COMBINATION OF MULTIPLEX ISOTHERMAL AMPLIFICATION WITH AN IMMUNOCHROMATOGRAPHIC STRIP FOR SUBTYING INFLUENZA A VIRUS

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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 infected by influenza A virus were 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.

KEYWORDS: Immunochromatographic strip, Influenza A virus, Multiplex RT-LAMP, Subtyping

INTRODUCTION
Considering that influenza A virus has detrimental effect on human being and livestock over the world, early diagnosis of influenza virus is critical to prevent economic and social damages [1,2]. Commercial kit for detecting influenza A virus is based on an immunochromatographic strip (ICS) due to simplicity, rapidity, and cost-effectiveness [3]. However, the commercial ICS was only designed to identify influenza A virus, so it is impossible to subtype influenza A virus whose information important for medical treatment. Moreover, an immunoassay based strip sensor still needs to improve the detection sensitivity and multiplexity. To overcome such limitations, we developed multiplex reverse-transcription loop-mediated isothermal amplification (RT-LAMP) and multiplex RT-LAMP amplicon detection on an ICS for subtyping influenza A virus (A/H1N1, A/H3N2, and A/H5N1).

EXPERIMENTAL
The ICS consists of four parts: a buffer loading pad, a conjugate pad, a detection region, and an absorbent pad. Streptavidin coated gold nanoparticle (AuNP) was concentrated in the conjugate pad to capture the biotin labeled RT-LAMP amplicons. Anti-digoxigenin, anti-texas red, and biotin are immobilized in the detection region (Figure 1a). H1 gene of influenza A H1N1 virus was isothermally amplified by texas red-labeled primers and biotin-dUTP, while M gene was isothermally amplified by digoxigenin-labeled primers and biotin-dUTP at 66 °C (Figure 1b). After RT-LAMP, 1 µL of an amplicon solution was injected to the conjugation pad window and 45 µL of a running buffer was introduced to the buffer loading reservoir (Figure 1c,d). After 15 min, multiplex amplicons could be captured by the hapten (texas red and digoxigenin) of RT-LAMP amplicons and the immobilized anti-hapten interaction, and visualized by naked eyes (Figure 1e). For subtyping influenza A virus, H1, H3, and H5 were targeted, and LF and LB primers were labeled with texas red hapten (Figure 2a-c). To confirm the influenza A virus, the conserved region of M gene was targeted, and the LF primer was
labeled with digoxigenin (Figure 2d). Universal M gene expression in the three virus strains was confirmed by showing the same pattern of the RT-LAMP product (Figure 2e).

Figure 1: (a) Schematic illustration of a lateral flow strip for multiplex detection of RT-LAMP amplicons. (b) Multiplex RT-LAMP system for subtyping influenza A virus. (c) A RT-LAMP product loading. (d) A running buffer loading. (e) A detection result on an ICS.

Figure 2: Primer designs for multiplex RT-LAMP. (a) H1 gene of Influenza A/H1N1. (b) H3 gene of A/H3N2. (c) H5 gene of A/H5N1. (d) Primer design for conserved M gene of influenza A virus. (e) Gel electropherogram for M gene expression in all the influenza A virus strains.

RESULTS AND DISCUSSION

Figure 3a shows the monoplex and multiplex RT-LAMP detection on an ICS for three influenza A virus strains whose results were perfectly matched with those of gel electropherograms (Figure 3b). We also evaluated the RT-LAMP reaction time from 60 min to 20 min, and multiplex H1 and M amplicons were successfully detected even at 30 min with 10^6 copy of influenza A/H1N1 viral templates (Figure 4). Limit of detection test was performed using 10^6 to 10 copy templates with 40 min of an RT-LAMP reaction time, and multiplex targets (H1 and M gene) were identified even with 10 copy of RNA templates (Figure 5). Three clinical samples were also analyzed by multiplex RT-LAMP and ICS analysis, whose results were confirmed by a conventional real-time RT-PCR (Figure 6).

Figure 3: Monoplex and multiplex RT-LAMP detection of influenza A viruses (HA and M gene) by (a) ICS and (b) gel electrophoresis.
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 (A/H1N1, A/H3N2, and A/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 (A/H1N1, A/H3N2, and A/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.

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