Original Article

Reverse transcription loop-mediated isothermal amplification combined with lateral flow device (RT-LAMP-LFD) for swine influenza virus detection

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Abstract

Swine influenza virus (SIV) causes respiratory disease in pigs leading to economic losses in swine production. Some SIV subtypes can also infect human and considered as a zoonotic pathogen. The importants of swine influenza are not only impact on the economic losses but also the human health. The objectives of this study were to develop a reverse transcription loop-mediated isothermal amplification combined with lateral flow device assay (RT-LAMP-LFD) for the detection of SIVs and to evaluate the performance, sensitivity and specificity of the assay. Our result showed that RT-LAMP-LFD assay was developed with six newly designed primers and with optimum condition at 63^{o}C and 30 minutes. The detection limit of the assay was 7.8 pg/µl, which is comparable to real time RT-PCR assay. RT-LAMP-LFD assay is a specific method for the detection of SIV, in which there were no cross reaction with other important viruses of pigs (PRRSV, PCV2, PRV and PEDV). When evaluation with reference SIVs, field SIVs and other viruses (n=59), the newly developed RT-LAMP-LFD assay has good performance with 100% sensitivity, 100% specificity and perfect percentage of agreement (Kappa=1) compared to reference assays. In conclusion, this RT-LAMP-LFD assay has a potential for a rapid diagnostic test for SIV detection and could be developed for low-cost or in house assay. The RT-LAMP-LFD assay could be implemented and applied at first point of care of disease outbreaks to help prevent and control the spread of SIV in pig farms.

Keywords: Detection, LFD, Performance, RT-LAMP, Swine Influenza

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Received October 5, 2020.

Accepted December 22, 2020.

doi: 10.14456/tjvm.2021.9

Introduction

Swine influenza virus (SIV) or swine-origin influenza virus (S-OIV) causes endemic respiratory disease in pigs. SIV is an influenza A virus of the family *Orthomyxoviridae*. There are three main SIV subtypes circulating in pigs worldwide including SIV-H1N1, H3N2 and H1N2. SIV infection in pigs causes high morbidity rate (probable 100%), while mortality rate is usually low. Clinical signs of SIV infection are mild respiratory problems, which lead to weight loss in pigs causing a delayed time to reach the market weight. These make SIV infection as one of the important hidden causes of economic losses in pig farms. There is a major concern about risk of SIV for human health. Due to pigs play an important role as "a mixing vessel" of influenza viruses, pigs can support infection and replication of influenza viruses of avian, human and swine origins (Brown *et al*., 1997; Campitelli *et al*., 1997; Qi and Lu, 2006; Webby *et al*., 2000). Several studies showed swine-origin influenza virus (S-OIV) seropositivity in humans especially those who have close contact with pigs (Myers *et al*., 2007; Myers *et al*., 2006; Olsen *et al*., 2002).

There are several methods for influenza A virus detection such as reverse transcription-PCR (RT-PCR), real time RT-PCR, multiplex RT-PCR assay, nucleic acid sequence-based amplification (NASBA), mismatch amplification mutation assay (MAMA), and DNA/RNA microarray. However, those methods require expensive equipment and skilled technicians, thus they do not suitable in the field-setting or in laboratories of developing countries (Ge *et al*., 2013). Another disadvantage of PCR is a false positive due to DNA/RNA contamination during PCR procedure. Thus, the rapid and accurate assay and adaptable for field application for the detection of influenza A virus especially swine influenza is necessary.

Loop-mediated isothermal amplification (LAMP) has been widely used and employed in isothermal amplification research. LAMP is a highly specific and sensitive method operated under constant temperature between 60 – 65^{o} C, within 30-45 minutes. It is an efficient technique and can be process with less equipment (only water bath). Combined with reverse transcription reaction (RT), RT-LAMP assay has been used for detection of several influenza subtypes such as H5, H7, H9 and H10 (Chen *et al*., 2008; Imai *et al*., 2006; Luo *et al*., 2015; Poon *et al*., 2005; Zhang *et al*., 2013). The interpretation of RT-LAMP assay can be performed in many ways, by measure the turbidity, by gel electrophoresis, by visual detection of fluorescence from the integration with SYBR Green intercalating dye or by lateral flow device (Chen *et al*., 2010; Ge *et al*., 2013; Mori *et al*., 2004). Lateral flow device (LFD) is one of the rapid and easy to interpret assays. Briefly, LFD dipstick can detect biotinylated LAMP-amplicons which hybridized with fluorescent amidite (FAM)-labeled DNA probe. Hybridized LAMP product and gold-labeled anti FAM antibody on conjugated pad of the LFD can form complexes as signal or color on test line. Non-target products could not form complexes, therefore signal or color could not be observed (Mallepaddi *et al*., 2018; Yongkiettrakul *et al*., 2014). Several studies have proven the combination of LAMP assay with LFD to be easy and field-capable (Ge *et al*., 2013; Khunthong *et al*., 2013; Yongkiettrakul *et al*., 2014). In this study, we developed RT-LAMP-LFD assay for rapid and sensitive detection of swine influenza virus. The newly developed RT-LAMP-LFD assay was evaluated for the sensitivity, specificity and performance for swine influenza virus detection. This developed RT-LAMP-LFD assay is a simple rapid assay and suitable for the use in-farm setting or field application in the future.

Materials and Methods

Development of RT-LAMP-LFD assay for the detection of swine influenza viruses

Reference swine influenza virus subtype H1N1 (SIV-H1N1): The reference SIV-H1N1 virus used in this study was A/swine/Thailand/CU-CB1/2006 (SIV-06CB1). The SIV-06CB1 is an endemic swine influenza virus subtype H1N1, which whole genome seqeunces of the virus was submitted to the GenBank database (accession number HM142752). The virus titer from cell culture in this study was 10^4 TCID50/ml and RNA concentration used in the study was 78.4 ng/ul. The virus was acquired from the culture collection of the Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals (CU-EIDAs) at the Faculty of Veterinary Science, Chulalongkorn University and has previously identified and confirmed as SIV-H1N1 by using viral isolation (egg inoculation), real time RT-PCR and whole genome seqeuncing. This study was condcuted under the approval protocol of Chulalongkorn University's insititute biosafety committee (IBC#1831065) and instittute of animal care and usage (IACUC#1831104).

Development of an RT-LAMP-LFD assay

LAMP primer design for swine influenza virus detection: In this study, 6 primers (F3, B3, FIP, BIP, loop F, and loop B) were designed for specific detection of swine influenza virus based on M gene. In brief, reference sequences of M gene of SIVs were obtained from the GenBank database to generate the consensus of M gene sequences suitable for primer design. All the nucleotide sequences were aligned by MegAlign program and conserved regions (approximately 200 base pairs) were determined to use as target for primer design. The LAMP primers consist of six specific primers including two internal primers (FIP and BIP), two outer primers (F3 and B3) and two loop primers (loop F and loop B) were designed. The primer design was performed by using PrimerExplorer V4 program a LAMP primer designing software (Fujitsu limited, Japan). The primer design was performed with criteria as following: the Tm of primers were about 57^{o}C (55 - 59^{o}C) for F3 and B3, about 63^{o}C (60 – 65^{o}C) for loop F and loop B primers, about 80^{o}C (78 - 82^{o} C) for FIP and BIP primers. The free energy (ΔG) was -4 kcal/mol or less and the GC content was between 40 – 60%.

Optimization of RT-LAMP protocol for swine influenza virus detection: The RT-LAMP assay was performed in a total of 50 µl of reaction mixture containing 10 µM each of the internal primers (FIP and

BIP), 10 µM each of the outer primers (F3 and B3), 25 µM each of loop primers (Loop F and Loop B), 10X ThermoPol buffer (NEB, MA, USA), 0.4 mM dNTPs, 8 mM $MgSO_4$, 5M Betaine, 8U *Bst* DNA Polymerase (MERCK, Dramstadt, Germany), 10X AMV buffer, 10U AMV reverse transcriptase (MERCK, Dramstadt, Germany), 4 µl of template RNA and distilled water upto 50 µl. Optimization of RT-LAMP protocol was conducted by variation of temperature and time. Temperature for RT-LAMP was conducted with three different values (60°C, 63°C, and 65°C) for 45 minutes and inactivates reaction at 80°C for 5 minutes. After the optimal temperature was obtained, time variation for RT-LAMP was conducted with 30, 40, 50, 60, 70 minutes, respectively. The visualization was done by gel electrophoresis with Redsafe™ stain. The optimized temperature and time was used throughout the study.

Optimization of RT-LAMP combined with LFD protocol for swine influenza virus detection: In order to combine RT-LAMP with LFD, primers loop F and loop B were tagged with biotin and fluorescein amidite (FAM) at 5'end, respectively. With this labeled loop F and B, the LAMP products could be detected by lateral flow device (LFD). The LFD is commercially available acquired from Milenia® Genline, Germany. Briefly, the amplified product (8 µl) was mixed with 100 µl of HybriDetect Assay Buffer, per company recommendations. Then the dipstick with the sample application area is placed into the solution and incubated for 5-10 minutes. At the end of incubation period, the dipstick could be removed from the assay solution and interpret the results immediately. For LFD interpretation positive result was formed two lines (control line and test line) and negative result was shown only one line (control line).

Evaluation of the sensitivity, specificity and performance of RT-LAMP-LFD assay

Evaluation of sensitivity and specificity of RT-LAMP-LFD assay: Analytical sensitivity or another term "the detection limit" of RT-LAMP-LFD assay was assessed by using ten-fold serial dilution of reference SIV-H1N1 (SIV-O6CB1). The viral RNA was diluted using RNAase free water. RT-LAMP-LFD assay was performed in each serial dilution in tripication. The result was interpreted as the strength of RT-LAMP-LFD assay to detect SIV. The sensitivity of RT-LAMP-LFD assay was compared with RT-LAMP with gel eletrophoresis and realtime RT-PCR. The specificity of RT-LAMP-LFD assay was assessed against other important swine viruses (PRRSV, PEDV, PCV2 and Pseudorabies virus). For specificity test, the RT-LAMP-LFD assay was evaluated by testing RNA or DNA of each virus. The result was interpreted as the specificity of RT-LAMP-LFD assay to differentiate SIV from other swine viruses.

Evaluation of performance of RT-LAMP-LFD assay: To evaluate the performance of RT-LAMP-LFD assay or another term 'diagnostic sensitivity and specificity", the result of RT-LAMP-LFD was compared to other standard assays including RT-LAMP with gel eletrophoresis and realtime RT-PCR assay. In this study, 59 samples of different types of viruses, (SIV (n=30), PRRSV (n=8), PEDV (n=20) and Pseudorabies virus (n=1) were randomly blinded to the researchers. All samples have previously identified by viral isolation (egg inoculation) and/or real time RT-PCR and coventional PCR. The samples were then tested with RT-LAMP-LFD, RT-LAMP with gel eletrophoresis and realtime RT-PCR assay, to assess the performance of both assays. The results were un-blinded and compared by two-by-two table with standard test. The diagnostic sensitivity and specificity were calculated following the previous study (Ahn *et al.*, 2019). The agreement of the RT-LAMP-LFD assay with standard was assessed by using Kappa statsitic (McHugh, 2012).

Results

Development of RT-LAMP-LFD assay for the detection of swine influenza viruses: In this study, the RT-LAMP-LFD assay was developed by designing the primers and optimizing the protocol. Six primers were designed by using PrimerExplorer V4 program. A set of primers has been designed based on the specific target (M gene) of influenza virus. There were six primers including two internal primers (FIP and BIP), two outer primers (F3 and B3) and two loop primers (Loop F and loop B). In order to combine RT-LAMP with LFD, primers loop F was tagged with biotin and loop B was tagged with fluorescein amidite (FAM) at 5'end. The locations of each primer on the target sequence are shown in Table 1 and Figure 1.

Table 1 Properties and nucleotide sequence of primers for the LAMP-LFD assay in this study

Primer	Position* (bp)	Length (mer)	Tm (°C)	GC content (%)	Sequence
F3	358 - 375	18	58.4	61.11	5'-GGCCAAGGAGGTGTCACT-3'
B3	530 - 549	20	56.4	45	5'-TGCCTGATTAGTGGATTGGT-3'
FIP:		42	79.9	50	
F1c	431 - 452	22			5'-CAGCTTCTGTGGTCACTGTTCC-3'
F2	380 - 399	20			5'-TATTCAACTGGTGCACTTGC-3'
BIP:		42	80.9	52.38	
B1c	458 - 479	22			5'-GGTCTAGTGTGTGCCACTTGTG-3'
B2	510 - 529	20			5'-GGTAGTAGCCATCTGTCTGT-3'
Loop F	400 - 421	22	62.1	50	5'-Biotin-GTATATGAGGCCCATGCAACTG-3'
Loop B	489 - 509	23	62.9	47.83	5'-FAM-ATTGCTGATTCACAGCATCGGTC-3'

* nucleotide position of consensus sequence of M gene (KJ162040).

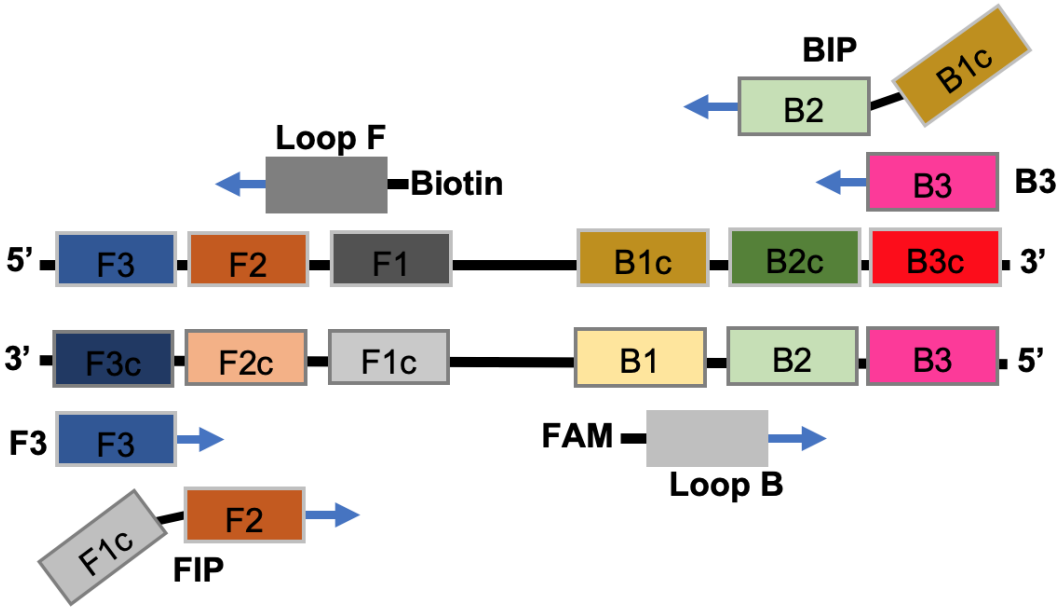


Figure 1 Schematic presentation of position of LAMP primers designed for specfic detection of M gene of SIV in this study

In this study, RT-LAMP protocol was assessed for optimum temperature and time for the detection of SIV. Our result showed that RT-LAMP products can be visualized in all range of temperature 60° C, 63° C, and 65° C (Figure 2A). In this study, temperature of 63°C was chosen for optimized RT-LAMP protocol. With the optimum temperature, the variations of time 30, 40, 50, 60 and 70 minutes were evaluated. Our result showed that RT-LAMP product can be visualized within all range of time variation (Figure 2B). In this study, at temperature of 63°C for 30 minutes was chosen for optimized RT-LAMP protocol and throughout the study due to this condition provide best visulalized LAMP products. Optimization of RT-LAMP with LFD protocol was performed with the optimized RT-LAMP condition at 63°C for 30 minutes. Our result showed that RT-LAMP detection by LFD visualization was well performed, which positive RT-LAMP result showed 2 lines in both control and test lines, while negative RT-LAMP result showed only one control line (Figure 2C).

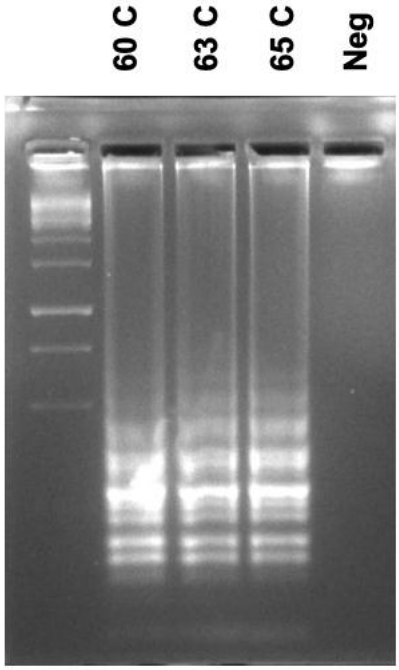
Evaluation of the sensitivity, specificity and performance of developed RT-LAMP-LFD assay: RT-LAMP-LFD assay was evaluated for the sensitivity, specificity and performance of the assay. In this study, RT-LAMP-LFD assay has an analytical sensitivity (minimum detection limit) at $78x10^{-4}$ ng/µl (7.8 pg/µl) or 1.0 TCID50/ml. Our results showed that RT-LAMP-LFD assay have comparable analytical sensitivity with realtime RT-PCR and RT-LAMP by using agarose gel electrophoresis (Table 2 and Figure 3). For specificity test, RT-LAMP-LFD assay has high specificity which no cross-reaction with other important viruses in pigs (PRRSV, PCV2, PEDV and PRV).

Performance of RT-LAMP-LFD was evaluated with 59 samples of randomly blinded RNA/DNA of reference and field viruses (SIV, PRRSV, PEDV and PRV). Performance assessment of RT-LAMP-LFD assay was described as diagnostic sensitivity, diagnostic specificity and the measure of agreement (Kappa statistic). The RT-LAMP-LFD assay were assessed with blinded samples of RNA/DNA viruses (n=59) comparing with RT-LAMP by agarose gel electrophoresis and realtime RT-PCR (Table 3). The measurement of agreement (Kappa statistic) of RT-LAMP-LFD compared with reference assays by two-by-two table for diagnostic sensitivity and diagnostic specificity were calculated (Table 4). Our result showed that RT-LAMP-LFD assay have a good performance with 100% sensitivity, 100% specificity and perfect percentage of agreement (Kappa = 1).

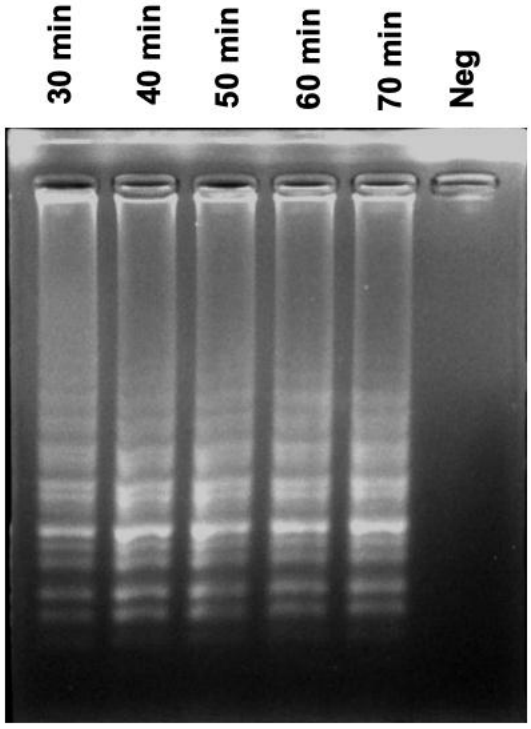
Discussion

Swine influenza in pigs causes high morbidity and low mortality. The disease spreads rapidly and causes the delay of pig weight to reach market leading to economic losses in swine production. Pigs play important role as "mixing vessel" of influenza viruses. Pigs can support infection and replication of influenza viruses from avian, human and swine origins. Some SIV subtypes can infect human and considered as a zoonotic pathogen (Brown *et al.*, 1997; Campitelli *et al.*, 1997; Qi and Lu, 2006; Webby *et al.*, 2000). These lead to major concerns of the risk of swine influenza which are not only impact on the economic lossess but also the human health. There are three main SIV subtypes co-circulating in pigs including H1N1, H1N2, H3N2 with variety of their reassortants. With high density of pigs population in Thailand, gives more opportunity of SIVs infection and subsequently genetic reassortment and the risk of inter-species transmission. Previous studies had reported the reassortment between endemic Thai SIVs with pandemic H1N1 2009 (Hiromoto *et al.*, 2012; Nonthabenjawan *et al.*, 2015) and an outbreak of infection by pandemic H1N1 2009 in commercial pig farm which possibility transmitted from human to pig (Sreta *et al.*, 2010). Therefore, the need of accurate and rapid diagnosis for SIV is critical to minimize further spreading of the viruses in pig population and to reduce the risk of multiple infections of influenza viruses.

2A.



2B.



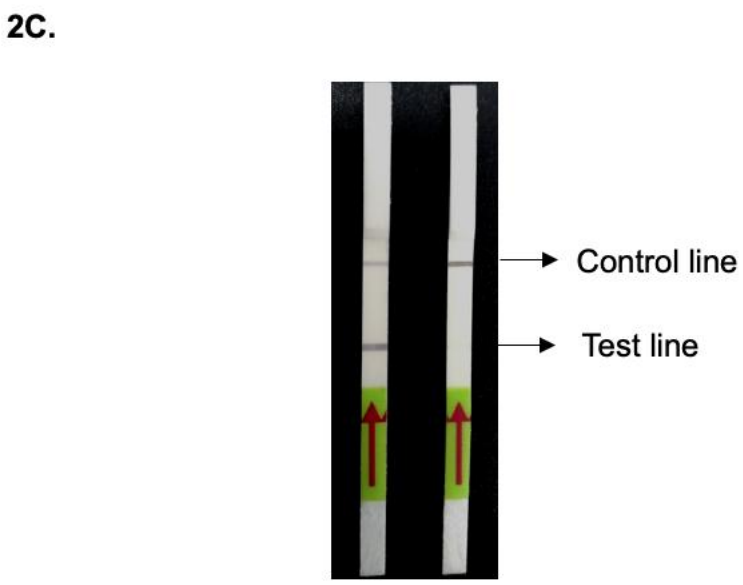


Figure 2 A) Effect of temperature in RT-LAMP assay. Lane 1: marker, lane 2: 60° C, lane 3: 63° C, lane 4: 65° C and lane 5: negative control (no template). B). Effect of time in RT-LAMP assay. Lane 1: 30 minutes, lane 2: 40 minutes, lane 3: 50 minutes, lane 4: 60 minutes, lane 5: 70 minutes and line 6: negative control (no template). C) RT-LAMP assay with LFD visualization. 1: positive SIV (2 lines at control line and test line), 2: negative SIV (1 line at control line).

Table 2 A). The analytical sensitivity (detection limit) of the RT-LAMP-LFD assay compared with real time RT-PCR.

Serial dilution	Assay			viral titer (TCID50/ml)	RNA concentration	
	RT-LAMP with agarose gel electrophoresis	RT-LAMP with LFD assay	Real time RT-PCR (Ct)*		ng/µl	pg/ µl
10^{-2}	+	+	+ (21.61)	100	0.78	780
10^{-3}	+	+	+ (27.85)	10	0.078	78
10^{-4}	+	+	+ (33.42)	1	0.0078	7.8
10^{-5}	-	-	suspected (38.57)	0.1	0.00078	0.78
10^{-6}	-	-	-	0.01	0.000078	0.078
10^{-7}	-	-	-	0.001	0.0000078	0.0078
10^{-8}	-	-	-	0.0001	0.00000078	0.00078
Negative control (no template)	-	-	N/A	N/A	N/A	N/A

* The interpretation of real time RT-PCR result was in Ct value, where value under 36 considered as positive, from 37 to 40 as suspected and above 40 as negative.

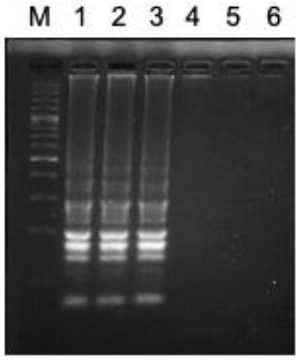
In this study, we have developed an RT-LAMP-LFD assay which is the combination of reverse transcription loop-mediated isothermal amplification (RT-LAMP) with lateral flow device (LFD) for SIV detection. The primers for RT-LAMP assay was newly designed based on M gene of SIV, the primers can be applied in RT-LAMP assay and make it possible to detect three common subtypes of SIVs (H1N1, H1N2, and H3N2). Previous studies have also been reported that RT-LAMP assay can be used for SIV detection such as: SIV-H3N2 (Gu *et al*., 2010), pandemic (H1N1) 2009 (Kubo *et al*., 2010), and swine origin influenza A H1N1 (Parida *et al*., 2008). In this study, six primers were used for RT-LAMP assay including two internal primers (FIP and BIP), two outer primers (F3 and B3) and two loop primers (loop F and loop B). Since the loop primers were used, the RT-LAMP reaction was enhancing for the speed and specificity (Nagamine *et al*., 2002). This speculation agreed with our result which the RT-LAMP reaction required only 30 minutes to process and provides suitable condition for RT-LAMP assay. It was noted that LAMP assay without loop primers (only two internal and two outer primers) requires longer time (60 minutes) (Notomi *et al*., 2000).

Unlike PCR, LAMP assay can be performed under isothermal temperature due to the use of DNA polymerase. In this study, we used *Bst* polymerase large fragment where the optimal temperature are between 60°C and 65°C (Notomi *et al*., 2000). Our developed RT-LAMP assay can work under all three temperatures (60°, 63° and 65°C). The temperature 63°C was selected as our optimum condition for RT-LAMP assay. RT-LAMP assay is a sensitive method in several studies. For example, RT-LAMP assay has either comparable detection limit with real time RT-PCR (Kubo *et al*., 2010) or 10-fold higher than real time RT-PCR (Parida *et al*., 2008). Some studies reported that the RT-LAMP assay has detection limit 100 fold higher than conventional RT-PCR (Chen *et al*., 2010). In this study, our developed RT-LAMP assay has comparable detection limit with real time RT-PCR when using agarose gel electrophoresis and LFD. In general,

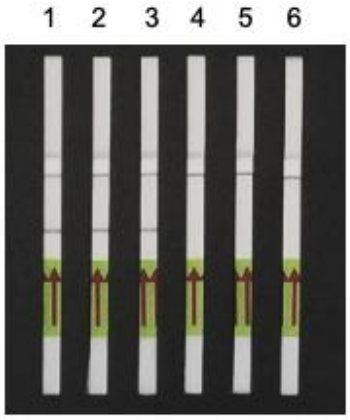
visualization of RT-LAMP by using agarose gel electrophoresis contains several steps from preparing gel, loading LAMP products into the wells, and then running gel in electrophoresis chamber. These steps provide opportunities of contamination and time consuming. On the other hands, by using LFD for LAMP visualization, the assay provides limit contamination and less time which only take 5-10 minutes. Thus, the time of overall process of RT-LAMP-LFD assay from RNA extraction until LFD visualization can be redueced and achieved within 40-45 minutes.

3A. Analytical sensitivity

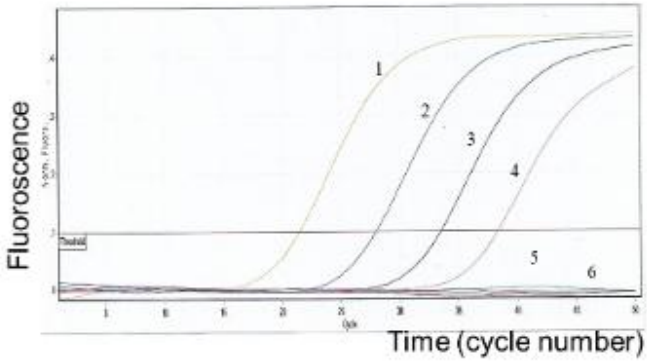
A. RT-LAMP-gel



B. RT-LAMP-LFD



C. RT-PCR



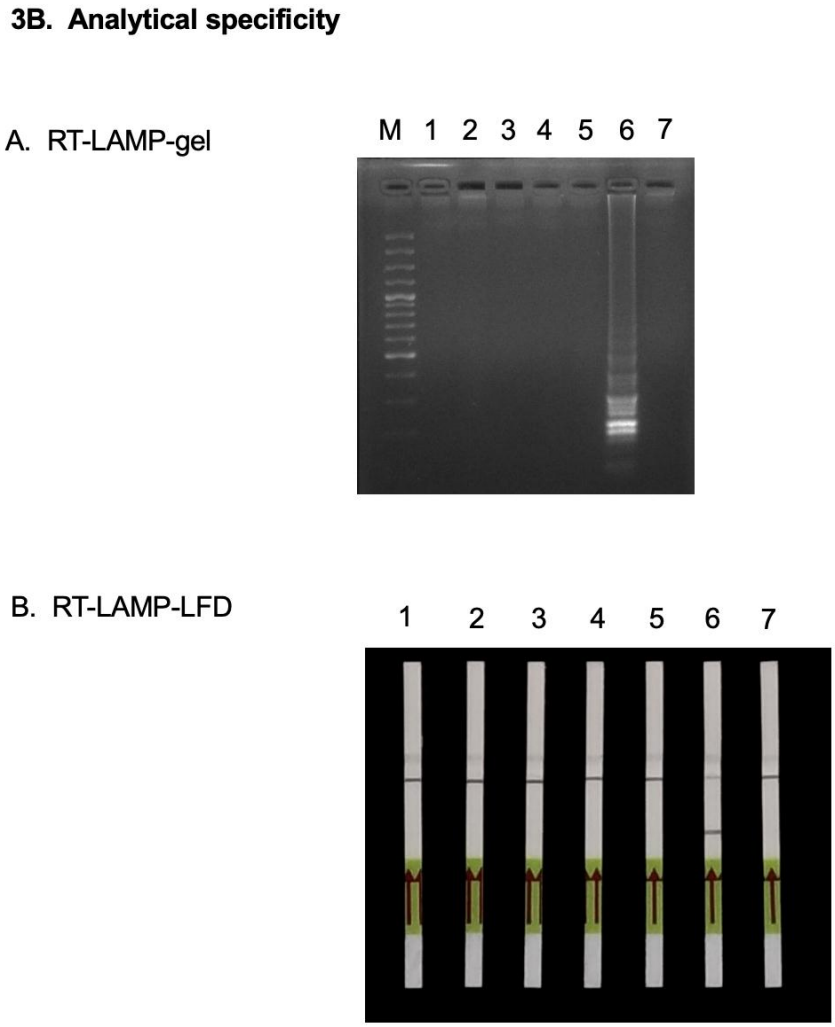


Figure 3 A). The analytical sensitivity (detection limit) of the RT-LAMP-LFD compared with RT-LAMP (gel) and real time RT-PCR. M: Marker, 1: SIV dilution 10^{-2}, 2: SIV dilution 10^{-3}, 3: SIV dilution 10^{-4}, 4: SIV dilution 10^{-5}, 5: SIV dilution 10^{-6}, 6: Negative control (no template). B). Specificity test by RT-LAMP assay: A. visualization by agarose gel electrophoresis, B. visualization by LFD. M: Marker, 1: Pseudorabies virus, 2: PCV2, 3: PRRSV EU, 4: PRRSV US, 5: PEDV, 6 : SIV, 7: Negative control (no template).

In previous reports, LAMP assay have been developed and used to detect other respiratory viruses in pigs such as PRRSV (Chen *et al*., 2010; Li *et al*., 2009), PCV2 (Zhao *et al*., 2011) and Pseudorabies virus (En *et al*., 2008). In this study, analytical specificity was conducted with assessed RT-LAMP-LFD assay against several important viruses in pigs including SIV, PRRSV, PCV2, PRV and PEDV. As result, RT-LAMP-LFD assay capable to detect SIV and there was no cross-reaction with other viruses. The six primers were designed to recognize eight distinct region of target gene, which expected to have high specificity. Moreover, the loop primers (loop F and B) that have been tagged with FAM and biotin, ensure that only targeted amplicons could show visible band in test band of LFD. To evaluate the performance of RT-LAMP-LFD assay, we conducted SIV detection by RT-LAMP-LFD with blinded samples. After blinded samples were decoded and compared with RT-LAMP-LFD result, the diagnostic sensitivity, diagnostic specificity and percentage of agreement by Kappa statistic were calculated. Our result showed that RT-LAMP-LFD assay provide 100% sensitivity, 100% specificity with Kappa = 1. This result indicated that the newly developed RT-LAMP-LFD has high sensitivity and specificity with perfect percentage of agreement. Similar performance of previous study where RT-LAMP-LFD assay have been developed for the detection of Influenza A (H7N9) virus with 100% sensitivity and 100% specificity (Ge *et al*., 2013).

With the combination of RT-LAMP with LFD, this assay is simple, rapid and low cost with less time than PCR based method (conventional PCR). The time of overall process of RT-LAMP-LFD assay from RNA until LFD visualization can be achieved within 40-45 minutes. However, despite of the high sensitivity of RT-LAMP-LFD could offer, it gives chances of false positive result. Even with only a single trace of DNA/RNA could create contamination. RT-LAMP assay need preparation of mixing several reagent such as thermoPol buffer, dNTPs, $MgSO_4$, betaine, *Bst* DNA polymerase, AMV buffer, AMV RT, and H_2O. Therefore it need extra cautious in handling the preparation of master mix and should be in sterile condition. The combination of RT-LAMP assay with LFD helps to avoid contaminations in post-amplifications operations.

Table 3 Performance test of RT-LAMP-LFD and real time RT-PCR

No	Virus	Strain name/sample ID	Source	Type of sample	Confirmation test			Virus titer	Previous reference test	Detection assay[d]		
					Screening[a]	Virus isolation[b]	Sequencing[c]			RT-LAMP-gel	RT-LAMP-LFD	rRT-PCR
1	SIV	SF 21625 N	Field sample	Nasal swab	yes	no	no	n/a	+	+	+	+
2	SIV	SF 22300 N	Field sample	Nasal swab	yes	no	no	n/a	+	+	+	+
3	SIV	SF 21298 N	Field sample	Nasal swab	yes	no	no	n/a	+	+	+	+
4	SIV	SF 21299 N	Field sample	Nasal swab	yes	no	no	n/a	+	+	+	+
5	SIV	SF 21305 N	Field sample	Nasal swab	yes	no	no	n/a	+	+	+	+
6	SIV	SF 20218 E1	Field sample	Allantoic fluid	yes	yes	yes	n/a	+	+	+	+
7	SIV	SF 20226 E1	Field sample	Allantoic fluid	yes	yes	yes	n/a	+	+	+	+
8	SIV	SF 21302 E1	Field sample	Allantoic fluid	yes	yes	no	n/a	+	+	+	+
9	SIV	SF 21304 E1	Field sample	Allantoic fluid	yes	yes	yes	n/a	+	+	+	+
10	SIV	SF 21307 E1	Field sample	Allantoic fluid	yes	yes	no	n/a	+	+	+	+
11	SIV H3N2	NRU 3488	Field sample	Allantoic fluid	yes	yes	no	n/a	+	+	+	+
12	SIV H1N1	NRU 3336	Field sample	Allantoic fluid	yes	yes	no	n/a	+	+	+	+
13	SIV-eH1N1	CU-S3334	Reference	Nasal swab	yes	yes	yes	n/a	+ (14.12)	+	+	+
14	SIV-rH1N1	CU-S3629	Reference	Nasal swab	yes	yes	yes	n/a	+ (15.25)	+	+	+
15	SIV-rH1N1	CU-S3795	Reference	Nasal swab	yes	yes	yes	n/a	+ (12.08)	+	+	+
16	SIV-rH1N2	CU-S3631	Reference	Nasal swab	yes	yes	yes	n/a	+ (14.59)	+	+	+
17	SIV-rH3N2	CU-S3474	Reference	Nasal swab	yes	yes	yes	n/a	+ (13.95)	+	+	+
18	SIV-rH3N2	CU-S3673	Reference	Nasal swab	yes	yes	yes	n/a	+ (22.61)	+	+	+
19	SIV-rH3N2	CU-S14129	Reference	Allantoic fluid	yes	yes	yes	n/a	+ (25.43)	+	+	+
20	SIV-rH3N2	CU-S14252	Reference	Allantoic fluid	yes	yes	yes	n/a	+ (13.76)	+	+	+
21	SIV-H1N1	*SIV 06CB1	Reference	Allantoic fluid	yes	yes	yes	10^4TCID50/ml	+ (14.61)	+	+	+
22	SIV	SF 21626 N	Field sample	Nasal swab	yes	no	no	n/a	+ (22.87)	+	+	+
23	SIV	SF 21970 N	Field sample	Nasal swab	yes	no	no	n/a	+ (20.23)	+	+	+
24	SIV	SF 22117 N	Field sample	Nasal swab	yes	no	no	n/a	+ (24.04)	+	+	+
25	SIV	SF 22323 N	Field sample	Nasal swab	yes	no	no	n/a	+ (34.1)	+	+	+
26	SIV	SF 22325 N	Field sample	Nasal swab	yes	no	no	n/a	+ (21.64)	+	+	+
27	SIV	SF 22317 N	Field sample	Nasal swab	yes	no	no	n/a	+ (23.59)	+	+	+
28	SIV	SF 22629 N	Field sample	Nasal swab	yes	no	no	n/a	+ (34.93)	+	+	+
29	SIV	SF 22630 N	Field sample	Nasal swab	yes	no	no	n/a	+ (29.3)	+	+	+
30	SIV	SF 22362 N	Field sample	Nasal swab	yes	no	no	n/a	+ (23.49)	+	+	+
31	PRRSV	S1803 074	Field sample	Serum	yes	no	no	n/a	-	-	-	-
32	PRRSV	S1803 110	Field sample	Serum	yes	no	no	n/a	-	-	-	-
33	PRRSV	P97/61 1.0	Field sample	Serum	yes	no	no	n/a	-	-	-	-
34	PRRSV EU	01KB1	Reference	Cell	yes	yes	no	10^3TCID50/ml	-	-	-	-
35	PRRSV US	01NP1	Reference	Cell	yes	yes	yes	10^3TCID50/ml	-	-	-	-
36	PRRSV	Pool 1	Field sample	Serum	yes	no	no	n/a	-	-	-	-
37	PRRSV	Pool 2	Field sample	Serum	yes	no	no	n/a	-	-	-	-
38	PRRSV	Pool 5	Field sample	Serum	yes	no	no	n/a	-	-	-	-
39	PEDV	S 5005	Field sample	Intestine	yes	no	no	n/a	-	-	-	-
40	PEDV	S 5032	Field sample	Intestine	yes	no	no	n/a	-	-	-	-

41	PEDV	S 5039	Field sample	GI organ	yes	no	no	n/a	-	-	-	-
42	PEDV	S 5043	Field sample	GI organ	yes	no	no	n/a	-	-	-	-
43	PEDV1	PED Vaccine	Reference	n/a	n/a	n/a	n/a	n/a	-	-	-	-
44	PEDV	S5381	Field sample	Feces	yes	no	no	n/a	-	-	-	-
45	PEDV	S5383	Field sample	Feces	yes	no	no	n/a	-	-	-	-
46	PEDV	S5385	Field sample	Feces	yes	no	no	n/a	-	-	-	-
47	PEDV	S5386	Field sample	Feces	yes	no	no	n/a	-	-	-	-
48	PEDV	S5411	Field sample	Feces	yes	no	no	n/a	-	-	-	-
49	PEDV	S5412	Field sample	Feces	yes	no	no	n/a	-	-	-	-
50	PEDV	S5414	Field sample	Feces	yes	no	no	n/a	-	-	-	-
51	PEDV	S5415	Field sample	Feces	yes	no	no	n/a	-	-	-	-
52	PEDV	S5443	Field sample	Intestine	yes	no	no	n/a	-	-	-	-
53	PEDV	S5446	Field sample	Intestine	yes	no	no	n/a	-	-	-	-
54	PEDV	S5447	Field sample	Intestine	yes	no	no	n/a	-	-	-	-
55	PEDV	S5448	Field sample	Intestine	yes	no	no	n/a	-	-	-	-
56	PEDV	S5449	Field sample	Intestine	yes	no	no	n/a	-	-	-	-
57	PEDV	S5450	Field sample	Intestine	yes	no	no	n/a	-	-	-	-
58	PEDV	S5451	Field sample	Intestine	yes	no	no	n/a	-	-	-	-
59	ADV	NIAH	Reference sa	Cell	yes	yes	no	10^4TCID50/ml	-	-	-	-

[a] The samples have been previously identified by using PCR or real time RT-PCR-

[b] The samples have been previously isolated by using egg inoculation (SIV) or tissue culture (PRRSV and ADV)

[c] The samples sequences have been previously identified by next generation sequencing (NGS)

[d] The samples were blinded and tested by each detection assay; RT-LAMP-gel, RT-LAMP-LFD, realtime RT-PCR

Table 4 Diagnostic sensitivity, diagnostic specificity and the measure of agreement (Kappa value) of RT-LAMP-LFD with RT-LAMP-gel and realtime RT-PCR

		Gold standard (Reference test)		
		Positive	**Negative**	**Total**
RT-LAMP-gel	Positive	30	0	30
	Negative	0	29	29
		39	29	59
				Sensitivity 100%
				Speciificity 100%
				Kappa = 1
		Positive	Negative	Total
RT-LAMP-LFD	Positive	30	0	30
	Negative	0	29	29
		39	29	59
				Sensitivity 100%
				Speciificity 100%
				Kappa = 1
		Positive	Negative	Total
rRT-PCR	Positive	30	0	30
	Negative	0	29	29
		39	29	59
				Sensitivity 100%
				Speciificity 100%
				Kappa = 1

In conclusion, RT-LAMP-LFD assay was developed with newly designed primers. The RT-LAMP-LFD has high analytical sensitivity with minimum detection at 7.8 pg/µl. There were no cross reaction with other viruses of pigs. The newly developed RT-LAMP-LFD has good performance with 100% sensitivity, 100% specificity and perfect percentage of agreement compared to reference assays. With these conclusions, RT-LAMP-LFD can become a simple assay for the detection of SIV and suitable for the field or in-farm setting. The RT-LAMP-LFD assay for SIV detection could be applied at first point of care in outbreak situation to prevent and control the disease as well as health management of the pigs.

Ethics Statement: The study was conducted under the approval of the Chulalongkorn University's insititute biosafety committee (IBC#1831065) and the Institute for Animal Care and Use Protocol of the CU-Vet, Chulalongkorn University (IACUC#1831104).

Declaration of conflicting interests: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgements

This research was supported by the 90th Anniversary of Chulalongkorn University Scholarship under the Ratchadaphisek Somphot Fund (GCUGR1125622102M-102). We thank Chulalongkorn University for supporting the scholarship program for neighboring countries to the first author (AU). Chulalongkorn University support the Center of Excellence for Emerging and Re-emerging Infectious Disease in Animals and the OneHealth Research Cluster. The Thailand Research Fund (TRF) support the TRF Senior Scholar to corresponding author (RTA6080012).

Funding: This research was supported by Chulalongkorn University, Ratchadaphisek Somphot Fund (GCUGR1125622102M-102), the Center of Excellence for Emerging and Re-emerging Infectious Disease in Animals, the OneHealth Research Cluster and the TRF Senior Scholar (RTA6080012).

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