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# Rapid pre-clinical detection of classical swine fever by reverse transcription loop-mediated isothermal amplification

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ABSTRACT

The usefulness of reverse transcription loop-mediated isothermal amplification (RT-LAMP) for rapid pre-clinical detection of classical swine fever virus (CSFV) infection was evaluated. The RT-LAMP reaction could be finished in 60 min under isothermal condition at 65 °C by employing a set of four primers targeting the 5′ untranslated region of CSFV. The RT-LAMP assay of CSFV showed higher sensitivities than that of RT-PCR, with a detection limit of 5 copies per reaction. No cross-reactivity was observed from the samples of other related viruses including porcine circovirus type 2, porcine parvovirus, porcine pseudorabies virus, Japanese encephalitis virus, and porcine reproductive and respiratory syndrome virus. The detection rates of CSFV RT-LAMP, RT-PCR and virus isolation for samples including blood, tonsil, nasal and rectal swabs from uninoculated pigs without any clear clinical symptom were 89%, 78% and 71%, respectively. Furthermore, all of the assays showed higher sensitivity for blood and tonsil swabs samples than nasal and rectal swabs. These results indicate that the CSFV RT-LAMP assay is a valuable tool for its rapid, cost-effective detection and has potential usefulness for rapid pre-clinical detection and surveillance of classical swine fever in developing countries.



## 1. Introduction

Classical swine fever (CSF) is a highly contagious disease affecting swine and resulting in severe economic losses, characterized by fever, neurological disorders, hemorrhages, and high mortality rates. The etiological agent, classical swine fever virus (CSFV), is a member of the genus *Pestivirus*, which belongs to the family Flaviviridae [1,2]. CSFV is a small, enveloped virus with a 12.5-kb positive, single-stranded RNA genome containing a single large open reading frame encoding a polyprotein precursor, which is cleaved co- and post-translationally by cellular and viral proteases into structural and nonstructural proteins [3]. Both ends of the genome are flanked by 5′ and 3′ untranslated regions (UTR), which are highly conserved among all of the virus isolates [4–7]. The 5′ UTR functions as an internal ribosomal entry site for translation initiation of the pre-polyprotein and genome replication [8].

The disease was endemic in many areas of the world during the last decades. Although some countries applied an eradication policy for infected animals, this strategy decreased the risks rather than eliminated CSF. Furthermore, prenatal infection of fetuses could lead to persistently infected animals which shed virus over a long period of time, as did pigs which exhibit the chronic form of disease or no clear clinical symptoms [9,10]. A rapid diagnosis based solely upon clinical signs is difficult and can result in late detection [11]. Rapid and pre-clinical laboratory diagnosis of CSFV is therefore a matter of urgency in order to prevent and control the epidemics. Current methods for diagnosis of CSFV rely on virus isolation [12], fluorescent antibody technique [13], enzyme-linked immunosorbent assay [14,15], RT-PCR [7,16–20] and real-time RT-PCR [21]. RT-PCR and real-time RT-PCR procedures are generally considered to be the most sensitive in vitro method for detecting CSFV infection. However, this technique requires centralized laboratory facilities and clinical specimen submissions, which delay disease diagnosis, thus affecting the efficiency of emergency disease management.

Loop-mediated isothermal amplification (LAMP), a novel amplification method, had been originally developed by Notomi et

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al. [22]. The most significant advantage of LAMP is the ability to amplify specific DNA sequences under an invariable temperature between 63 °C and 65 °C and enable visual judgment within 60 min, besides its highly sensitivity and specificity. The method had been successfully applied to detect human influenza A virus, severe acute respiratory syndrome coronavirus and Japanese encephalitis virus [23–25]. But RT-LAMP for detecting CSF has not been reported so far and a rapid pre-clinical diagnosis at the site of any suspected disease outbreak would be extremely useful for controlling CSF in endemic countries. In this study, we investigated the potential of RT-LAMP for rapid pre-clinical detection of CSFV infection.

## 2. Materials and methods

### 2.1. Viral strains and clinical samples

The reference strains of CSFV-C from Chinese Veterinary Microorganism Conservation Center were used to standardize the RT-LAMP method in this study. The other strains including field isolates of CSFV-LT, CSFV-GS, porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), pseudorabies virus (PRV), Japanese encephalitis virus (JEV), and porcine reproductive and respiratory syndrome virus (PRRSV) were identified by conventional PCR or RT-PCR and sequencing.

A total of 483 samples including blood, tonsil, nasal and rectal swabs were collected from uninoculated pigs without clear clinically syndrome in different pig farms at same area in Northwest China. The samples were maintained in Hanks' balanced salts solution with antibiotics of 0.25 μg/ml amphotericin B, 50 μg/ml gentamicin and 50 μg/ml sodium benzyl penicillin. All clinical samples were subjected to both virus isolation and RT-PCR. Among 483 samples, 11 of blood, 12 of tonsil swabs, 14 of nasal swabs and 8 of rectal swabs were positive by RT-PCR or virus isolation. The other 438 samples were confirmed as negative by both virus isolation and RT-PCR (Table 1).

### 2.2. Virus isolation

One hundred microliters of sample suspensions were inoculated onto confluent PK15 cells, seeded in a 24-well plate. Following the initial isolation, five to eight consecutive passages on cell cultures were carried out using each time 300 μl of the supernatants collected previously as starting material. Then the supernatant was collected and the plate was washed five times with water. Subsequently, the plates were dried at 37 °C for 15 min. Next, 500 μl isopropanol was added to the wells and was left to incubate for 15 min at 4 °C. After removal of the isopropanol, the plates were dried at 37 °C for 15 min. Finally, 500 μl FITC-labelled polyclonal antibodies were added to the wells following an incubation of 1 h at 37 °C. The plates were washed three times with water and the excess was removed with absorbent paper. The plates were examined using a UV-microscope.

### 2.3. RNA and DNA extraction

Total RNA was extracted from blood, tonsil, nasal and rectal swabs sample and cell culture of JEV and PRRSV infection, respectively, using Trizol reagent (Invitrogen) according to the manufacturer's instructions. DNA was extracted directly from PCV2, PPV and PRV by using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. After extraction, DNA or RNA was eluted in 60 μl of elution buffer and stored at −20 °C until further use.

### 2.4. Primers design and reaction regime of RT-LAMP and RT-PCR

The highly conserved sequence in the 5′ untranslated regions was selected as the target sequence of RT-PCR and RT-LAMP. P1 and P2 primers were applied in RT-PCR. A set of 4 primers were designed for RT-LAMP according to alignment analysis of CSFV genomic sequences in GenBank (Accession number DQ127910, AY775178, AF333000, D49532, and AF099102). The primers for RT-PCR and RT-LAMP were shown in Table 2.

Total RNA was extracted and RT-PCR was conducted using a one-step RT-PCR kit (Takara, Corp., Japan). RT-PCR was performed in 25 μl of reaction mixture consisting of PCR buffer, 250 μM deoxynucleoside triphosphate, 5 mM $MgCl_2$, 20 U of RNAase inhibitor, 2.5 U of AMV RTase XL, 2.5 U of AMV-Optimized Taq polymerase, 0.4 μM P1 and P2 primers and 5 μl of RNA. Reaction conditions were set at 50 °C for 45 min and 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 70 °C for 45 s. A total of 10 μl of RT-PCR product was then analyzed by 2.5% agarose gel electrophoresis in tris-buffer, and target bands were visualized by staining with ethidium bromide.

The RT-LAMP reaction was carried out in a conventional water bath by mixing 2.0 μM each of FIP and BIP primer, 0.2 μM each of F and B primer, 1.0 mM each deoxynucleoside triphosphate, 1 U of the THERMO-X reverse transcriptase (Invitrogen) and 8 U of Bst DNA polymerase (New England Biolabs) using the supplied 10×buffer containing 2 mM of MgSO4, 0.8 M betaine and 1 μl of template RNA or DNA. The amplification reaction was performed at 65 °C for 60 min and heated at 80 °C for 10 min to terminate the reaction. RT-LAMP products were analyzed by 2.5% agarose gel electrophoresis.

### 2.5. Detection of RT-LAMP amplification

In order to discriminate the false positive reaction producing similar ladder-like bands, the products of the reaction were also

**Table 1**
Comparative evaluation of RT-LAMP assay with RT-PCR and virus isolation for 483 samples obtained from uninoculated pigs without clear clinical signs.

| Specimen type | No. of samples tested | No. of positive samples[a] | No. (%) of samples with indicated result by: | | |
|---|---|---|---|---|---|
| | | | Virus isolation | RT-PCR | RT-LAMP |
| Blood | 124 | 11 | 8 (73) | 9 (82) | 10 (91) |
| Tonsil swabs | 98 | 12 | 10 (83) | 11 (92) | 12 (100) |
| Nasal swabs | 154 | 14 | 10 (71) | 10 (71) | 12 (86) |
| Rectal swabs | 107 | 8 | 4 (50) | 5 (63) | 6 (75) |
| Total | 483 | 45 | 32 (71) | 35 (78) | 40 (89) |

[a] Positive samples were confirmed by virus isolation or RT-PCR and sequencing.

**Table 2**
Details of RT-PCR and RT-LAMP primers designed for detection of 5' UTR sequences of CSFV.

| Primer name[a] | Genome position | Sequence |
|---|---|---|
| P1 | 1–20 | 5′-GTATACGAGGTTAGTTCATT-3′ |
| P2 | 480–500 | 5′-TTGTGGGTGTACCTCACTGG-3′ |
| F | 114–134 | 5′-ACTAGCAAACGGAGGGACTA-3′ |
| B | 360–380 | 5′-CTCCATGTGCCATGTACAGC-3′ |
| FIP | 180–200 | 5′-AGCTCCCTGGGTGGTCTAAG+TTTT+ |
| FIP | 146–166 | CGTCGAACTACTGACGACTG-3′ |
| BIP | 239–259 | 5′-GCCTCTGCAGCGCCCTATCA+TTTT+ |
| BIP | 312–332 | GCCTCTGCAGCGCCCTATCA-3′ |

[a] The primers of F, B, FIP and BIP were for RT-LAMP and each inner primers of RT-LAMP has two binding regions connected by a TTTT spacer. Primers of P1 and P2 were applied in RT-PCR.

inspected by the naked eye. Follow amplification, the tubes were inspected for white turbidity using the naked eye after a pulse spin to deposit the precipitate in the bottom of the tube. The observation of color changed was also conduced following the addition of 1 μl of SYBR Green I dye to the tube. In the case of positive amplification, the original orange color of the dye would change into green that can be judged under natural light. In case there is no amplification, the original color of the dye would be retained. This change of color is permanent and can be kept for record purpose.

### 2.6. Sensitivity and specificity of RT-LAMP for CSFV

To determine the sensitivity of the CSFV RT-LAMP assay, the CSFV-C RNAs were prepared in vitro transcription and quantitated using UV spectrophotometry at 260 nm (UNICAM 3000, US). A series of 5×dilutions, spanning from 1 to $5^5$ copies/tube were used to test the sensitivity of RT-LAMP reactions and compared with RT-PCR by using the same templates at identical concentrations. In addition, to assess the specificity of the RT-LAMP for CSFV, the different DNAs from tissue of PCV2, PPV and PRV and RNA from PRRSV, JEV and CSFV were extracted and used as the template for CSFV RT-LAMP, respectively. The cross-reactions of RT-LAMP with DNA of PCV2, PPV, PRV and RNAs of PRRSV, and JEV were carried out. The CSFV-GS and CSFV-LT strain genomic RNAs were used as positive control and RNAs extracted from healthy swine blood were used as negative control.

### 2.7. RT-LAMP assay for CSFV with clinical samples

All clinical samples were detected with CSFV RT-LAMP. The sensitivities of CSFV RT-LAMP were calculated and compared with conventional RT-PCR and virus isolation.

### 2.8. DNA sequencing

All of the PCR products were determined with an automated ABI model 373 A Stretch DNA sequencer. DNAStar software was used to align the sequences and combine with the BLAST program search of GenBank for a homology check with known 5′ untranslated region sequence of CSFV.

## 3. Results

### 3.1. Detection limit of RT-LAMP method compared to RT-PCR

A successful RT-LAMP reaction with CSFV-specific primers at 65 °C in 60 min produced many bands of different sizes upon agarose electrophoresis, since RT-LAMP products consisted of several inverted-repeat structures. The amplification by RT-LAMP showed a ladder-like pattern, whereas RT-PCR products were a specific DNA band. Sequencing result indicated that the amplified partial 5′ UTR sequence of RT-PCR was a 500 bp amplicon. The detection limit of CSFV RT-LAMP was 5 copies per reaction and that of CSFV RT-PCR was 125 copies per reaction (Fig. 1). Thus, the comparative sensitivity of RT-LAMP and RT-PCR indicated that the detection limit of RT-LAMP for CSFV was 25 times less than that of conventional RT-PCR.

### 3.2. Analytical cross-reaction of CSFV RT-LAMP method

Agarose gel electrophoresis analysis indicated that no DNA band of PCV2, PPV, PRV, JEV, and PRRSV was observed by CSFV RT-LAMP, only CSFV-GS and CSFV-LT RNAs demonstrated specifically positive reaction (Fig. 2).

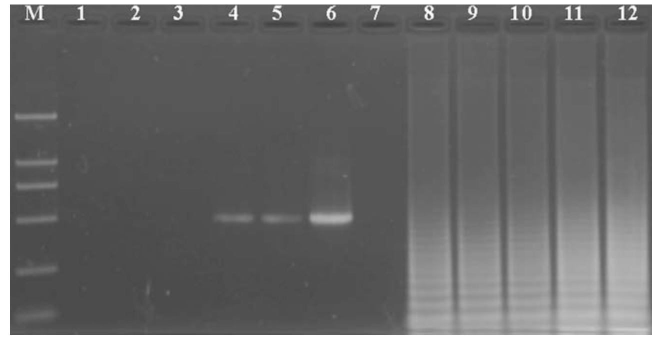


**Fig. 1.** Comparative sensitivities of RT-LAMP and RT-PCR for the detection of CSFV by agarose gel electrophoresis analysis. From left to right: Lane M, DNA Marker DL-2000 (Takara); Lanes 1–6, different CSFV RNAs copy numbers of RT-PCR (1, $5^1$, $5^2$, $5^3$, $5^4$ and $5^5$ copies/tube, respectively); Lanes 7–12, different CSFV RNAs copy numbers of the RT-LAMP assay (1, $5^1$, $5^2$, $5^3$, $5^4$ and $5^5$ copies/tube, respectively). RT-PCR products showed a specific amplification for CSFV with a detection limit of $5^3$ copies per tube, whereas detection limit of RT-LAMP is 5 copies per tube.

### 3.3. Comparative sensitivity of detection from clinical sample by RT-LAMP and RT-PCR

In order to evaluate the ability of pre-clinical detection for CSFV by RT-LAMP, the detection rates of virus isolation, RT-PCR and RT-LAMP for blood, tonsil, nasal and rectal swabs were analyzed, respectively. The general detection rates of CSFV RT-LAMP, RT-PCR and virus isolation for above mentioned different clinical samples were 100%, 83% and 72%, respectively. In general, all of the assays showed higher sensitivity for blood and tonsil swabs samples than nasal and rectal swabs (Table 1).

## 4. Discussion

Previous researchers have reported that CSFV, PPV, PRV and PRRSV may cause diseases with similar clinical symptoms [26,27]. In addition, CSFV co-infection with PCV2, PRRSV, PRV and PPV played an important role in reproducing typical postweaning multisystemic wasting syndrome [28–30]. Taxonomically, JEV belonged to the same Flaviviridae family as CSFV. Therefore, the development of a simple and rapid diagnostic tool that can detect CSFV and differentiate it from PCV2, PPV, PRV and PRRSV in the same samples would be of significant importance in the epidemiologic surveillance and the prediction of severity of economically important viral diseases in swine herds.

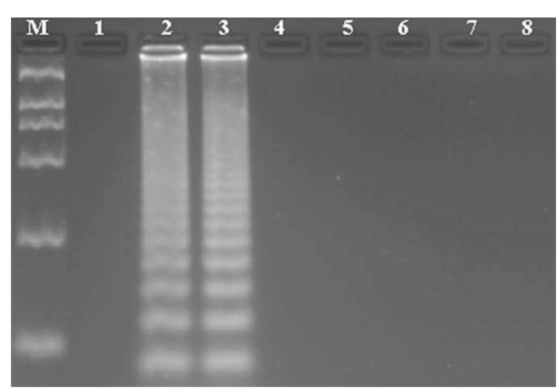


**Fig. 2.** Electrophoresis analysis of cross-reaction of CSFV RT-LAMP with PCV2, PPV, PRV, JEV and PRRSV. Lane M, DNA Marker DL-2000. Lane 1, negative control. Lanes 2, RNA of CSFV-LT. Lane 3, RNA of CSFV-GS. Lane 4, DNA of PCV2. Lane 5, DNA of PPV. Lane 6, DNA of PRV. Lane 7, RNA of PRRSV. Lane 8, RNA of JEV.

RT-LMAP operation is quite simple, requiring only a conventional water bath or heat block for incubation at isothermal conditions. Another useful feature was RT-LAMP products could be directly observed by the addition of SYBR Green I dye to the amplified products [31]. Therefore, this technique is effective due to the high specificity and amplification efficiency, and may facilitate the application of RT-LAMP, especially in the place as a dental chair-side.

The sensitivity of CSFV RT-LAMP in this study was greater than that of the RT-PCR method, which is in accordance with the sensitivity of other RT-LAMP methods for detection of porcine circovirus type 2, Japanese encephalitis virus, mumps virus and West Nile virus [23,32–34]. There is no cross-reaction with of CSFV RT-LAMP with PCV2, PPV, PRV, JEV, FMDV and PRRSV suggested that the method is highly specific among the viral strains we tested. In addition, RT-LAMP is a simple and timesaving procedure, allowing the results to be obtained within 1 h, whereas RT-PCR method requires 2–4 h. Some positive samples detected by RT-LAMP were missed by RT-PCR. Compared with RT-PCR, RT-LAMP method appears to be a fast and sensitive tool in the clinical diagnosis of CSFV infection.

CSFV can be detected in many tissues, such as blood, lymph nodes, spleen, kidney and tonsil swabs following experimental infections [21,35]. In this experiment, we evaluated the applicability of CSFV RT-LAMP for different samples including blood, tonsil, nasal and rectal swabs. Blood and tonsil swabs seemed to be the preferable samples for the pre-clinical diagnosis of CSFV. This was in agreement with the result of real-time RT-PCR assay by Ralph et al. [36]. And 18 of 172 samples were positive for CSFV suggested some sub-clinical infection exist in the swine herd. Importantly, the pre-clinical detection of CSFV suggests two potential uses in disease control for the assay: as a surveillance tool in areas free of the disease and as a screening assay for monitoring a disease outbreak. Nonetheless, the reliability of this assay should be further evaluated by large-scale investigation.

In conclusion, CSFV RT-LAMP is a simple, highly sensitive, rapid and reliable method that would be more suitable for the pre-clinical detection of CSFV infection in developing countries.

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