 10 copies.

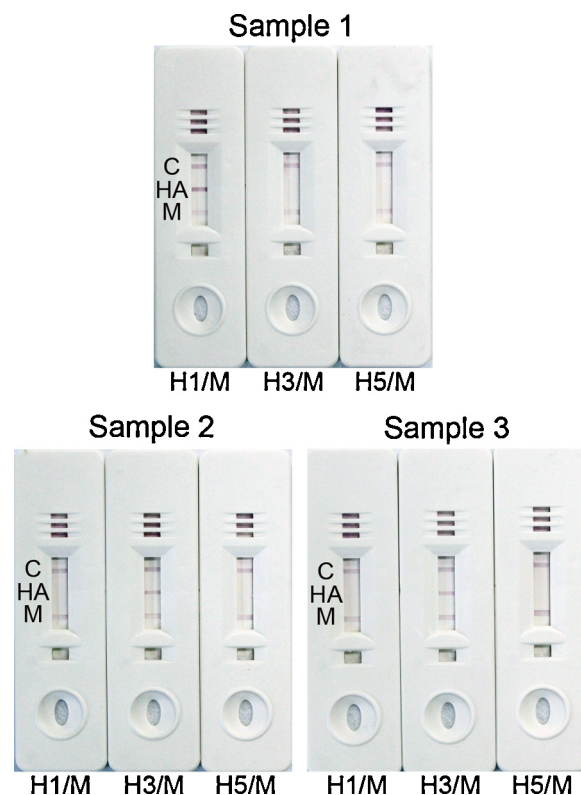


Fig. 7. Clinical sample analysis by combining the multiplex RT-LAMP and ICS. Sample 1 shows the H1 and M gene expression, while samples 2 and 3 reveal the H3 and M gene expression.

simplicity and accuracy. Multiplex RT-LAMP and ICS were performed in 40 min and 15 min, respectively, so the total process time was completed in 55 min.

4. Conclusion

We successfully demonstrated high performance of the combined multiplex RT-LAMP and the ICS for identifying influenza A viruses. Three influenza A virus strains (H1N1, H3N2, and H5N1) were subtyped by genotyping HA and conserved M genes simultaneously under the optimized RT-LAMP conditions. Texas Red and Digoxigenin hapten labels were used for detecting HA and conserved M gene on the ICS through the hapten and anti-hapten interaction. Our methodology is capable of pathotyping and subtyping influenza A virus (H1N1, H3N2, and H5N1) even with 10 copies of viral RNAs, and the clinical samples were accurately analyzed in 55 min. The combination of the multiplex RT-LAMP and ICS can provide an advanced diagnostic platform for on-site early influenza virus detection with high speed, sensitivity, and accuracy.

Acknowledgments

This work was supported by Center for BioNano Health-Guard funded by the Ministry of Science, ICT & Future Planning (MSIP) of Korea as Global Frontier Project" (H-GUARD_2013M3A6B2078964) and National Research Foundation of Korea (NRF) (Grant NRF-2014-009799).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aca.2014.10.020>.

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