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Original Article

Development of One-Step Reverse Transcription Loop-Mediated Isothermal Amplification (LAMP) as a Screening tool for Influenza A Viruses

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Abstract

Influenza A virus is a major cause of influenza pandemics and can infect several host species including humans and animals. The objective of this study was to develop a one-step reverse transcription loop-mediated isothermal amplification assay (LAMP) for the detection of genetically diverse influenza A viruses from both human and animal hosts. First, a set of two inner and two outer primers were designed based on the conserved region of the matrix (M) gene of influenza A viruses. The amplification reaction was optimized at 63°C for 60 min and performed in a simple heat block. The amplicons could be visualized either by gel electrophoresis or by visual analysis upon addition of SybrGreen. The developed LAMP assay was tested with 50 influenza A isolates including H1N1, H1N2, H3N2, H5N1 and H7N4 from swine, avian and human hosts. In sensitivity test, the assay detection capability was ten times more sensitive than conventional RT-PCR and comparable to real time RT-PCR. In summary, this assay is a rapid, simple and sensitive assay suitable for less-equipped laboratories and thus can be utilized in the field as a screening test.

Keywords: Influenza A, reverse transcription loop-mediated isothermal amplification

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Introduction

Influenza viruses are segmented negative-sense single stranded RNA viruses that belong to the family *Orthomyxoviridae*. Influenza viruses can be divided into types A, B or C. Influenza A virus can infect different animal hosts, including domestic birds, and wild birds, domestic mammals and wild animals as well as humans. Type A influenza viruses can be divided into different subtypes according to surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Webster et al., 1992).

Detection of influenza A viruses has been accomplished by using various polymerase chain reaction (PCR)-based tests such as reverse transcription-PCR (RT-PCR) (Ellis and Zambon, 2002), multiplex RT-PCR assay (Wu et al., 2008), real-time RT-PCR (Playford and Dwyer, 2002), nucleic acid sequence-based amplification (NASBA) (Collins et al., 2003), mismatch amplification mutation assay (MAMA) PCR (Hata et al., 2007) and DNA or RNA microarray (Dawson et al., 2007). However, these methods require expensive equipment and reagents. These prerequisites may not be readily available, particularly in laboratories in developing and underdeveloped countries or in frontline laboratories. In contrast to other molecular techniques, loop-mediated isothermal amplification (LAMP) is a novel technique based on nucleic acid amplification under isothermal conditions. It is a rapid and efficient technique (Notomi et al., 2000).

The principle of reverse transcription (RT)-LAMP is an isothermal reaction forming a stem-loop structure so that a target gene can be amplified with high specificity and sensitivity (Imai et al., 2006; Li et al., 2009). The technique does not require expensive equipment such as thermal cycler. Amplified products can be analyzed by agarose gel electrophoresis or a colorimetric method, involving a visible color change of fluorescent dye added to the amplified product (Notomi et al., 2000). It has been successfully applied to detection of numerous viruses such as West Nile virus (Parida et al., 2004), Viral Hemorrhagic Septicemia virus (VHS) (Soliman and El-Matbouli, 2006), Spring viremia of carp virus (Liu et al., 2008), Respiratory Syncytial Virus (RSV) (Ushio et al., 2005), H3 swine influenza virus (Gu et al., 2009), Porcine Reproductive and Respiratory Syndrome virus (PRRSV) (Chen et al., 2010), Infectious Bursal Diseases virus (Xue et al., 2009), Hepatitis E virus (Lan et al., 2009) and Newcastle Disease virus (Chen et al., 2008). Moreover, RT-LAMP has been used for the detection of influenza A viruses, including H5, H7 and H9 subtypes (Poon et al., 2005; Imai et al., 2006; Chen et al., 2008). In this study, we developed a one-step RT-LAMP as a screening assay to detect influenza A viruses from both animals and humans. The detection limit of the developed assay was evaluated with that of conventional RT-PCR and real time RT-PCR.

Materials and Methods

Influenza A virus isolates: Fifty isolates of influenza A virus (H1, H3, H5, H7) were included for analysis in this study. Seventeen avian influenza isolates including H3N2, H5N1, H7N4 and eleven swine influenza isolates including H1N1, H1N2, H3N2 were obtained from Veterinary Diagnosis Laboratory (VDL), Chulalongkorn University, and twenty-two human influenza isolates (seasonal H1N1 and H3N2, pandemic H1N1 2009) were kindly provided by the Center of Excellence for Clinical Virology (CECV), Chulalongkorn University, Bangkok, Thailand. All viruses were isolated by standard viral isolation method (Cottey et al., 2001; Woolcock, 2008) and confirmed by real time RT-PCR based on the matrix gene (Spackman and Suarez, 2008). An avian influenza isolate [A/chicken/Thailand/CU-K2/2004 (H5N1); CU-K2] was used as a reference strain in order to optimize the assay. A list of the influenza A viruses used in the study is shown in Table 1.

Table 1 Influenza A isolates from different hosts used in this study

Host	Subtypes	Number of isolates
Chicken	H5N1	14
Duck	H3N2	1
	H5N1	1
	H7N4	1
Swine	H1N1	3
	H1N2	6
	H3N2	2
Human	H1N1 (seasonal)	3
	H3N2 (seasonal)	3
	H1N1 (pandemic 2009)	16
Total		**50**

RT-LAMP primers and assay optimization: To design the RT-LAMP primers, the matrix (M) gene sequences of Influenza A viruses from both human and animal hosts originating from different geographical locations were obtained from the GenBank database. All nucleotide sequences were aligned by the computer program MEGA 4.0 and conserved regions for each group were determined. One set of primers from each group, with each set having four specific primers including two inner primers and two outer primers, was constructed. The set of primers was designed manually in accordance with the standard parameters of the primer explorer LAMP primer design software (Primer Explorer V4, Fujitsu limited, Japan) to recognize the conserved region of six distinct genomic sequences of the M gene of influenza A virus.

To optimize the RT-LAMP assay, viral RNA was extracted from known titers allantoic fluid of viral stock using QIA amp viral RNA mini kit (Qiagen GmBH, Hilden, Germany) in accordance with the manufacturer's recommendation. Viral RNA concentration was determined with an ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The stock RNA sample of the CU-K2 isolate was serially diluted ten-fold. RT-LAMP was carried out in 50 ul reaction mixture comprising 1x ThermoPol buffer, 0.4mM dNTPs, 8mM of $MgSO_4$, 0.2 μM each of forward outer primer (F3) and reverse outer primer (B3), 1.6 μM of forward inner primer (FIP) and reverse inner primer (BIP), 0.04 M betaine, 5U cloned AMV reverse transcriptase, 8U *Bst* DNA polymerase and 2μl of template RNA. Normal saline is used as negative

control used for assay optimization in this study. Reaction temperature (60, 63, and 65°C) was optimized in a heating block for 60 min (predetermined time) and at 80°C for 10 min to terminate the reaction. The reaction time (30, 40, 50, 60 and 70 min) was optimized (predetermined temperature 63°C). The optimization concentrations of MgSo4 (2, 4, 6, and 8 mM) and *Bst* DNA polymerase (0.25, 0.5, 0.75, and 1 Unit) were evaluated and the predetermined minimum requirement for LAMP amplification are 6 mM of MgSO4 and 0.75U of *Bst* DNA polymerase.

Sensitivity and specificity of RT-LAMP assay: The stock CU-K2 virus was titrated by inoculation into influenza A-specific antibody negative (SAN) embryonated eggs and the median embryo infectious dose (EID50/ml) was calculated according to Reed and Muench formula. Ten-fold serial dilution of the reference influenza A strain (CU-K2) was prepared and RNA extraction from each dilution was conducted. Detection limit of RT-LAMP for influenza A virus detection was performed by testing ten-fold serial dilution of the reference influenza A strain (CU-K2). The detection limit of the one-step RT-LAMP assay was also compared with that of the conventional RT-PCR assay and real time RT-PCR for detecting influenza A virus using the same ten-fold serial dilutions of RNA templates. Both RT-PCR and real time RT-PCR were performed using one-step RT-PCR kit (Qiagen GmBH, Hilden, Germany) and QuantiFast Probe RT-PCR kit (Qiagen GmBH, Hilden, Germany). The primers and hybridization probe used in this study are those recommended by the World Health Organization (Spackman and Suarez, 2008). Spiked reference influenza A strain (CU-K2) into cloacal and tracheal swabs and excess fecal samples with VTM was prepared and extracted for viral RNA. Normal saline was used as negative control for spike sample test.

Results

RT-LAMP primers and assay optimization: In this study, the set of primers for RT-LAMP was designed to recognize the conserved region of six distinct genomic sequences of the M gene of influenza A virus. The primer sequences and locations are shown in Figure 1 and Table 2.

To optimize the RT-LAMP assay, the avian influenza isolate [A/chicken/Thailand/CU-K2/2004 (H5N1); CU-K2] was used as the reference strain. Result showed that the RT-LAMP assay for influenza A virus detection could be activated at all three temperatures (60, 63 and 65°C) (Fig 2A). After 60 min an RT-LAMP product could be visualized upon gel electrophoresis as a ladder-like formation (Fig 2B). For visual analysis, addition of 1µl of SYBR Green I (Invitrogen, USA) to the amplified LAMP product facilitated result interpretation as green coloration indicates positive results, while orange coloration indicates negative results (Fig 3).

Sensitivity and specificity of RT-LAMP assay: The detection limit of the RT-LAMP assay was first evaluated by testing ten-fold serial dilution of the reference influenza A strain (CU-K2). Viral RNA was extracted from the ten-fold serially diluted virus samples using RNAse free water and RT-LAMP was

Table 2 Reverse transcription loop-mediated isothermal amplification (RT-LAMP) primer sets used in the study

Primer	Location	Sequences (5′-3′)
F3	33-51	TCTCTCTATCATCCC(R)TCA
B3	244-263	CCATTTCCATTTAGGGC(R)TT
FIP (F1c + F2)	68-127	TGAGAGCCTC(R)AGATCGGTG-AGATCGCGCAGA(R)ACTTG
BIP (B1c + B2)	160-263	CCTCTGACTAAAGGGATTTTGGGA-CTGGACAAAGCGTCTACG

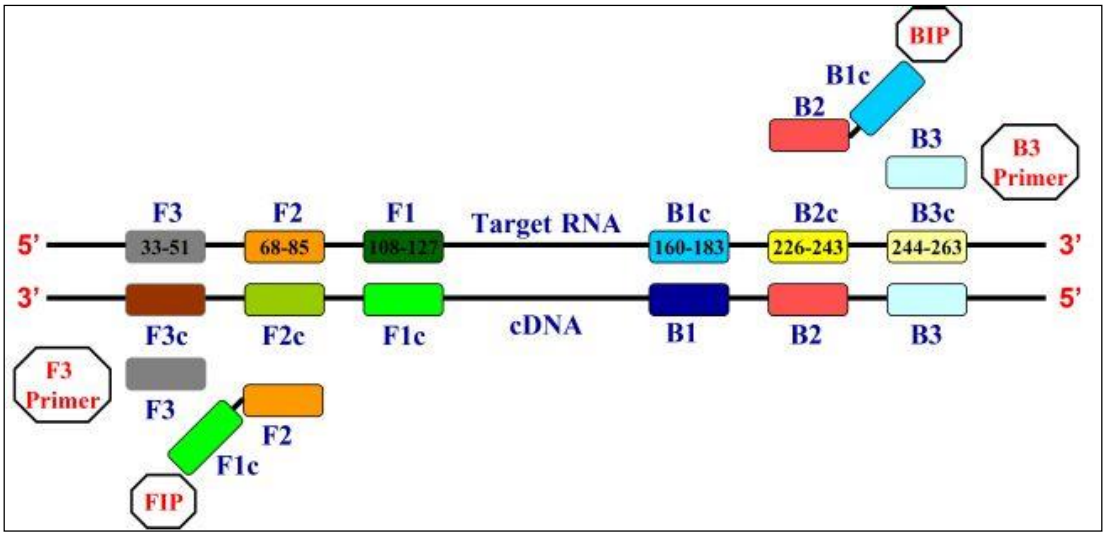

Figure 1 Design of RT-LAMP primers for influenza A virus detection. The figure shows locations of primers on target template

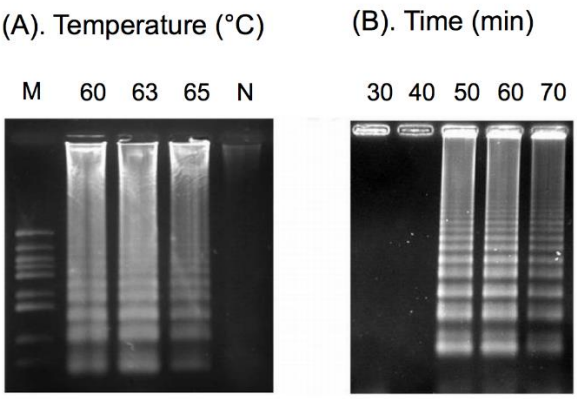

Figure 2 Effect of temperature and amplification time on LAMP amplification. (A) The effect of temperature (60, 63 and 65°C), M= marker, N= negative control. (B) The effect of reaction time (30, 40, 50, 60 and 70 min)

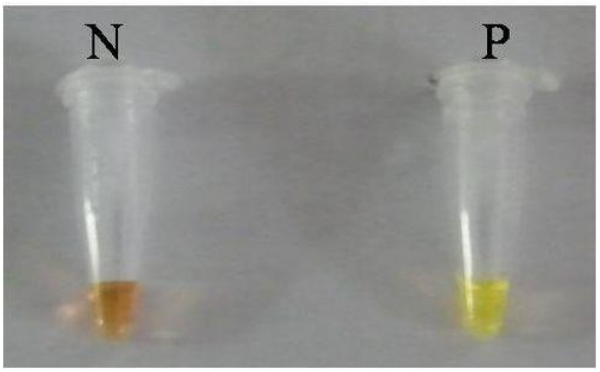

Figure 3 Visual detection of products from RT-LAMP reaction by adding SybrGreen I: P= positive (green color) and N= negative (orange color).

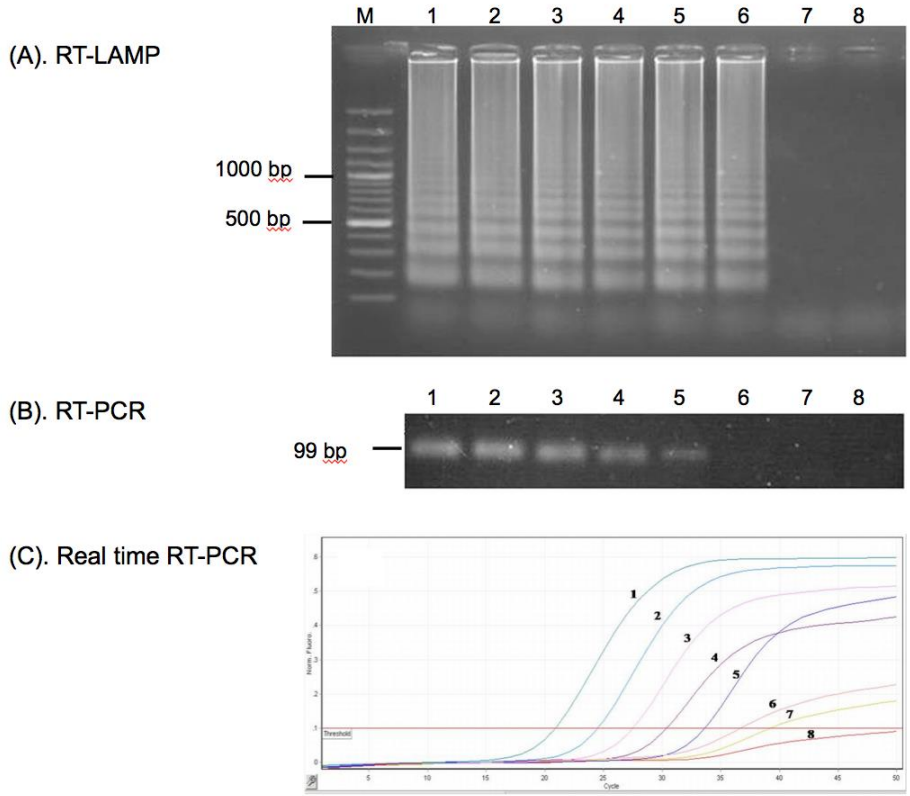

Figure 4 Comparison of detection capability between three molecular assays (one-step RT-LAMP, conventional RT-PCR and real time RT-PCR). Reactions were carried out with ten-fold serial dilution of RNA from reference influenza A virus (CU-K2). The same RNA concentrations per reaction were applied in every technique. (A) Detection limit of one-step RT-LAMP (upper panel) and conventional RT-PCR (lower panel). M: marker, lanes 1-7: 10^7 EID50 to 10^1 EID50 and lane 8: negative control. (B) Real Time PCR. Lines 1-7: 10^7 EID50 (Ct 21), 10^6 EID50 (Ct 24), 10^5 EID50 (Ct 27), 10^4 EID50 (Ct 30), 10^3 EID50 (Ct 33), 10^2 EID50 (Ct 36) and 10^1 EID50 (Ct 39) and Line 8: negative control

carried out with each virus dilution. Results showed that the assay could detect the viral RNA at concentrations as low as 1.7 pg of RNA per assay (approximately 10^2 EID50/ml), as determined by electrophoresis yielding LAMP-specific ladder-shaped bands. Then, we compared the detection limit of the one-step RT-LAMP assay with that of the conventional RT-PCR assay and real time RT-PCR for detecting influenza A virus using the same ten-fold serial dilutions of RNA templates. In this study, the detection limit of one-step RT-LAMP assay was approximately to 1.7 pg of RNA (approximately 10^2 EID50/ml), while the RT-PCR reaction and the real time RT-PCR reaction had a detection limit of 10^3 EID50 and 10^2 EID50, respectively. This result suggested that the sensitivity of the one-step RT-LAMP assay for detecting influenza

A virus was approximately ten times higher than that of the conventional RT-PCR and comparable to the real time RT-PCR (Fig 4).

The specificity of the RT-LAMP assay was determined by applying the assay with 50 isolates of different influenza A subtypes and other animal viruses commonly found in avian, swine and human hosts. The viruses used for testing the assay's specificity included porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), infectious bronchitis virus (IBV), Newcastle disease virus (NDV) and human influenza B virus. The assay showed positive results for all 50 influenza A strains, whereas the results were negative for all other viruses. Our result indicated that the RT-LAMP assay was highly specific and devoid of cross-reaction with the other RNA viruses. Furthermore, this developed RT-LAMP assay was able to detect diverse strains of influenza A virus from different host species.

To test the ability of RT-LAMP on swab samples, the virus positive allantoic fluid was spiked into viral transport media (VTM) with cloacal and tracheal swabs and fecal samples from pathogen free chickens. Viral RNA was extracted from all spiked samples and tested with the one-step RT-LAMP assay in duplication. All spiked samples could be successfully detected by our developed assay except for one of the duplicated virus spiked fecal samples (Fig 5). This indicates that fecal contamination in test samples may lower the test's capability and thus cloacal and tracheal swab samples are preferable over fecal samples.

Discussion

Influenza A virus is an important respiratory disease. To prevent and control the outbreak of influenza, rapid diagnosis is required. In general, the influenza outbreaks of specific subtypes (H5 and H7) are reported to World Health Organization (WHO) and World Animal Organization (OIE). However, in some developing countries, there are still limitations in applying the recommended molecular assay for influenza detection because the requirements of thermocycler and complicated methods of analysis. Therefore, this study intended to develop the one-step RT-LAMP assay to overcome this gap.

RT-LAMP is a very sensitive PCR based technique and can be applied without any special equipment. The primers in this study is designed based on the conserved M gene and can detect most of the influenza viruses from both human and animals including pandemic H1N1 2009. The RT-LAMP assay have been used for HA gene detection (Ito et al., 2006). However, HA gene is very diverse among influenza subtypes, therefore it may not be the suitable target for influenza A detection.

In this study, the effect of MgSO4 on the RT-LAMP reaction was examined. The amplicons could be detected by gel electrophoresis with a minimum of 4 mM of MgSO4 but the best result was showed at 6-8 mM of MgSO4. The effect of Bst DNA polymerase concentration on our developed assay and its compatibility with designed primers were tested. Our result showed that the RT-LAMP assay could work with the presence of a minimum of 0.75 Units of Bst DNA polymerase. The optimum concentration of Bst DNA polymerase was 1 unit, which agrees with the standard recommendation. From our finding, all three temperatures (60, 63 and 65^{o}C) tested provided positive amplicons and the optimum temperature for RT-LAMP amplification was 63^{o}C. This result agrees with another report done for shrimp viruses (Puthawibool et al., 2009). Our result also showed that the amplification time of 60 min provided enough quantity of amplicons for gel electrophoresis and visual detection similar to previous reports (Boldbaatar et al., 2009; Newhouse et al., 2009).

LAMP is a very sensitive technique in which high amount of amplicons can be obtained with the presence of ten copies of pathogen genome (Notomi et al., 2000). Our result indicated that the lower detection limit of RT-LAMP assay was 1.7 Pico grams of RNA (Ct 36.68) and 10 times more sensitive than conventional RT-PCR. However, some LAMP assays can be 100 to 1000 times more sensitive (Imai et al., 2006). To replace the use of gel electrophoresis for detecting amplicons, the colorimetric assay was used. The intercalating dye

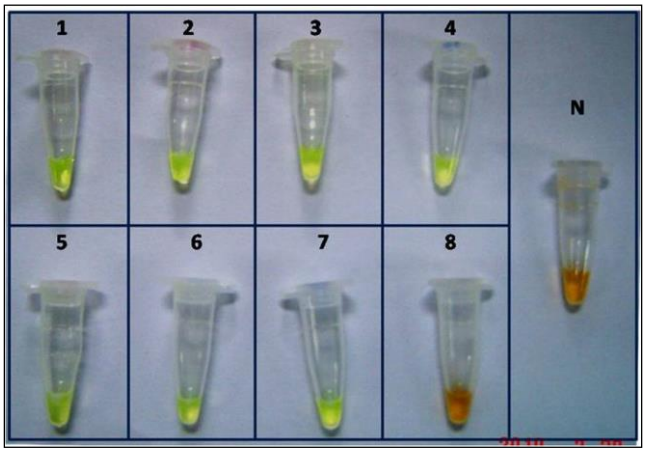

Figure 5 One-step RT-LAMP assay on virus spiked samples. 1 and 5: viral spiked in normal saline, 2 and 6: viral spiked in cloacal swab with VTM, 3 and 7: viral spiked in tracheal swab with VTM, 4 and 8: viral spiked in excess fecal samples with VTM, N: negative control

"SYBR Green I" could detect the amplicons by naked eyes and the sensitivity was similar to gel electrophoresis. This result indicated that it would be very suitable for the amplicon detection in field situation. The RT-LAMP assay in this study was very specific for influenza A and no false positive with other RNA viruses such as PRRSV, CSFV, NDV, IBV, and Influenza B viruses was found. In the spiked sample tests, we compared the ability of LAMP to detect the viruses from different sample sources such as cloacal and tracheal swabs. Most of the samples could be detected by the developed assay, however one viral spiked sample in the excess fecal samples could not be detected. It may be because of the interference of natural inhibitor in fecal material from RNA extraction process.

The advantage of one-step RT-LAMP is that the assay is simple, avoids cDNA synthesis and reduces RNA contamination. Since influenza A virus genome is highly diverse, the use of two primer pairs corresponding to M gene target sequences provides high specificity of the assay. LAMP has been proven to be more powerful than conventional PCR, and applied to samples with inhibitory substances such as blood and feces (Poon et al., 2006) and samples with no extraction step such as cell culture (Poon et al., 2006; Kaneko et al., 2007). This simplified procedure can be further used in a field situation.

In conclusion, a rapid, simple, sensitive and specific one-step RT-LAMP assay that can successfully detect various influenza A viruses from different animal species as well as humans was developed. Different types of samples including tracheal and cloacal swabs can be used; however, natural inhibitors in fecal samples can interfere with the sensitivity of the assay. Further studies to simplify the viral RNA extraction method may help enhance this assay for future use in the field. Overall, the one-step RT-LAMP method can be applied for screening of both human and animal influenza A viruses.

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บทคัดย่อ

การพัฒนาวิธี One-step reverse transcription loop-mediated isothermal amplification (LAMP) เพื่อใช้ตรวจคัดกรองไวรัสไข้หวัดใหญ่ชนิด A

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เชื้อไวรัสไข้หวัดใหญ่ชนิดเอเป็นสาเหตุของโรคไข้หวัดใหญ่ที่ระบาดทั่วโลก สัตว์หลายชนิดรวมทั้งคนสามารถติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอได้ วัตถุประสงค์ของการศึกษาครั้งนี้ เพื่อพัฒนาวิธี one-step reverse transcription loop-mediated isothermal amplification assay (LAMP) เพื่อตรวจหาเชื้อไวรัสไข้หวัดใหญ่ชนิดเอที่มีพันธุกรรมที่หลากหลาย ทั้งที่แยกได้จากคนและสัตว์ ขั้นตอนการทดลองประกอบด้วย การออกแบบ primers ที่จำเพาะกับบริเวณที่มีความคงตัวสูงของยีน matrix (M) (ภายใน 2 เส้นและภายนอก 2 เส้น) การปรับภาวะที่เหมาะสมของวิธี LAMP ได้แก่ การใช้อุณหภูมิที่ 63°C เวลา 60 นาที การอ่านผลโดยการผ่าน gel electrophoresis และการเปลี่ยนสีเมื่อเติม SybrGreen วิธี LAMP ที่พัฒนาขึ้นนี้ได้นำมาทดสอบกับเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ H1N1, H1N2, H3N2, H5N1 และ H7N4 จำนวน 50 ตัวอย่างที่แยกได้จากสุกร สัตว์ปีก และคน จากการทดสอบพบว่าวิธี LAMP มีความไวมากกว่าวิธี RT-PCR ปกติ 10 เท่า แต่มีความไวเทียบเท่ากับวิธี real time RT-PCR โดยสรุปวิธี LAMP นี้เป็นวิธีที่ง่าย รวดเร็ว และมีความไวสูง เหมาะสำหรับนำไปใช้ในห้องปฏิบัติการที่ไม่ต้องการเครื่องมือ และสามารถนำไปใช้ในภาคสนามเพื่อตรวจคัดกรองโรคต่อไป

คำสำคัญ: ไข้หวัดใหญ่ชนิดเอ ปฏิกริยาลูกโซ่พอลิเมอเรสแบบย้อนกลับชนิดแลมป์

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