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Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of Japanese Encephalitis Virus in Swine and Mosquitoes

[1,2]Hao Liu, [2]Hui-Jun Lu, [2,3]Zhen-Jiang Liu, [1,2]Jie Jing, [1,2]Jing-Qiang Ren, [1,2]Yan-Yu Liu, [2,3]Huan-Huan Guo, [2,3]Min Fan and [1,2]Ning-Yi Jin
[1]College of Animal Science and Veterinary Medicine, Jilin University, 130062 Changchun, P.R. China
[2]Genetic Engineering Laboratory of PLA, Academy of Military Medical Sciences of PLA, 130122 Changchun, P.R. China
[3]College of Animal Science, Jilin Agricultural University, 130118 Changchun, P.R. China

Abstract: Japanese Encephalitis (JE) can infect many agriculturally important animals and humans and has a high incidence in Asia. One of the natural hosts of the mosquito-borne JE Virus (JEV) is domestic pigs which act as amplifier hosts. Porcine infection results in fatal encephalitis, abortion and stillbirth in pregnant sows and hypospermia in boars. In this study, a rapid JEV Detection Method for swine and mosquitoes was developed based upon Reverse Transcription Loop-Mediated isothermal Amplification (RT-LAMP) targeting the nucleocapsid (*E*) genes of JEV genotype I (lineage K94PO5) and genotype III (lineage SA14-14-2). About 56 swine blood samples and 20000 mosquitoes were used to evaluate the method, compared to conventional RT-Polymerase Chain Reaction (PCR) and real-time RT-PCR. RT-LAMP had detection limits of 2.57 and 2.34 copies/μL for JEV I and III, respectively. Assay sensitivity was similar to real-time RT-PCR but was 10 fold higher than conventional RT-PCR. Assay specificity was high, showing no cross-reactivity to other flaviviruses. Finally, the JEV RT-LAMP assay was simpler and less time consuming than conventional RT-PCR or real-time RT-PCR, since the amplification step could be completed in a single tube within 50 min at 63°C. In conclusion, the newly-developed RT-LAMP assay is an accurate and convenient method for rapid and sensitive diagnosis of JEV in swine and mosquitoes and may prove to be a practical molecular tool for surveillance and epidemiologic investigations.

Key words: Diagnosis, Japanese encephalitis virus, LAMP, real-time RT-PCR, RT-PCR

INTRODUCTION

Japanese Encephalitis Virus (JEV) is a mosquito-borne flavivirus that causes severe encephalitis in humans and many countries in Asia (Lindenbach *et al*., 2007; Vaughn and Hoke, 1992). In mainland China, the JEV genotypes I and III are the most prevalent (Solomon *et al*., 2003). The JEV transmission cycle involves a mosquito vector and two natural hosts, namely wild birds which act as the maintenance host and domestic pigs which act as the amplifier host. As such, pigs are the primary source of JEV for naive mosquito infection and transmission to humans. The *Culex tritaeniorhynchus* mosquito has been identified as the most important vector for human and animal transmission in China. Similar to many of the vector-borne viral diseases, JEV transmission rates vary temporally and geographically (Samuel *et al*., 2010). The global annual incidence of JE in humans has been estimated at 30000-50000 cases (Erlanger *et al*., 2009) and the rates in animals are certainly much higher. Animal infection has devastating biological and economic impact; in swines, JEV disrupts sow and boar reproduction processes and causes death in piglets (Graeber *et al*., 1998).

Diagnosis of JE by clinical symptoms is not feasible due to non-specific signs at both the early and acute stages of infection. In addition, JEV isolation from peripheral blood is precluded by the low level transient viremia which persists even in the acute stage of the disease (Shope and Meegan, 1997). Currently, the most commonly used methods of swine diagnosis are laboratory-based Enzyme-Linked Immunosorbent Assays (ELISAs) that detect Immunoglobulin (Ig) M or IgG antibodies in serum or cerebrospinal fluid taken from suspect herds (Yang *et al*., 2006). Unfortunately, these serological assays have considerable cross-reactivity with

Corresponding Author: Ning-Yi Jin, Genetic Engineering Laboratory of PLA, Academy of Military Medical Sciences of PLA, Liuying West Road 666, 130122 Changchun, P.R. China

other flaviviruses (Holbrook *et al*., 2004). Human JEV infection is clinically diagnosed by a variety of methods including Reverse Transcription Loop-Mediated isothermal Amplification (RT-LAMP).

The RT-LAMP assay has been reported as effective for detecting JEV in cerebrospinal fluid samples from patients with clinical diagnosis of acute encephalitis (Parida *et al*., 2006). However, this approach has yet to be applied to a swine population. Genome-based JEV diagnostic assays have been previously developed for swine and include the conventional RT-Polymerase Chain Reaction (RT-PCR) and real-time RT-PCR. While both of these assays are specific and sufficiently sensitive, they require expensive thermal cycling equipment and technical expertise.

Therefore, researchers sought to develop a technically simple and highly accurate genome-based JEV diagnostic procedure for swine that is inexpensive and also easily applicable outside of the laboratory setting. To this End, the nucleocapsid (*E*) genes of JEV I (lineage K94PO5) and JEV III (lineage SA14-14-2) were selected as the detection targets in an RT-LAMP assay. The efficiency of this novel diagnostic procedure was comparatively analyzed with conventional RT-PCR and real-time RT-PCR for detecting JEV I and JEV III in swine and mosquitoes.

MATERIALS AND METHODS

Viruses: JL0801 and YN0901 strains of JEV belong to the K94P05 and SA14-14-2 lineages, respectively and they were selected as positive standards based upon earlier isolations from swine and mosquitoes.

Working stocks of JL0801 and YN0901 strains were propagated in the mosquito cell line, C6/36. Porcine stool specimens that Contained Porcine Circovirus (PCV), Porcine Reproductive and Respiratory virus (PRRS), Classical Swine Fever Virus (CSFV), Bovine Viral Diarrhea Virus (BVDV) or Swine Hepatitis E Virus (SHEV) were obtained from swine with laboratory-confirmed infections. A 10% (w/v) suspension of the respective stools were made and stored at -80°C until use. *C. tritaeniorhynchus* infected with sindbis virus, Chikungunya Virus (CHIKV), or Yellow Fever Virus (YFV) were confirmed and selected for study.

Sample collection: About 56 swine blood samples including 46 acute-phase serum samples and 10 negative serum samples were obtained from different farms in the Li Shu county of Jilin province (latitude 22°00′N and longitude 100°47′E) and were analyzed using molecular biological methods. *C. tritaeniorhynchus* adult female mosquitoes were collected during dusk hours from cattle sheds and hog pens in the Jing Hong canton of Yun Nan province (latitude 43°18′N and longitude 124°20′E). A total of 200 tubes of mosquitoes (100 mosquitoes/tube) were identified and sorted according to species and only the *C. tritaeniorhynchus* females were used for these experiments.

Study methods: Gene amplification-based molecular techniques such as RT-PCR have emerged as a preferred method to make viral diagnoses using infected host tissue and fluid samples (Deubel *et al*., 1990; Tanaka, 1993). However, since these methods require expensive equipment and technical expertise they have not been adopted by many small and/or frontline laboratories. In addition, the characteristic low copy number of viral RNA in the blood of JEV-infected swine makes it difficult to extract sufficient amounts of RNA for such methods (Chuang *et al*., 2003).

RT-LAMP is a relatively new nucleic acid amplification method that can amplify DNA rapidly, efficiently and with high specificity under isothermal conditions (Notomi *et al*., 2000). RT-LAMP relies on autocycling strand displacement DNA synthesis which is carried out by Bst DNA polymerase large fragments with high strand displacement activity. The reaction is conducted under isothermal conditions that range from 60-65°C and the amplified products can be readily analyzed by naked-eye visualization or digital imaging after electrophoresis through an agarose gel. In addition, detection can be carried out by evaluating the turbidity properties of the samples, caused by the large amounts of by-product magnesium pyrophosphate from the amplification reaction or by a colorimetric method that involves a visible color change produced by the addition of SYBR Green I to the amplification reaction (Mori *et al*., 2001; Nagamine *et al*., 2002).

Design of primers for RT-LAMP, real-time RT-PCR and conventional RT-PCR of JEV I and JEV III: For RT-LAMP amplification of the E gene sequences of JEV, four primers were designed based on alignment analysis of various JEV strains' genomic sequences (GenBank: Beijing-1, CJN-L1, JaOArS982, SA14-14-2, TL Taiwan, K94P05, ThCMAr4492). The Primer Explorer Version 3 Software was used (http://primerexplorer.jp/lamp3.0.0/index.html). Each set of primers included an outer pair (F3, B3) and an inner pair (FIP, BIP). The resultant YG1/YG2, YG3/YG4, E1/E2 and E3/E4 primer (Table 1) were used for real-time RT-PCR and conventional RT-PCR amplification of the *JEV I* and *JEV III E* genes.

RNA extraction from JL0801 and YN0901 strains and reverse transcription to cDNA: Genomic viral RNA of JEV was extracted from inoculated JL0801 and YN0901

Table 1: List of primers used for RT-LAMP, real-time RT-PCR and conventional RT-PCR amplification of the *JEV I* and *JEV III E* gene

Primer name	Sequence 5'-3'
JEV1-F3	GGCTCCATTGGAGGGGTAT
JEV1-B3	GTGGCCAGAAAAGCCAGG
JEV1-FIP	AAGACATTCCCCCGAAGAGCG-G-GGAAAGCTGTTCACCAAGT
JEV1-BIP	CACACAAGGACTAATGGGGGCC-ATT-GACCGGTTTCGTGCG
JEV3-F3	GGACTGAACACTGAAGCGT
JEV3-B3	CTGTGACCCAAGAGCAACAA
JEV1-FIP	CCAGGGGAGAGCGAGGTCATACCGT-GGGGTCAAAGTCAT
JEV3-BIP	TTCGAGCACAGCGTGGAGAAACGGAC-TGTTTTGTGGCGT
JEVYG1	CGTACGAATGTCCGAAGCTTG
JEVYG2	TTTTCATGAGGTATCGCGTGG
JEVYG3	GGCAAACGACAAACCAACATT
JEVYG4	ATCAGCTCGCTTCTCGTTGTG
JEV E1	CCATGACCTTTCTCTTCCCTG
JEV E2	TAATCTGCTTGTCCCCTCTTC
JEV E3	GCGCACGCCACAAAACAGTCC
JEV E4	GCGTGCTTCCAGCTTTGTGCC
R1	TGCACATTGGTCGCTAAAAAC
R2	ATGTTAACTTCACTGAGCTTG

strains that were propagated in C6/36 cells by using the QIAamp Viral RNA Mini kit (Qiagen, Germany) in accordance with the manufacturer's protocol. In order to evaluate the detection limit of the assays, the amount of JEV RNA was determined by spectrophotometry and converted to molecular copies using a earlier published formula (Krieg, 1990).

The 10 fold serial dilutions of the JEV RNA (molecular copies from 10^{6}-10^{-1}) were used as templates in reverse transcription reactions to synthesize cDNA. The 30 μL RT synthesis reaction for JEV consisted of 9.0 μL of DEPC H_2O, 1.0 μL of 50 ng μL^{-1} genotype-specific reverse primer R1 or R2 and 5.0 μL of total RNA. The mixture was incubated at 75°C for 5 min then chilled on ice for at least 5 min. Afterwards, the following reagents were added to each of the reactions: 9.0 μL of DEPC H_2O, 1.0 μL of 200 U μL^{-1} M-MLV RT (Promega, USA), 1.0 μL of 40 U μL^{-1} ribonuclease inhibitor (Promega), 2.0 μL of 5×RT buffer and 2 μL of 10 mM dNTP mix. The reaction mixture was incubated at 42°C for 50 min and 75°C for 5 min.

The resultant cDNA products were detected concentration by a NanoDrop 2000 spectrophotometer (Thermo, USA) and used as template in RT-LAMP reactions.

RT-LAMP reaction and product detection: RT-LAMP was carried out in a 25 μL reaction mixture that contained 1.0 μL of 5 μM each of the F3 and B3 primers, 1.0 μL of 40 μM each of the BIP and FIP primers, 2.5 μL of 10×ThermoPol reaction buffer, 1.0 μL of 8 U μL^{-1} Bst DNA polymerase (New England Biolabs, USA), 2.5 μL of 10 mM dNTP mix (TaKaRa, Japan), 4.0 μL of 5 M Betaine (Sigma-Aldrich, USA), 5.0 μL of 30 mM $MgSO_4$, (Invitrogen, USA), 4.0 μL of target cDNA and 2 μL of nuclease-free water. The amplification reaction was performed at 65°C for 60 min and terminated by heating to 80°C for 10 min. Sterile water was used as a negative control template. RT-LAMP products were resolved by 2.0% agarose gel electrophoresis and visualized by ethidium bromide staining and Ultraviolet (UV) light detection; the positive reaction mixtures showed a characteristic ladder-like pattern. In an alternative protocol, the products were observed directly with the naked-eye by adding 1.0 μL of SYBR Green I (Invitrogen) to the reaction mix. The solution turned green in the presence of RT-LAMP amplification products but stayed orange in the absence of the amplicon.

Optimization of the RT-LAMP assay: The RT-LAMP reaction mixtures were incubated at 60, 61, 62, 63, 64 or 65°C for 60 min to determine the optimal reaction temperature. Subsequently, the optimal reaction time was determined by performing RT-LAMP at 63°C for 30, 35, 40, 45, 50 or 60 min. The reactions were terminated by heat inactivation at 80°C for 10 min. The amplified RNA products from the RT-LAMP assays were visualized as described above by agarose gel electrophoresis or SYBR Green I.

Determination of the RT-LAMP assay detection limit: To determine the sensitivity of the RT-LAMP assay, the 10-fold serial dilutions of the JEV I and JEV III RNA molecular copies (from 10^{6}-10^{-1}) were reacted. The various reaction mixtures were incubated at 63°C for 50 min, followed by termination at 80°C for 10 min. RT-LAMP products were analyzed by the electrophoresis and SYBR Green I detection methods.

Determination of the real-time PCR assay detection limit: Real-time PCR was performed with the JEV I and JEV III-specific primers (JEVYG1/YG2 and JEVYG3/YG4, respectively). Amplification was carried out in a 25 μL total reaction volume consisting of 12.5 μL of SYBR Green Real-Time PCR Master Mix (Toyobo, Japan), 8.0 μL of nuclease-free water, 1.0 μL of 0.4 μM forward and reverse primers and 5 μL of the cDNA sample (molecular copies from 10^{6}-10^{-1}). The following real-time RT-PCR thermal cycling program was run on an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, USA): one cycle of 50°C for 2 min and 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 50 sec. The data were analyzed with the accompanying ABI 7500 Software package, Version 2.0.5.

Determination of the PCR assay detection limit: PCR was performed with the JEV I and JEV III-specific primers that targeted the respective *E* genes. The reaction was carried out in a 20 μL total reaction volume that contained 1.0 μL of 5 U μL^{-1} ExTaq polymerase (TaKaRa), 0.5 μL of 10 mM dNTP mix, 0.5 μL of each 10 μM primer JEV E1 and E2 or JEV E3 and E4, 2.0 μL ExTaq buffer, 4.0 μL cDNA and 11.5 μL nuclease-free water. The PCR reaction was carried out on a TC-512 instrument (Techne, USA) using the following thermal cycling conditions: one cycle of 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec and a final extension cycle of 72°C for 10 min. The products were analyzed by 1.0% agarose gel electrophoresis with ethidium bromide staining and UV light-assisted visualization. The expected amplicon sizes were 475 bp (JEV I) and 524 bp (JEV III).

Specificity of the RT-LAMP assay: To analyze the specificity of the established RT-LAMP assay, JEV I and JEV III, PCV, PRRSV, CSFV, BVDV, SHEV, CHIKV, YFV and Sindbis virus were used as templates and subjected to RT-LAMP as described earlier. The reaction was performed at 63°C for 50 min.

RESULTS AND DISCUSSION

RT-LAMP amplification of the *E* gene from JEV I and JEV III and assay specificity: JEV RNA and the genotype-specific primers that targeted the virus *E* gene from JL0801 and YN0901 strains were included in an RT-LAMP assay that was performed at in a 65°C water bath for 60 min. The agarose gel electrophoresis analysis indicated that the amplified DNA products produced a characteristic laddering pattern with multiple bands which indicated that the final RT-LAMP products were mixtures of stem-loop DNAs with various stem lengths (Fig. 1a and b). In contrast, the negative control did not produce the characteristic multi-band laddering pattern.

Optimization of the RT-LAMP assay: The optimal reaction temperature and time of the RT-LAMP assay were investigated. As shown in Fig. 2a and b, the products of the RT-LAMP assay at different temperatures produced the characteristic multi-band laddering pattern. However, the intensity of bands in the ladder was the strongest from the reactions carried out at 63°C indicating the optimal temperature for RT-LAMP amplification of JEV E genes from JL0801 and YN0901. The RT-LAMP assay was then performed at the 63°C optimal temperature for different lengths of time. The subsequent results indicated that the DNA products with the highest intensity were produced using a 50 min reaction time

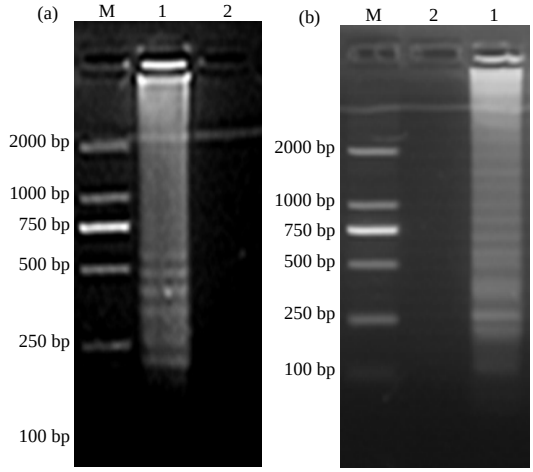

Fig. 1: Amplification of the *E* gene from a) JEV I and b) JEV III by RT-LAMP. JEV RNA was used as template and four genotype-specific primers targeting the virus *E* gene were used in an RT-LAMP assay performed at 65°C for 60 min. Lanes M: DL2000 marker; 1: RT-LAMP product; 2, negative control

(Fig. 2c and d). Therefore, the optimal reaction conditions of the RT-LAMP assay for detecting JEV was determined to be 63°C for 50 min.

Detection limit of the RT-LAMP assay compared with conventional RT-PCR: The sensitivity of the E-specific RT-LAMP assay was then compared with that of conventional PCR using E-specific primers. The JEV I and JEV III RNA initial concentration was converted to 2.57×10^6 and 2.34×10^6 copies/μL, respectively. Then, 10 fold serial dilutions of the JEV I and JEV III RNA molecular copies (from 10^6-10^{-1}) were used to determine assay sensitivity.

As observed by 2% agarose gel electrophoresis, the JEV I and III detection limit of the RT-LAMP assay was estimated to be 2.57 and 2.34 copies/μL, respectively (Fig. 3a and b). The RT-LAMP assay was more sensitive than the conventional RT-PCR assay (Fig. 3c and d). In addition, visual inspection of amplification products using SYBR Green I stain was better for the RT-LAMP assay than the conventional PCR assay (RT-LAMP detection sensitivity: 2.57 JEV I and 2.34 JEV III copies/μL vs. conventional RT-PCR; both $p<0.05$) (Fig. 3e and g). However, the SYBR Green I staining method was inferior to the electrophoresis detection method. Using UV transillumination allowed for RT-LAMP detection limits of 2.57 JEV I and 2.34 JEV III copies/μL (Fig. 3f and h) (Table 2).

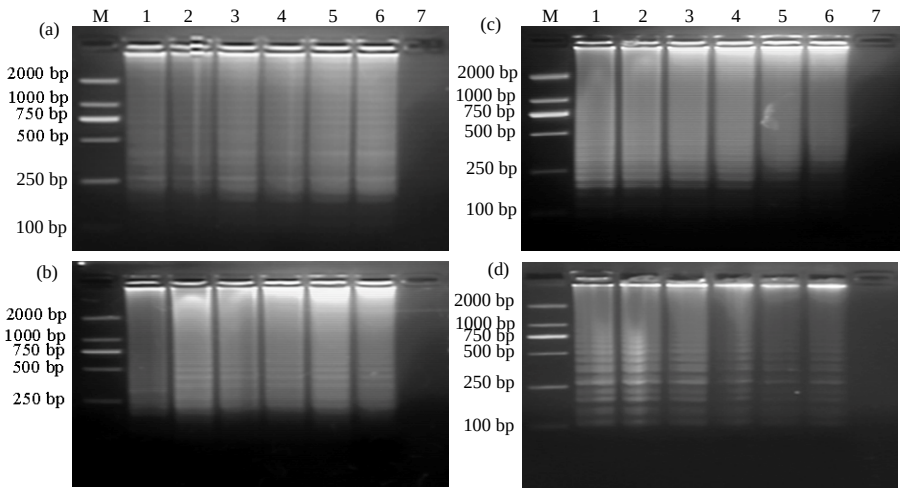

Fig. 2: Determination of optimal conditions for RT-LAMP detection of a, c) *JEV I* and b, d) *JEV III E* genes. The optimal temperature of the RT-LAMP assay was analyzed by performing the reaction at 60, 61, 62, 63, 64 or 65°C for 60 min. The reaction results are shown in Lanes 1-6, respectively, for a) JEV I and b) JEV III. The optimal reaction time for the RT-LAMP assay was analyzed by performing the reaction at 65°C for 60, 50, 45, 40, 35, 30 or 20 min. The reaction results are shown in Lanes 1-7, respectively for c) JEV I and d) JEV III. Lane M: DL2000 DNA marker; Lane 7: negative control

Table 2: Comparisons of RT-PCR Methods' detection sensitivity for JEV I and JEV III RNA

	Test							
Methods	Result	Result	Result	Result	Result	Result	Result	Result
RT-PCR RT-LAMP	+	+	+	+	+	-	-	-
Agarose gel analysis	+	+	+	+	+	+	+	-
Naked-eye inspection	+	+	+	+	+	+	+	-
UV transillumination	+	+	+	+	+	+	+	-

Concentrations of JEV I and JEV III RNA (copies μL^{-1}): JEV I (2.57×10^{6}, 2.57×10^{5}, 2.57×10^{4}, 2.57×10^{3}, 2.57×10^{2}, 2.57×10^{1}, 2.57×10, 2.57×10^{-1}); JEV III (2.34×10^{6}, 2.34×10^{5}, 2.34×10^{4}, 2.34×10^{3}, 2.34×10^{2}, 2.34×10^{1}, 2.34×10, 2.34×10^{-1}); +: positive reaction; -: negative reaction

Table 3: Comparison of real-time RT-PCR Methods' detection sensitivity for JEV I and JEV III RNA

	Test							
Methods	Result	Result	Result	Result	Result	Result	Result	Result
Real-time RT-PCR RT-LAMP	+	+	+	+	+	+	+	-
Agarose gel analysis	+	+	+	+	+	+	+	-
Naked-eye inspection	+	+	+	+	+	+	+	-
UV transillumination	+	+	+	+	+	+	+	-

Concentrations of JEV I and JEV III RNA (copies μL^{-1}): JEV I (2.57×10^{6}, 2.57×10^{5}, 2.57×10^{4}, 2.57×10^{3}, 2.57×10^{2}, 2.57×10^{1}, 2.57×10, 2.57×10^{-1}); JEV III (2.34×10^{6}, 2.34×10^{5}, 2.34×10^{4}, 2.34×10^{3}, 2.34×10^{2}, 2.34×10^{1} ,2.34×10 ,2.34×10^{-1}); +: positive reaction; -: negative reaction

Detection limit of the RT-LAMP assay compared with real-time RT-PCR: Serial 10 fold dilutions of JEV I and JEV III RNA were tested in parallel by real-time RT-PCR. The detection limits of conventional real-time RT-PCR were 2.57 and 2.34 copies/μL, respectively (Fig. 4a and b). Figure 5 shows the standard curves for JEV I (Fig. 5a) and JEV III (Fig. 5b) which were constructed using the known concentrations of the respective 10 fold serial dilutions. Melting curve analysis showed that specific amplification melting temperatures were 87.3°C (for JEV I; Fig. 6a) and 86.6°C (for JEV III, Fig. 6b). According to the amplification and dissociation plots, the amplified products could be identified as specific genomic sequences. As compared to products from the RT-LAMP assay, the detection sensitivities by agarose gel electrophoresis and SYBR Green I staining were equivalent with those of real-time RT-PCR. The details are shown in Table 3. Moreover, the RT-LAMP amplification procedure was completed within 50 min which was remarkably <2.5 h required for the real-time RT-PCR procedure.

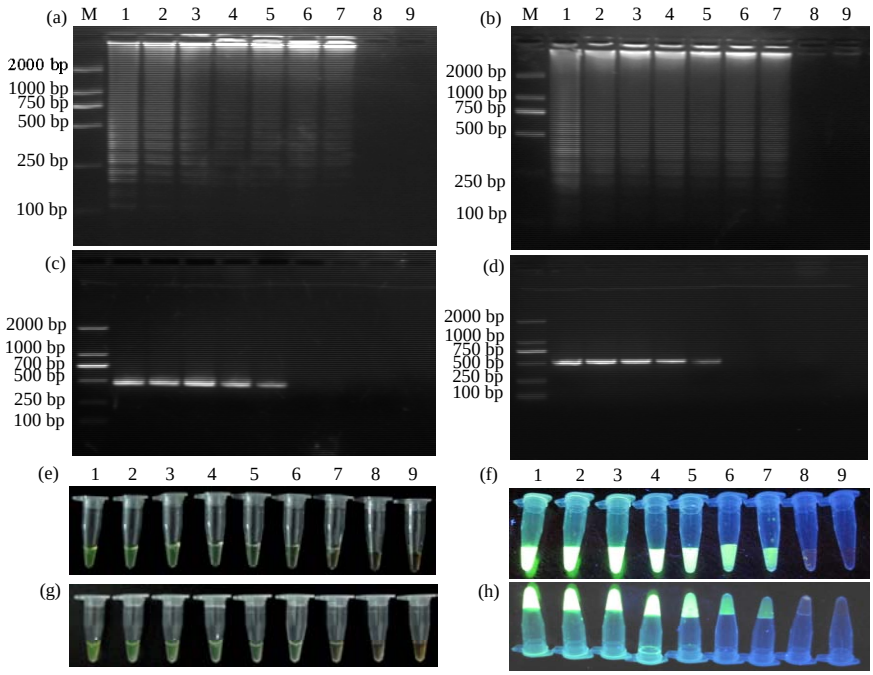

Fig. 3: Sensitivity of the RT-LAMP assay for JEV I and JEV III E genes as detected by electrophoresis and SYBR Green I staining. The RT-LAMP 2% on agarose gel electrophoresis detection limit for a) JEV I and b) JEV III. LAMP was 2.57 and 2.34 copies/μL, respectively. Lanes: 1-8: JEV I RNA concentrations from 2.57×10^6 to 0.257 copies/μL; JEV IIII RNA concentrations from 2.34×10^6 to 0.234 copies/μL; M: 100 bp DNA Ladder marker; 9: negative control. The conventional RT-PCR assay 1.5% agarose gel electrophoresis detection limit for c) JEV I and d) JEV III was 257 and 234 copies/μL, respectively. Lanes: 1-8, JEV I RNA concentrations from 2.57×10^6 to 0.257 copies/μL; JEV III RNA concentrations from 2.34×10^6 to 0.234 copies/μL; M: DL2000 DNA marker; 9: negative control. Naked-eye inspection of the RT-LAMP reaction stained with e, g) SYBR Green I under normal light and f, h) UV transillumination. For tubes 1-8, the concentrations of e, f) JEV I and g, h) JEV III RNA range from 2.57×10^6 to 0.257 copies/μL and from 2.34×10^6 to 0.234 copies/μL, respectively. Only positive amplifications are shown (the original orange color of SYBR Green I is green in the online version of this study (e, g). When visualized under UV transillumination the reaction appears as a bright green fluorescence (f, h). The detection limits of JEV I and JEV III RT-LAMP are respectively, 2.57 and 2.34 copies/μL by naked-eye inspection (in the online version) and 2.57 and 2.34 copies/μL under UV transillumination. Tube 9: negative control

Table 4: JEV RT-LAMP assay with swine blood samples

	No. of positive or negative samples (%)		
Results	RT-LAMP	RT-PCR	Real-time RT-PCR
JEV I-positive	2 (3.6)	0 (0.0)	1 (1.8)
JEV I-negative	54 (96.4)	56 (100.0)	55 (98.2)
JEV III-positive	44 (78.5)	38 (67.8)	43 (76.7)
JEV III-negative	12 (21.4)	18 (32.1)	13 (23.2)

Table 5: JEV RT-LAMP assay with mosquito samples

	No. of positive or negative samples (%)		
Results	RT-LAMP	RT-PCR	Real-time RT-PCR
JEV I-positive	3 (1.5)	1 (0.5)	3 (1.5)
JEV I-negative	197 (98.5)	199 (99.5)	197 (98.5)
JEV III-positive	38 (19.0)	24 (12.0)	34 (17.0)
JEV III-negative	162 (81.0)	176 (88.0)	166 (83.0)

Specificity of the RT-LAMP assay: Figure 7 shows that the RT-LAMP assay with JEV genotype-specific primers produced no positive reactions when using PCV, PRRSV, CSFV, BVDV, SHEV, CHIKV, YFV and Sindbis virus as templates.

Validation of the JEV RT-LAMP assay with swine blood samples and mosquito samples: Researchers tested the field applicability of the RT-LAMP assay by using acute-phase serum samples and mosquitoes taken from local agricultural hog pens with ongoing JEV epidemic. The results were compared with those from conventional RT-PCR and real-time RT-PCR. The LAMP assay demonstrated exceptionally higher sensitivity than conventional RT-PCR but had similar sensitivity to real-time RT-PCR (Table 4 and 5). All 10 negative serum

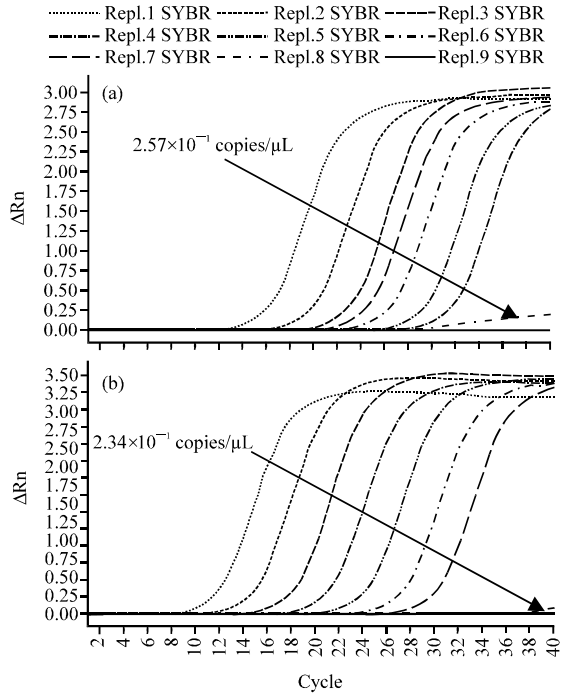

Fig. 4: Detection sensitivity of the real-time RT-PCR assay for a) JEV I and b) JEV III RNA. The amplification plots for detection of JEV I and JEV III show that the detection limit of the assay is 2.57 and 2.34 copies/µL, respectively. Replicates 1-8 represent the RNA concentration ranges for JEV I from 2.57×10^{6} to 2.57×10^{-1} copies/µL and for JEV III from 2.34×10^{6} to 2.34×10^{-1} copies/µL of initial quantity log copies. Replicate 9: negative control

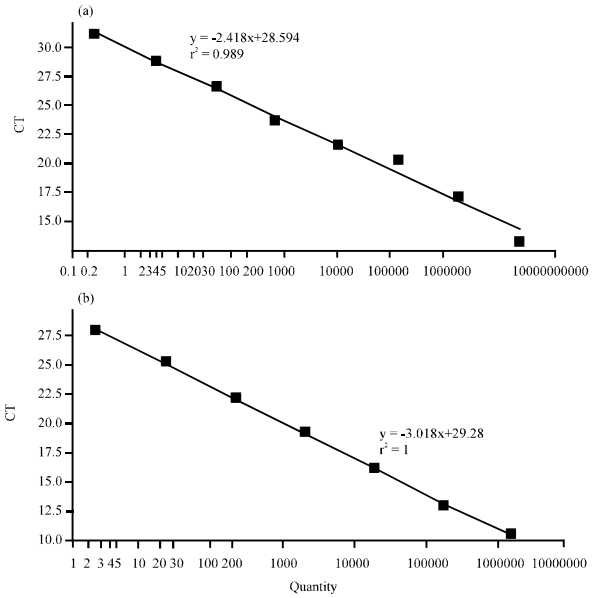

Fig. 5: Standard curve of real-time RT-PCR for detecting a) JEV I and b) JEV III. The assay standard curve was generated by the known concentration of virus RNA in serial 10 fold dilutions

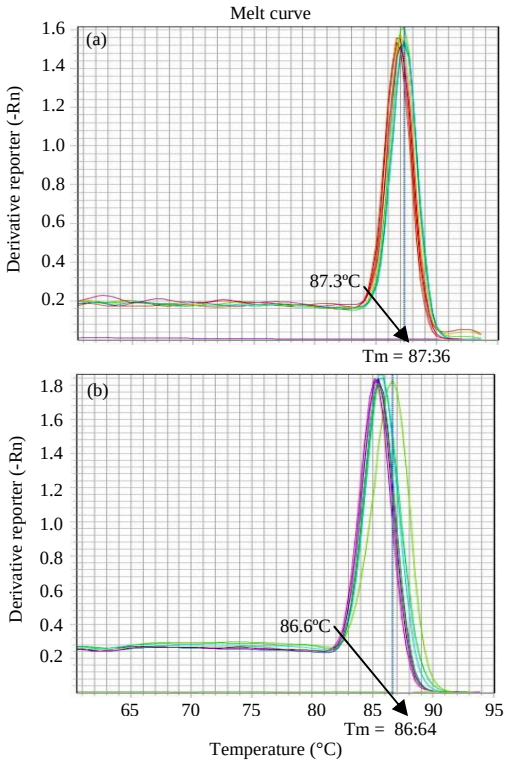

Fig. 6: Dissociation curve of real-time RT-PCR for detecting a) JEV I and b) JEV III using the sequence-specific DNA melting temperatures of 87.3 and 86.6°C, respectively

samples were also negative by all of the tests thereby ruling out the possibility of false positives. None of the RT-PCR or real-time RT-PCR-positive samples was missed by RT-LAMP, thereby indicating the higher sensitivity of the RT-LAMP assay.

JEV is an important pathogen of humans and agricultural animals. JEV infection in pigs causes severe reproductive disorders in both male and female pigs. Accurate diagnosis of JE in suspected encephalitis infections is essential for optimal disease management and protection of the remaining herd. However, the application of conventional assays such as RT-PCR, real-time RT-PCR, oligonucleotide microarray and ELISA has been limited by their requirements for laboratory-based operations, skilled technicians and expensive equipment. Considering the needs for a high-throughput method that can be readily adapted for use on routine pig farms, especially in remote rural areas where there is usually a high risk of JEV infection, a rapid, specific and sensitive test is urgently needed for effective surveillance of new has the advantages of reaction

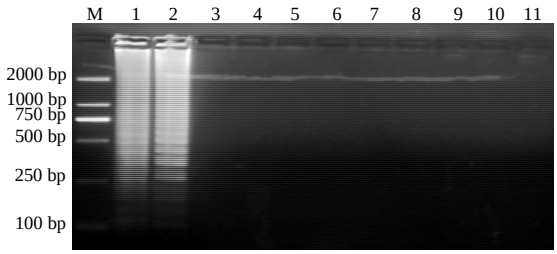

Fig. 7: Specificity of the RT-LAMP assay for detecting *JEV I* and *JEV III E* genes. JEV and other selected viruses were used as templates and subjected to RT-LAMP performed at 65°C for 60 min. Lanes M: DL2000 DNA marker; 1-10, RT-LAMP results from templates JEV I, JEV III, PCV, PRRSV, CSFV, BVDV, SHEV, CHIKV, YFV and Sindbis virus, respectively; 11: negative control

simplicity and detection sensitivity as the reaction is carried out in a single tube and by isothermal incubation; here, the optimal reaction parameters for *JEV E* gene detection were 63°C and 50 min. In contrast to conventional RT-PCR, real-time RT-PCR, oligonucleotides microarray and ELISA, the RT-LAMP Method does not rely on expensive or sophisticated facilities or equipment such as thermal cyclers. The amplification products of the reaction produced characteristic ladder-like patterns by agarose gel electrophoresis or were detectable by naked-eye inspection with SYBR Green I under normal light or UV transillumination.

Herein, researchers describe the establishment of a novel two-step RT-LAMP assay that can rapidly detect the *E* genes of JEV I and JEV III in swine and mosquitoes and which has higher sensitivity than conventional RT-PCR and real-time RT-PCR. In addition, the JEV RT-LAMP assay showed good specificity as no positive results were found in the negative control samples which included PCV, PRRSV, CSFV, BVDV, SHEV, CHIKV, YFV and Sindbis virus assayed under the same experimental conditions. However, since only Chinese JEV strains were tested, more research is required to determine how the RT-LAMP assay performs with all known strains of JEV.

CONCLUSION

In this study, researchers describe a LAMP Method for the detection of JEV I and JEV III in swine that is rapid, sensitive and specific. From a practical point of view this method is more suitable than the currently available methods for use as a routine diagnostic tool and can accommodate the large numbers of clinical samples required by active pig farms.

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