ORIGINAL ARTICLE

DEVELOPMENT OF REVERSE-TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD USING A DUAL INDICATOR FOR THE VISUAL DETECTION OF DUCK TEMBUSU VIRUS

Nguyen Huu Huan, DVM, MS[1], Dang Huu Anh, DVM, PhD[1], Huynh Thi My Le, DVM, PhD[1], Nguyen Van Giap, DVM, PhD[1], Tran Thi Huong Giang, DVM, PhD[1], Le Van Phan, DVM, PhD[1], Mai Thi Ngan, DVM, PhD[1*], Satoshi Sekiguchi, DVM, PhD[2,3] and Wataru Yamazaki, DVM, PhD[4*]

[1]Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi 100000, Vietnam; [2]Department of Veterinary Science, Faculty of Agriculture; [3]Center for Animal Disease Control, University of Miyazaki, Miyazaki 889-2192, Japan; [4]Center for Southeast Asian Studies, Kyoto University, Kyoto 606-8501, Japan

ABSTRACT

The disease caused by the Tembusu virus (TMUV) is characterized by egg-drop syndrome in ducks. In recent years, TMUV has become a concern due to the expansion of host species and increasing number of infection records in Asia. This study aimed to develop a sensitive and rapid reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method to target highly conserved regions using a colori fluorescent indicator (CFI) for TMUV detection. Two primer sets for RT-LAMP, including loop primers, were designed from the conserved region of the NS5 gene to accelerate amplification efficacy. The specificity and sensitivity of the RT-LAMP method were evaluated using the TMUV FX2010-180P vaccine strain and other duck-related viruses. RT-PCR and RT-LAMP concordance was evaluated based on results of 64 clinical samples. The developed RT-LAMP method was specific for TMUV. The sensitivity of the RT-LAMP method was 100 times higher than that of the two-step RT-PCR. The concordance between RT-PCR and RT-LAMP was 92.19%. A reliable, rapid, and simple RT-LAMP method for TMUV detection was successfully developed. This is the first RT-LAMP-based TMUV detection method with the addition of two loop primers for higher sensitivity and rapidity that could provide a potential strategy for screening TMUV infection.

Keywords: *CFI, NS5 gene, RT-LAMP, RT-PCR, TMUV*

Philipp. J. Vet. Med., 59(1): 15-23, 2022

INTRODUCTION

Tembusu virus (TMUV, genus *Flavivirus*, family *Flaviviridae)* is a single-stranded positive-sense RNA virus with a genomic length of about 11 kb. It has only one open reading frame encoding a polyprotein which is processed into three structural proteins namely capsid (C), membrane (M), and envelope (E); seven non-structural (NS) proteins, are encoded by the remainder of the genome, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Baltimore, 1971). Among these proteins, NS5 is the most conserved protein and plays an essential role in viral genome capping and replication processes (Baltimore, 1971; Chambers *et al.*, 1990).

TMUV was first isolated in Kuala Lumpur, Malaysia from *Culex tritaeniorhynchus* mosquitoes in 1955 (Platt *et al.*, 1975). In 2010, duck TMUV was first identified as the causative agent of the egg-drop syndrome in China, characterized by decrease in egg-laying, growth retardation, and paralysis (Yan *et al.*, 2011). Then, it spread quickly to most provinces of China with more than 10 million ducks infected causing significant economic losses of several billion dollars to the duck industry (Lei *et al.*, 2017; Zhang *et al.*, 2017). Thereafter, in 2013, TMUV was recorded as the severe causative

***FOR CORRESPONDENCE**:
(e-mail: mtngan@vnua.edu.vn;
yamazaki@cseas.kyoto-u.ac.jp)

agent of the egg-drop syndrome in duck farms in Thailand (Fu *et al.*, 2016). Based on phylogenetic analysis, the TMUV transmission to South Asia is believed to have originated from China (Baltimore, 1971). Moreover, TMUVs were detected in other species including geese, chicken, pigeons, and sparrows (Liu *et al.*, 2012; Liu *et al.*, 2013; Tang *et al.*, 2013). Newly reported duck infections in Asia indicated the need for the development of an accurate and fast diagnosis method of TMUV detection to effectively prevent and control TMUV (Zhang *et al.*, 2018).

There are several methods which are effective for the TMUV detection such as virus isolation, conventional PCR, and real-time PCR (Yan *et al.*, 2011; Yan *et al.*, 2011; Yan *et al.*, 2012). Virus isolation usually takes a few days to a few weeks. PCR methods are sensitive, specific, and effective. However, PCR technologies are expensive, time-consuming, and impractical in resource-limited laboratories. Loop-mediated isothermal amplification (LAMP) is an isothermal amplification method that has been shown distinct advantages with regards to specificity, sensitivity, detection time, as well as a simple process for rapid detection of pathogens (Notomi *et al.*, 2000; Nagamine *et al.*, 2002). Several TMUV detection methods using RT-LAMP have been reported (Tang *et al.*, 2012; Yan *et al.*, 2012; Tang *et al.*, 2016).

However, the published RT-LAMP methods for TMUV detection use only four primers or utilize the E gene with high genetic variability which may cause false negatives due to low sensitivity or primer mismatches. Pre-adding a dual indicator by a combination of hydroxy-naphthol blue (HNB) and Gelgreen as for visual detection of LAMP result for human African trypanosomiasis detection has been proved to be a stable indicator and did not inhibit amplification efficiency (Hayashida *et al.*, 2015). Therefore, this study aimed to develop and evaluate the performance of a more sensitive and rapid RT-LAMP method that targets the NS5 gene, a highly conserved region and includes loop primers with a dual colori fluorescent indicator (CFI) for TMUV detection.

MATERIAL AND METHODS

Primers

To identify the most conserved genetic region of the TMUV strains, 106 complete TMUV genome sequences were retrieved from the DDBJ/EMBL/GenBank databases (www.ncbi.nlm.nih.gov/nucleotide/). The alignment of complete ORFs was conducted by the BioEdit V7.2.5 software and the comparison of the selected conserved region was performed using the using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo). Then, to specify conserved regions in the TMUV genomes, aligned results were used for similarity plotting analysis with the Simplot program V3.5.1. The NS5 gene was found as the most highly conserved region, and therefore, was chosen as the optimal target for the RT-LAMP primer designing. All primers for RT-LAMP including outer primers (F3, B3), inner primers (FIP, BIP), and loop primers (LF, LB) were designed from the conserved region of the NS5 gene sequence of the TMUV PY-2013 strain (GenBank accession number: KX686576) using an online program, the Primer Explorer V5 (http://primerexplorer.jp/lampv5e/index.html). In silico analysis, specificities of the designed primers were further confirmed using a BLAST search in the NCBI nucleotide database. Considering the genetic diversity of TMUV, two primer sets were employed. Primer set 1 targeted the conserved region from 9,521 to 9,880 and primer set 2 targeted from 9,401 to 9,640 (corresponding to KX686576) as shown in Table 1. To prevent false negatives due to mismatches, one location of degenerate oligonucleotides in the FIP primer for primer set 1 and in the LF primer for primer set 2 was used.

Viruses

TMUV FX2010-180P is a vaccine strain with tissue culture infective dose ($TCID_{50}$) of 10^7 (Shanghai Veterinary Research Institute, Shanghai, China) that served as the reference control for optimization. As negative controls, other duck related viruses were also used, including avian influenza virus (AIV, H5N1), duck hepatitis virus (DHV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and duck viral enteritis (DVE). Viral DNA/RNA was extracted from 250 µl supernatants of TMUV, other viruses, and diluted samples using TRIzol® LS reagent (Thermo Fisher, San Francisco, CA, USA) as the manufacturer's instructions.

RT-PCR method

The two-step RT-PCR was performed. Firstly, reverse transcription reaction incubated at 45 °C for 60 minutes then heated to 95 °C for 5 minutes (for denaturation) using random hexamers (Thermo Fisher Scientific, Waltham, MA, USA) and kit M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Then, the second strand cDNA synthesis and PCR amplification were performed by using GoTaq® Green Master Mix (Promega Corporation, WI, USA) as following thermal

Table 1. Primers used for RT-LAMP in this study

Primer	Sequence (5' to 3')	Length (bp)	Gene position
Set 1			
F3	CATCCAAGAATGGCTCCAA	19	9,594 – 9,613
B3	CGACATGGAACCACAATCT	19	9,855 – 9,837
FIP (F2 + F1c)	TGGCGAATCTGTCATCTGCTG	43	9,695 – 9,675
	GAGAACAGGTTGTCAGC*Y*		9,623 – 9,640
BIP (B2 + B1c)	GCACTTCCTCAACAGCATGTC	45	9,703 - 9,723
	TTCTTGCCAGTTTCTCCAAC		9,784 – 9,765
LF	GTTTCACCACACAATCATCCC	21	9,674 - 9,653
LB	AAGATACTCAGGAATGGAAGCC	22	9,735 – 9,756
Set 2			
F3	GATGTCATCTCGCGGGAAG	19	9,434 - 9,452
B3	GCTGACAACCTGTTCTCTCC	20	9,619 – 9,639
FIP (F2 + F1c)	AGCTGGACGCACAGGTTCGT	38	9,500 – 9,519
	ACCAGAGGGGAAGTGGAC		9,453 - 9,470
BIP (B2 + B1c)	AAGGGGAGGAGTTGCTGCTCC	40	9,534 – 9,554
	TGGAGCCATTCTTGGATGC		9,594 – 9,612
LF	AGT*R*TTGAGAGCATAAGTCACAACC	25	9,472 – 9,495
LB	AGCGCCTAAAGAAAGGAAAGG	21	9,567 – 9,588

Italic text indicates degenerate oligonucleotides, Y = C or T, R = A or G

condition: denatured at 95 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds (for denaturation), 55 °C for 30 seconds (for annealing), and 72 °C for 30 seconds (for extension); followed by 72 °C for 5 minutes for final extension. PCR products were analyzed by 1.5% agarose gel electrophoresis. The two-step RT-PCR used a previously published primer pair on the NS5 gene (Liu *et al.*, 2012).

RT-LAMP method

The RT-LAMP reaction was conducted in a final reaction volume of 25 µl consisting of 2 µl RNA template, FIP and BIP primers (1.6 µM each), Loop F and Loop B primers (0.8 µM each), F3 and B3 primers (0.2 µM each), 1.6mM dNTPs, 5M betaine, 20mM Tris-HCl (pH 8.8), 10mM KCl, 10mM $(NH_4)_2SO_4$, 4mM $MgSO_4$, 8 U of Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA), 0.15 U of AMV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 1 µl of CFI stock solution for visual detection. CFI stock solution consisting of 3 mM HNB (MP Biomedicals, Aurora, OH, USA) and 0.35% v/v GelGreen (10,000× Sol, Biotium, Hayward, CA, USA) was prepared with distilled water (Hayashida *et al.*, 2015). Amplification reactions were performed in a water bath at 65 °C for 60 minutes and were heated at 80 °C to terminate the reactions. RT-LAMP products were detected by naked eyes detection with color change or fluorescence observation under UV light. In addition, the RT-LAMP reaction was performed in the real time using the LightCycler® 96 System (Roche, Basel, Switzerland). The thermal cycling profile for real-time RT-LAMP proceeded as follows: 60 cycles of 65 °C for 30 seconds and 65 °C for 30 seconds. After collecting the fluorescence signal at the end of the second 65 °C x 30 s step of each cycle, the enzyme reaction was inactivated by holding at 95 °C for 2 minutes, followed by melting curve analysis from 65 °C to 97 °C at 0.15 °C /sec. The real-time RT-LAMP results were detected by fluorescence observation with the FAM channel. The cut-off time and effective Ta (temperature of annealing) values were set to 30 minutes and 85.5-86.5 °C for primer set 1 and 40 minutes and 82.5-83.5 °C for primer set 2, respectively. The sample that the fluorescence intensity of the amplified signal reached 1.0 within the cut-off time and still showed an effective Ta value was defined as positive. In addition, samples were also defined as positive if the purple reagent changed to sky blue and fluoresced under UV irradiation through naked eye observation.

Sensitivity and specificity of RT-LAMP

TMUV (vaccine strain FX2010-180P), AIV, DHV, NDV, IBV, and DVE were used as templates for RT-LAMP to analyse the specificity of RT-LAMP of both primer sets. Sterilized double distilled water was used as the negative

control. To evaluate the sensitivity of the RT-LAMP method of both primer sets, the limits of detection (LODs) were compared to the two-step RT-PCR by amplifying ten-fold serial dilutions from the cell culture of TMUV vaccine strain FX2010-180P with defined median $TCID_{50}$. RNA was then extracted from 250 µL media of each dilution and used as a template for RT-LAMP and RT-PCR as mentioned above. All dilutions were analysed in duplicate.

Detection of TMUV in clinical samples

In total, 64 tissue samples were collected from duck farms that have ducks with suspected clinical symptoms caused by TMUV as ataxia, paralysis, and severe egg-drop syndrome throughout Vietnam between April 2020 and November 2020. In this study, specimen collection requirements of National Technical Regulation QCVN 01-83:2011 were followed. Tissue samples including brain, heart, lung, liver, spleen, and ovary were collected from animals which died disease in these farms. Samples were then homogenized and prepared as a 10% (w/v) suspension in PBS (pH 7.2) and centrifuged at 2300 g at 4 °C for 10 minutes. RNA was extracted from 250 µl supernatants as mentioned above and used as a template for the detection of TMUV by RT-PCR and RT-LAMP. For RT-LAMP detection of clinical samples, a water bath set at 65 °C was used, assuming a simple on-site test in the field. Primer set 1 and 2 were performed by heating for 30 and 40 minutes, respectively. To prevent non-specific amplification due to residual heat, the results were judged by the naked eye immediately after the end of heating. A positive result was defined when the purple reagent changed to sky blue and fluoresced under UV irradiation through naked eye observation.

Statistical analysis

The kappa value, a statistical coefficient that represents the degree of accuracy and reliability in statistical classification, was calculated to measure the concordance between the RT-PCR and RT-LAMP results. All tissue samples analyzed in this study were collected from animals that had died for diagnostic purposes.

RESULTS

Specificity of the RT-LAMP

TMUV (vaccine strain FX2010-180P) and other related duck viruses (AIV, DHV, NDV, IBV, and DVE) were tested using the RT-LAMP method to evaluate the specificity. In both primer sets, only TMUV was positive (represented by the red amplification line), and no LAMP products were detected in the reactions from other relevant duck viruses or negative control (Figure 1A). The real-time RT-LAMP products were also evaluated by naked eye detection. The results stated that the RT-LAMP method was specific for TMUV and can be applied for distinguishing TMUV from other duck viruses. By pre-adding CFI to the LAMP reaction, the RT-LAMP results were observed directly after amplification by the naked eye in two ways. Firstly, the color change of HNB under natural light in the TMUV positive reaction turned from violet to blue as the Mg2+ ion concentration reduced by making insoluble $Mg_2P_2O_7$ (Figure 1B). Secondly, the LAMP results were visualized under UV light by DNA GelGreen dye. In the tube with amplified TMUV cDNA, the presence of a bright yellow-green color was considered a positive result, otherwise, the tube glowed red (Figure 1C).

Sensitivity of the RT-LAMP

The LOD of the RT-PCR was 10^4 TCID50/mL, whereas the LODs of the RT-LAMP method were 10^2 TCID50/mL in both primer sets, especially the same result in duplicate (Table 2). Hence, the sensitivity of the RT-LAMP method was 100 times higher than that of two-step RT-PCR. Moreover, the real-time fluorescence intensity from the reactions at all concentrations evaluated was high when the reactions were performed within 30 minutes for primer set 1 and 40 minutes for primer set 2 (Table 2). Therefore, the optimal reaction condition of the current RT-LAMP for TMUV was 65 °C for 30 minutes (primer set 1) and 40 minutes (primer set 2). In addition, the level for the change of color of HNB or the fluorescence signal by Gelgreen in different concentrations was still constant (Figure 2).

Detection of clinical samples

Of the 64 samples, 21 positive samples were detected by both RT-LAMP from two primer sets and RT-PCR, five samples were negative by RT-PCR but positive by both RT-LAMP. The remaining 38 samples resulted negative for both methods as well as both primer sets (Table 3). The Kappa value between the two methods was 0.833. The concordance rate between the two methods of RT-PCR and RT-LAMP is 92.19%. Furthermore, none of the positive results obtained by RT-PCR turned out to be negative by RT-LAMP. The twenty-one samples that were positive by RT-LAMP were the same as 21 samples that were positive by RT-PCR.

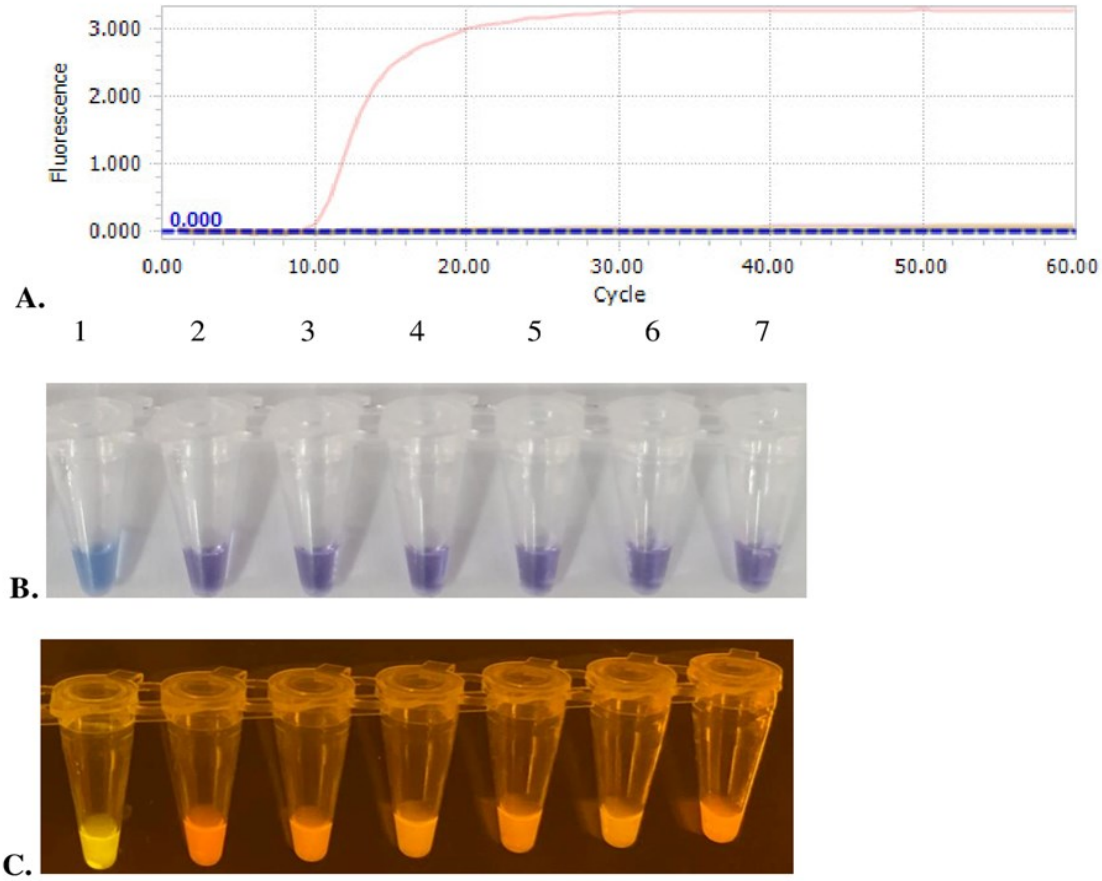

Figure 1. The specificity of the RT-LAMP method with primer set 1 for detecting the TMUV NS5 gene. (A) by real-time RT-LAMP, the positive curve represents TMUV; negative curves represent DVE, AIV, IBV, NDV, DHV and the negative control. (B) and (C) by naked eyes and UV light detection. From 1 to 7, RNA of TMUV, DVE, AIV, IBV, NDV, DHV, and negative control, respectively (Representative result by using primer set 1).

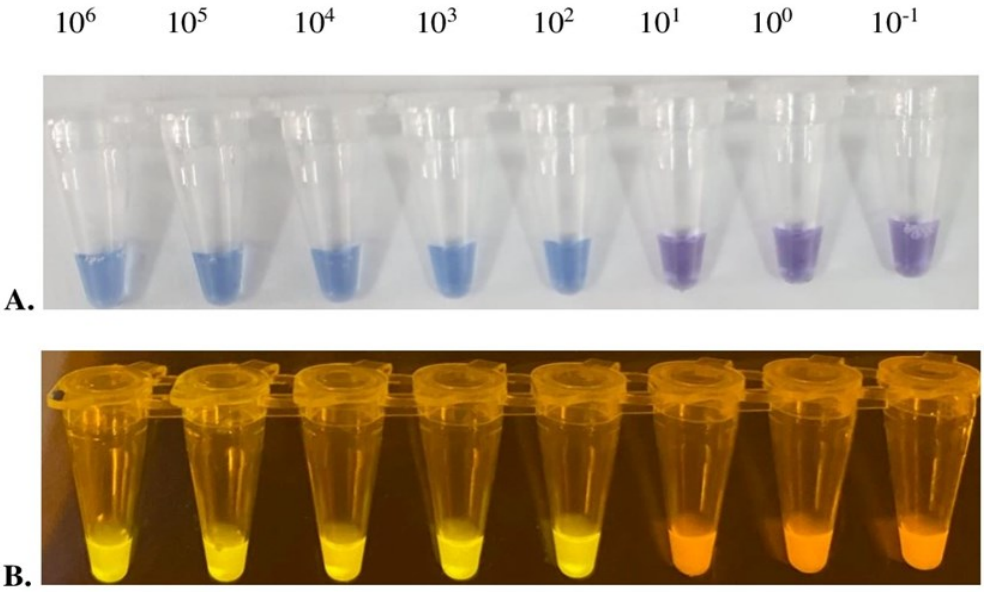

Figure 2. The sensitivity of the real-time RT-LAMP method with primer set 1 for detecting the TMUV was assessed based on visual inspection. (A) by the color change of HNB. (B) fluorescent of DNA Gelgreen dye under UV light. From 10^6 to 10^{-1}, RNA of ten-fold serial dilutions from the cell culture of 10^7 $TCID_{50}$ TMUV vaccine strain FX2010-180P (Representative result by using primer set 1).

Table 3. Detection of TMUV in clinical samples by RT-LAMP and two-step RT-PCR

		RT-PCR		
		Positive	Negative	Total
RT-LAMP Set 1	Positive	21	5	26
Kappa value: 0.833	Negative	0	38	38
Total		21	43	64
RT-LAMP Set 2	Positive	21	5	26
Kappa value: 0.833	Negative	0	38	38
Total		21	43	64

Table 2. Detection limit of two-step RT-PCR and real-time fluorescent RT-LAMP of FX2010-180P strain

$TCID_{50}$	**10^6**	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**	**10^0**	**10^{-1}**
RT-PCR	+	+	+	-	-	-	-	-
RT-LAMP								
Set 1	+	+	+	+	+			
(amplification time mm:ss)	(10:86)	(12:89)	(15:57)	(18:78)	(20:26)	-	-	-
Set 2	+	+	+	+	+			
(amplification time mm:ss)	(20:41)	(23:43)	(24:71)	(31:11)	(33:94)	-	-	-

DISCUSSION

In our study, an accurate, rapid, and simple RT-LAMP method of naked ,eye detection including loop primers from the NS5 gene, was successfully developed. The reaction condition of the RT-LAMP was optimized by selecting a primer set and simple incubation at 65 °C for 30 minutes. The sensitivity of the RT-LAMP method for TMUV detection was 100 times higher than that of two-step RT-PCR. Furthermore, the developed RT-LAMP method was evaluated with field samples and it exhibited almost perfect concordance with the two-step RT-PCR method.

In early 2010, TMUV was first detected as the causative agent of duck egg-drop disease in China. So far, the disease is one of the most harmful infectious diseases in the duck raising industry. A recent study reported a potential case of human infection with TMUV (Tang *et al.*, 2013). Therefore, the development of an easy and accurate diagnostic method is curial for successful TMUV infection control and prevention. LAMP is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions (Notomi *et al.*, 2000; Nagamine *et al.*, 2002). The application of LAMP for identifying TMUV infection has been previously published (Tang *et al.*, 2012; Yan *et al.*, 2012; Tang *et al.*, 2016). However, some of the developed RT-LAMP methods were based on the sequences of historical TMUV strains, making them unsuitable for detecting divergent TMUV strains from different years. In addition, genetic divergence between various TMUV strains isolated in different years was indicated in previous publication (Dai *et al.*, 2015). Moreover, in the published studies, loop primers were not used, E gen, which has a large genetic diversity, was utilized to design the primers. The preliminary tests found mismatches with field strains isolated in Vietnam in recent years. In addition, the previously reported primers published by Yan et al and Tang et al (Yan *et al.* (2012) and Tang *et al.* (2016) were

evaluated. However, for unknown reasons, the previously reported primers failed to amplify, contrary to our expectations (data not shown). Hence, new primers were newly developed for this study. These new primers can safeguard against the possibility of false negatives due to the appearance of mutant strains in the future. The LAMP method can halve the reaction time and increase the sensitivity more than ten-fold by adding loop primers (Nagamine *et al.*, 2002). In this study, the amplification time of the LAMP with and without loop primer was 19 minutes and 37 minutes, respectively. Hence, the two LAMP methods developed, both with the addition of two loop primers, can provide rapid and sensitive diagnosis.

Another important step in designing LAMP primer sets is to identify conserved regions in the genomic sequence of TMUV. Therefore, in the present study, the complete ORFs of 106 representative TMUV reference strains to find highly conserved regions were selected and NS5 was indicated based on the alignment result. The previously published article also indicated that NS5 is a highly conserved region and meets the requirement of LAMP primer set designing (Tang *et al.*, 2016). On the other hand, the NS5 sequence data of designing primers for RT-LAMP (KX686576.1) was chosen and compared with sequence data 10 TMUV reference strains from 2011 to 2020 retrieved from GenBank. In this study, all primers, including loop primers, were designed from the highly conserved NS5 gene of TMUV. Moreover, to achieve high specificity, the LAMP primers must have a strict match with their target sequences especially within nucleotide sequence 1-5 or 6 from the side of the reaction base, potentially leading to biased amplification results or even failure (Mai *et al.*, 2018). Furthermore, to achieve maximum sensitivity of detection for TMUV, the FIP in primer set 1 used included 1 degenerate oligonucleotide - Y (Y = C or T shared nucleotide identity with 97/106 and 9/106 available TMUV sequences in the GenBank, respectively). The LF in primer set 2 also included 1 degenerate oligonucleotide - R (R = A or G shared nucleotide identity with 81/106 and 25/106 available TMUV sequences in the GenBank, respectively).

The development of an easy and accurate diagnostic method is essential to the early prevention and control of the devastating disease. The LAMP method allows isothermal amplification using a water bath without the need for a dedicated thermal cycler. In addition, the reaction is rapid, simple, sensitive, and specific. The application of LAMP for TMUV detection has been published in previous studies in which TMUV can be visually detected by using electrophoresis or an intercalating dyestuff (SYBR Green I) (Tang *et al.*, 2012; Yan *et al.*, 2012; Tang *et al.*, 2015; Tang *et al.*, 2016). Gel electrophoresis is inexpensive but time-consuming for post-amplification manual procedures and requires an electrophoresis workstation. A DNA intercalating dyestuff is mixed into the solution after the reaction is finished for better visibility of the reaction result under UV light (Yan *et al.*, 2012). However, gel electrophoresis and colorimetric analyses applying intercalating dyestuff, may increase the chance of contamination of the next LAMP reaction solutions since the assessment of result requests the test tube opening. Pre-adding a dual indicator in LAMP reactions enabled rapid and sensitive detection of LAMP products with lower risk of contamination (Hayashida *et al.*, 2015). Similarly, in the present study, the color change of HNB was clearly visible in the positive tubes of various dilutions (Figure 2).

In this study, vaccine strain was used to evaluate the limit detection of RT-LAMP in comparison with two-step RT-PCR. The sensitivity test showed that RT-LAMP is 100 times more sensitive than two-step RT-PCR. Previous studies also indicate that the sensitivity of the RT-LAMP method was much higher than that of the RT-PCR method even with different strains in ELD_{50} (50% egg lethal dose), $TCID_{50}$ or copies (Wang *et al.*, 2011; Tang *et al.*, 2012; Yan *et al.*, 2012). The RT-LAMP method for TMUV was reported to be higher nearly 100 to 1000 times in sensitivity than the RT-PCR method (Wang *et al.*, 2011; Tang *et al.*, 2012). In addition, the sensitivity of real-time RT-LAMP also indicated that to be equal to that of the real-time RT-PCR, with a detection limit of 0.01 ELD_{50} (Yan *et al.*, 2012) or even 10 times more sensitive than real-time RT-PCR for TMUV detection (Jiang *et al.*, 2012). Moreover, based on the result of the sensitivity analysis, the duration of the RT-LAMP reaction could be done in a simple process within 30-40 minutes. Considering the difference in amplification time of each primer set, it is proposed that the cut off time be 30 minutes for primer set 1 and 40 minutes for primer set 2. Our developed RT-LAMP method showed as a simple process for rapid detection of TMUV, especially practical in poorly equipped laboratories.

A recent study indicated the presence of the TMUV by RT-PCR method combined with NS5B gene sequence analysis in the duck flocks in Vietnam (Dang *et al.*, 2020). In this study, 64 samples were collected in some duck farms that have

ducks with suspected clinical symptoms caused by TMUV. As shown in Table 3, the results of the field study showed that the concordance rate of the two methods, RT-PCR and RT-LAMP, was 92.19%, and of the 26 samples that were positive for RT-LAMP, five were negative for RT-PCR and positive for RT-LAMP. A previous study also indicated that the concordance between RT-LAMP and RT-PCR from NS5 gene in the detection of TMUV was 96.88% from 81 clinical samples (Tang *et al.*, 2016). In the experimental infection with TMUV, the detection rates of RT-PCR (targeting NS5 gene), semi-nested RT-PCR, real-time RT-PCR, and RT-LAMP were 52% (13/25 samples), 92% (23/25 samples), 88% (22/25 samples), and 88% (22/25 samples), respectively (Tang *et al.*, 2015). In other words, due to the difference in analytical sensitivity (LOD), samples with low viral load may show negative results for RT-PCR and positive results for RT-LAMP method.

Therefore, when the LAMP amplified products of these five discordant samples in the present study were measured by annealing, these were all within the defined positive Ta values (data not shown), and these five samples were interpreted as true positives rather than false positives due to non-specific reactions. These results may indicate that RT-LAMP could be further used as a screening and cost-effective test for the detection of TMUV infection. Further study will be required to confirm the effectiveness of the developed RT-LAMP method in different kinds of samples. Ultimately, the developed RT-LAMP methods were both found to be accurate, sensitive, and rapid in detecting the TMUV isolates prevalent in Vietnam. Using only a single primer set may result in false negatives due to mismatches against the design primers in a new outbreak of an unknown mutant isolates in the future. Therefore, as a safety measure, it is important to have multiple primer sets available for crisis management. Hence, two primer sets were developed and evaluated in this study.

In conclusion, a reliable, fast, and simple RT-LAMP method including loop primers from NS5 gene was successfully developed to detect TMUV. This method could be a powerful strategy for screening to control TMUV infections, and could be effective even in poorly equipped laboratories.

ACKNOWLEDGEMENT

This research was partially supported by Japan-ASEAN Platform for Trans disciplinary Studies, CSEAS, Kyoto University.

STATEMENT ON COMPETING INTEREST

The authors have no competing interest to declare.

AUTHOR'S CONTRIBUTION

NHH: investigation, manuscript writing; DHA: investigation, methodology; HTML: project administration and manuscript writing; NVG: investigation, data analysis; TTHG: investigation; methodology; LVP: funding acquisition, resources; SS: conceptualization; validation; MTN: data curation, methodology, data analysis, manuscript writing, WY: conceptualization, data curation, supervision, funding acquisition, resources and manuscript writing.

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