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Evaluation of Reverse-Transcription Loop-Mediated Isothermal Amplification Assay for Screening Influenza a Viruses from Different Animal Species

[1,2]Eun-Mi Kim, [3]Hyo-Sung Jeon, [3]Ji-Jung Kim, [4]Yeun-Kyung Shin,
[5]Youn-Jeong Lee, [1]Sang-Geon Yeo and [1,2]Choi-Kyu Park
[1]College of Veterinary Medicine and Animal Disease Intervention Center,
Kyungpook National University, 702-701 Daegu, Korea
[2]RAD Inc., 718-913 Daegu, Korea
[3]M Monitor Inc., 700-842 Daegu, Korea
[4]Virology Division, [5]Avian Disease Division,
Animal and Plant Quarantine Agency, 430-757 Anyang, Korea

Abstract: For efficient monitoring of infections caused by various subtypes of Influenza A Virus (IAV) in animal populations, it is necessary to provide a rapid, accurate and reliable moleculardiagnostic assay for detection of all IAV subtypes. This study aims to evaluate a previously reported Reverse-Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) assay for detection of animal IAVs from different species. The assay is capable of visually detecting 16 subtypes of Avian IAV (AIV), three subtypes of Awine IAV (SIV), two subtypes of Equine IAV (EIV), one subtype of Canine IAV (CIV) and two subtypes of human IAV but not influenza B virus. The detection limit of the assay was 10^{-2}, 10^{-3}, 10^{-1} and 10^{-1} tissue culture infective $dose_{50}$ for each tested AIV, SIV, EIV and CIV, respectively which was 10 fold higher than that of RT-Polymerase Chain Reaction (PCR) and the same as those of Real-Time RT-PCR (RRT-PCR) and RT-LAMP Methods for IAVs. The RT-LAMP Method detected eight samples as IAV-positive out of 589 field samples which was consistent with RRT-PCR and virus isolation results. The RT-LAMP assay evaluated in this study is applicable for rapid, user-friendly and reliable screening of IAV in animal populations and is expected to be an alternative method to RT-PCR or RRT-PCR, even in under-equipped laboratories.

Key words: Influenza a virus, loop-mediated isothermal amplification, hydroxy naphthol blue, matrix gene, assay

INTRODUCTION

Influenza A Viruses (IAVs) are enveloped, single-stranded RNA virusesof the family Orthomyxoviridae and are classified into subtypes based on the antigenic properties of the viral surface proteins Hemagglutinin (HA) and Neuraminidase (NA). Sixteen antigenically different HAs (H1-H16) and nine different NAs (N1-N9) have been recognized thus far with their combination designating the viral subtype. IAVs infect a large variety of animal species including humans, pigs, horses, sea mammals and birds (Alexander, 1982; Webster *et al*., 1992). Aquatic birds are the source of all IAV subtypes for domestic birds and mammals, notably humans, pigs, horses, seals, ferrets and mink (Olsen *et al*., 2006; Wright and Webster, 2001). IAV transmission between species is rare; however, its occurrence may have serious consequences. Introduction of animal influenza virus into the human population can initiate an influenza pandemic, provided the humans have no immunity to the virus and it spreads efficiently from person to person as was the caseforthe pandemic (H1N1) 2009 influenza virus that originated from reassortment between swine influenza viruses from North American and Eurasian lineages (Smith *et al*., 2009; Van Reeth, 2007).

Extensive surveillance, epidemiological investigation and genetic characterization of IAV in animal populations are necessary to monitor IAV evolution and control outbreaks and IAV epidemics in animal and human populations (Vincent *et al*., 2014). Rapid and accurate diagnostic methods for IAV infection are needed for surveillance, outbreak management and early infection control of emerging influenza viruses. Currently, several molecular diagnostic methods including Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and Real-Time RT-PCR (RRT-PCR) have been developed for

Corresponding Author: Choi-Kyu Park, Department of Infectious Diseases,
College of Veterinary Medicine and Animal Disease Intervention Center, Kyungpook National University,
702-701 Daegu, Korea

rapid IAV detection (Kim *et al*., 2013; Shin *et al*., 2011). Nevertheless, these techniques require sophisticated and expensive instrumentation and specialized technicians, thereby limiting the effectiveness of these assays in smaller and under-equipped laboratories in developed or developing countries.

Loop-mediated isothermal Amplification (LAMP) is a novel nucleic acid amplification method originally developed by Notomi *et al*. (2000). The LAMP assay employs four or six primers specific to multi-regions and Bst DNA polymerase which has a function involving strand displacement. Complete amplification can be accomplished within 30-90 min under isothermal conditions (~60-65 ou) and it does not require expensive instrumentsor equipment. The LAMP characteristics of rapidity, simplicity, high sensitivity and specificity make it a powerful tool for disease diagnostics that has been widely applied to human, animal and plant pathogen detection (Dhama *et al*., 2014; Mori and Notomi, 2009). Reverse Transcription LAMP (RT-LAMP) assay has successfully detected variable subtypes of animal and human IAV (Chen *et al*., 2008; Imai *et al*., 2007; Ito *et al*., 2006; Poon *et al*., 2005). However, for efficient monitoring of infections caused by various IAV-subtypes in animal populations, it is necessary to use a RT-LAMP assay that is effective in rapid detection of all IAV subtypes from different species. Recently, we developed a RT-LAMP assay for Swine IAV (SIV) detection that was very useful for detecting major subtypes of SIVs (H1N1, H1N2 and H3N2) (Kim *et al*., 2015). However, the usefulness of the RT-LAMP assay for detection of other animal IAVs was not evaluated, although, primers were designed for detection of all animal IAV subtypes. Therefore, we also evaluated the performance of the RT-LAMP assay with respect to animal IAVs including major subtypes of avian, canine and equine influenza viruses as well as field samples in the present study.

MATRIALS AND METHODS

Viruses: IAV reference strains isolated from different animals and humans were used to evaluate the RT-LAMP assay (Table 1). The Animal and Plant Quarantine Agency, Republic of Korea, provided inactivated animal IAVs and the Korea Centers for Disease Control and Prevention, Republic of Korea, provided human influenza viruses including IAV subtype H1N1 and H3N2 and influenza B virus.

RNA extraction: Viral RNAwas extracted from viruses and field samples by using RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Extracted nucleic acids were stored at -20°C until further use.

Table 1: Specificityof the Reverse-Transcription Loop-mediated isothermal Amplification (RT-LAMP) assay for the detection of influenza viruses

Viruses	Subtypes	RT-LAMP result[a]
Avian origin		
A/PR/8/34	H1N1	+
A/Singapore/1/57	H2N2	+
A/duck/Ukraine/1/63	H3N8	+
A/duck/Czechoslovakia/56	H4N6	+
A/duck/Hong Kong/820/80	H5N3	+
A/shear water/Australia/1/72	H6N5	+
A/wild duck/Kr/CSM42-34/11	H7N9	+
A/turkey/Ontario/6118/68	H8N4	+
A/turkey/Wisconsin/1/66	H9N2	+
A/wild duck/Kr/CSM42-9/11	H10N7	+
A/duck/Memphis/546/74	H11N9	+
A/duck/Alberta/60/76	H12N5	+
A/wild duck/Kr/SH38-45	H13N2	+
A/mallard/Gurjer/263/82	H14N5	+
A/Shear water/W.Austri/25761/79	H15N9	+
A/gull/Denmark/68110	H16N3	+
Swine origin		
A/Korea/D180-2/2009	H1N1	+
A/Korea/103/2009	H1N2	+
A/Korea/A18/2011	H3N2	+
Equine origin		
A/equi/Miami/1/63	H3N8	+
A/equi/Prague/56	H7N7	+
Canine origin		
A/canine/Korea/BD-1/2013	H3N2	+
Human origin		
A/Gyeongnam/1820/1/2009	H1N1	+
A/Gyeongnam/4251/11	H3N2	+
B/Wisconsin/1/2010	B	-

[a]Viral RNA amplification was evaluated by visually detecting color changesin the reaction tube from purple to sky-blue (+: RT-LAMP positive; -: RT-LAMP negative)

RT-PCR and RRT-PCR: One-step RT-PCR for detection of all IAV subtypes was performed using a one-step RT-PCR kit (Qiagen, Hilden, Germany) and a thermal cycler (Bioer Technology, Beijing, China) as previously described (Shin *et al*., 2011). The expected size of the RT-PCR product obtained using the IAV common primer set (M30F2/08-1 and M264R3/08-242) and the IAV matrix gene was 242 bp (Table 2). The 5 μL of RT-PCR products were analyzed by electrophoresis, using 1.5% agarose gel. Amplified products were visualized by ultraviolet light transilluminator (Bio-Rad Laboratories, Inc., Hercules, CA, USA) after staining with NEO green dye (Neoscience, Seoul, Korea). RRT-PCR for detection of all IAV subtypes was performed using a one-step Prime Script RT-PCR kit (Takara Bio, Inc., Shiga, Japan) and a real-time PCR instrument (Applied Biosystems, Waltham, MA, USA) as previously described (Kim *et al*., 2013). Primer sequences and probes for RRT-PCR are described in Table 2. The results of the RRT-PCR assay for the matrix gene showed high C_T values (>38) that were considered negative based on their proximity to the 40 cycle cutoff.

RT-LAMP: The RT-LAMP reaction was carried out using IAV matrix gene-specific primer sets (Table 2) as

Table 2: Primers and probesused in this study

Methods	Name of the primer	Sequence (5'-3')	Reference
RT-RAMP	IF3	AGTCTTCTAACCGAGGTCGA	Kim *et al.* (2015)
	B3	TGCAGTCCTCGCTCACTG	
	LF	GCAAAGACATCTTCAAGTCTCTGC	
	LB	GACTAAGGGGATTTTAGGGTTTGT	
	FIP(F1c+F2)	ACATCTTCAAGTCTCTGCGCGATC-ACGTTCTCTCTATCGTCCCG	
	BIP(B1+B2c)	AGACAAGACCAATCCTGTCACCTCT-TGCAGTCCTCGCTCACTG	
RT-PCR	M30F2/08-1	ATGAGYCTTYTAACCGAGGTCGAAACG	Shin *et al.* (2011)
	M264R3/08-242	TGGACAAANCGTCTACGCTGCAG	
RRT-PCR	M-Kr forward	AAGACCAATCCTGTCACCTCTGA	Kim *et al.* (2013)
	M-Kr reverse	CAAAGCGTCTACGCTGCAGTCC	
	M-Kr probe	FAM-TTTGTNTTYACGCTCACCGTGCC-TAMRA	

Table 3: Results of virus detection in clinical samples bythe Reverse-Transcription loop-mediated isothermal Amplification (RT-LAMP), Real Time Reverse Transcription-Polymerase Chain Reaction (RRT-PCR) and Virus Isolation (VI)

		Virus detection (number of positive samples)			
Animal sources	n	RT-LAMP	RRT-PCR	VI	Subtype (No. of subtyped viruses)
Wild duck	250	4	4	4	H1(1), H6(2), H5(1)
Domestic chicken	97	2	2	2	H9(2)
Swine	152	1	1	1	H3(1)
Equine	46	0	0	0	
Canine	44	0	0	0	
Total	589	7	7	7	

previously described (Kim *et al.*, 2015). Briefly, 5 μL viral RNA was added to a RT-LAMP premix containing 40 pmol each of the primers FIP and BIP, 5 pmol each of the outer primers F3 and B3, 20 pmol each of the loop primers LF and LB, 8 U of Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA), 10 U of AMV reverse transcriptase (Promega, Durham, NC, USA), 10 mM dNTPs (Takara Bio, Inc., Shiga, Japan), 250 mM betaine, 150 mM $MgSO_4$, 3 mM hydroxynaphthol blue [HNB, (Lemongreen, Shanghai, China)] and the final volume was adjusted to 25 μL with diet hylpyrocarbonate-treated water. The amplification reaction was performed at 58°C for 40 min which was followed by heating at 80°C for 2 min to terminate the reaction. After the RT-LAMP reaction, positive results were visually confirmed by color change from purple to sky-blue in the reaction tubes and detected by 1.5% agarose gel electrophoresis by observing LAMP-specific ladder-like DNA bands using an ultraviolet light transilluminator (Bio-Rad Laboratories, Inc., Hercules, CA, USA) after staining with NEO green dye (Neoscience, Seoul, Korea).

RT-LAMP sensitivity: To determine RT-LAMP sensitivity for other animal IAVs, RT-LAMP was performed using serially diluted IAVs including avian-origin A/duck/Hong Kong/820/80(H5N3), swine-origin A/Korea/103/2009(H1N2), equine-origin A/equi/Miami/1/63(H3N8) and canine-origin A/canine/Korea/BD-1/2013 (H3N2) reference strains, adjusted usingthe same viral titer [$10^{3.0}$ Tissue Culture Infective Dose $(TCID)_{50}$/0.1 Ml] and compared with RT-PCR and RRT-PCR results by using the same virus diluents as a template.

RT-LAMP evaluation: To evaluate the usefulness of the RT-LAMP assay for detection of all IAV subtypes, a total of 21 animal IAVs, including avian IAVs [AIVs (16 subtypes)], SIVs (three subtypes), equine IAVs [EIVs (two subtypes)], canine IAV [CIV (one subtype)], three human IAVs (two subtypes) and a human influenza B virus were tested (Table 1). RT-LAMP evaluationon animal field samples was performed on 589 field samples collected from different sources from 2013-2015 (Table 3). Wild duck fecal samples (n = 250) from migratory birds were collected from habitats in Jeonnam, Kyungpook and Kyungnam provinces in Southern parts of Korea as part of the national surveillance plan for wild birds. Domestic chicken (n = 97) and swine (n = 152) samples were collected from clinical cases from domestic farms and sent to our diagnostic laboratory from 2014 to 2015. Equine (n = 46) and canine (n = 44) nasal samples were collected from domestic horse-breeding farms and animal shelters located in Kyungpook Province, Korea. All samples were stored at -80°C and extracted RNAs were stored at -20°C until further use. For the clinical samples, virus isolation by egg inoculation was performed as previously described (Kim *et al.*, 2013) when each sample was received in our laboratory and RT-LAMP and RRT-PCR were performed as described above using RNAs extracted from the samples. For further evaluation of virus-positive samples, the HA protein was subtyped as previously described (Kim *et al.*, 2010).

RESULTS AND DISCUSSION

The RT-LAMP assay is recognized as a valuable method for the detection of various subtypes of animal and human influenza viruses owing to its rapidity, simplicity, high sensitivity and specificity (Chen *et al*., 2008; Imai *et al*., 2007; Ito *et al*., 2006; Poon *et al*., 2005). Recently, we developed a rapid and sensitive RT-LAMP Method for SIV detection using IAV matrix gene-specific primer sets (Kim *et al*., 2015). In this study, we reevaluated the utility of the assay by using additional animal reference IAVs and clinical samples obtained from different species including avian, equine, canine and human samples (Table 1).

Given the high mutation rate of AIV genes, it is difficult to design RT-LAMP primer setscapable of detecting all IAV subtypes from different species (Shivakoti *et al*., 2010; Webster *et al*., 1992; Yoshida *et al*., 2011). Six primer sets (F3, B3, LF, LB, FIP and BIP) that specifically target eight different regions that are highly conserved among all IAV subtypes were carefully designed by analyzing the IAV matrix gene deposited in the Influenza Sequence Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) during 2012-2014 (Kim *et al*., 2015). Using these primers, the RT-LAMP assay was capable of detecting all subtypes of AIVs, SIVs, EIVs, CIVs and human IAVs but not the human influenza B virus (Table 1), indicating that the assay was highly accurate and specific for all subtypes of different animal and human IAVs.

The RT-LAMP detection limit was 10^{-2}, 10^{-3}, 10^{-1} and 10^{-1}-$TCID_{50}$ for each tested AIV, SIV, EIV and CIV, respectively (Fig. 1) which was 10 fold higher than those observed from RT-PCR results and the same as those observed for RRT-PCR as predicted by previous reports (Kim *et al*., 2015). Sensitivity of RT-LAMP was comparable to those of previously reported RT-LAMP methods for SIV testing [~10-2 $TCID_{50}$ (Liu *et al*., 2012)]. Considering that the RRT-PCR detection limit using matrix gene-specific primers and probes was $10^{1.8}$ EID_{50} (Kim *et al*., 2013), the RT-LAMP detection limit was also estimated at approximately $10^{1.8}$ EID_{50} which was comparable to those of previously described RT-LAMP Methods for AIVs (Shivakoti *et al*., 2010; Yoshida *et al*., 2011).

RT-LAMP clinical performance was evaluated by using 589 field samples collected from different animal species. The RT-LAMP assay detected eight samples as IAV-positive which was consistent with RRT-PCR and virus isolation results and subtypes of IAV-positive cases were confirmed as H1, H3, H5, H6 or H9 subtype (Table 3). We note that the number of tested strains in this study was limited to some reference strains and field samples. Therefore, further validation using additional influenza

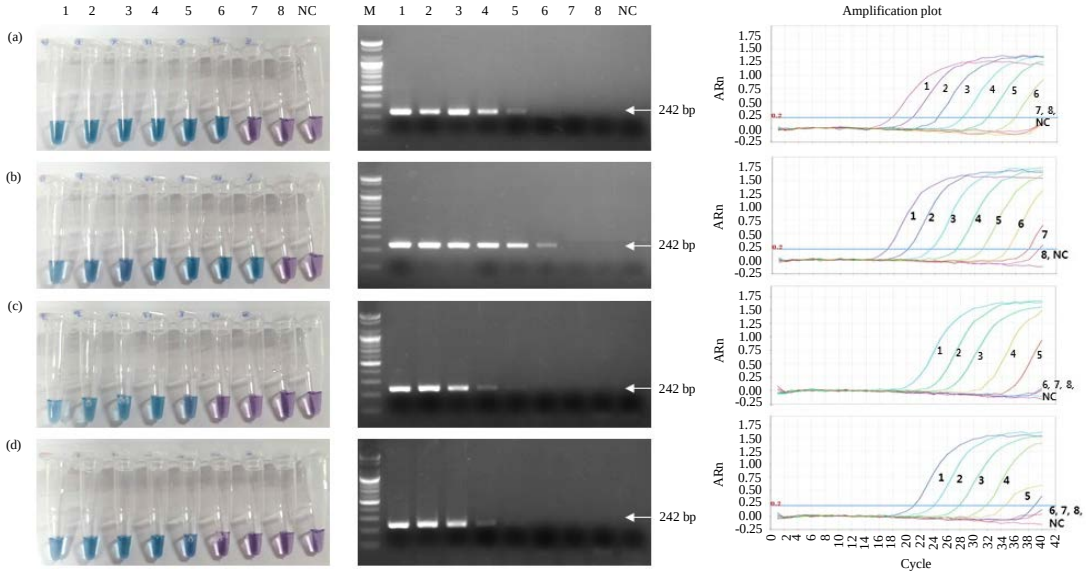

Fig. 1: Comparison of the detection limits of reverse transcription loop-mediated isothermal amplification assay, reverse transcription polymerase chain reaction and real-time reverse transcription polymerase chain reaction. a) AIV: A/duck/Hong Kong/820/80(H5N3; b) SIV: A/Korea/103/2009(H1N2); c) EIV: A/equi/Miami/1/63(H3N8); d) CIV: A/canine/Korea/BD-1/2013(H3N2). Lane M: 100 bp DNA marker; lane NC: Negative Control; lanes 1-8: 10 fold dilutions of each virus with initial viral titers of $10^{3.0}$ $TCID_50$/0.1 mL

isolates and clinical samples is needed to better define the usefulness of the assay. Additionally, continuous surveillance and genetic characterization of animal IAVs are required to guarantee the usefulness of primers for the RT-LAMP assay.

Previously, Shivakoti *et al.* (2010) and Yoshida *et al.* (2011) described the RT-LAMP assay using matrix gene-targeted primers for detection of all AIV subtypes. In those studies, viral RNA amplification was determined by turbidity detection system (Yoshida *et al.*, 2011) or fluorescence detection system (Shivakoti *et al.*, 2010). Although, the turbidity and fluorescence detection systems allow visual detection of results and are considered simple and cost-efficient methods for judging a positive or negative LAMP reaction, specific skills are required to accurately assessthe results obtained using these systems. Therefore, their use offers a potential risk for detection errors when the amount of amplified LAMP products is relatively small, requiring additional detection instruments such as a turbidometer or UV transilluminator, to provide more accurate results. Recently, a simple and more sensitive colorimetric assay was applied for visual detection of the LAMP reaction by adding metal indicators to the pre-reaction solution which reduced the chance of carryover contamination by the LAMP product (Goto *et al.*, 2009; Dhama *et al.*, 2014). In this study, we were able to visually detect RT-LAMP results by using HNB (Fig. 1) without any additional detection process. Considering these results, the RT-LAMP assay reevaluated in this study has been confirmed as a suitable method for screening different animal IAVs, especially in under-equipped laboratories or as an on-site rapid diagnostic tool.

CONCLUSION

Here, we evaluated our previously reported RT-LAMP assay for the detection of animal IAVs including major subtypes of avian, swine, canine and equine influenza viruses. The RT-LAMP assay was able to visually detect all subtypes of animal and human IAVs tested with high specificity and sensitivity. The RT-LAMP assay is applicable for rapid, user-friendly and reliable detection of IAVs in animal populations to screen for and control IAV infection and outbreaks, even in under-equipped laboratories.

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