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

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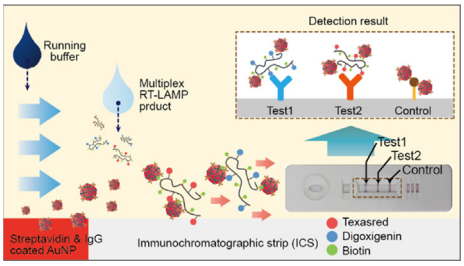
HIGHLIGHTS

- 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



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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 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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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 $MgSO_4$ 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.

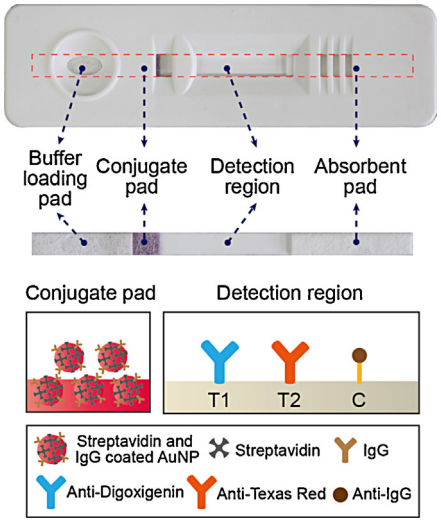

Fig. 1. Digital image of the packaged ICS and its structure. Streptavidin and IgG coated gold nanoparticles are immobilized in the conjugate pad. In the detection region, anti-Digoxigenin, anti-Texas Red and anti-IgG are immobilized on the test line 1, test line 2, and control line, respectively.