DETECTION OF NUCLEIC ACID OF CLASSICAL SWINE FEVER VIRUS BY REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP)

By

Kanokwan Wongsawat

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

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Department of Biology

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การตรวจกรดนิวคลีอิกของเชื้อไวรัสโรคอหิวาต์สุกรโดย Reverse Transcription Loop - Mediated Isothermal Amplification (RT-LAMP)

โดย
นางสาวกนกวรรณ วงษ์สวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ปีการศึกษา 2553

The Graduate School, Silpakorn University has approved and accredited the Thesis title of "Detection of nucleic acid of classical swine fever virus by reverse transcription loop-mediated isothermal amplification (RT-LAMP)" submitted by Miss Kanokwan Wongsawat as a partial fulfillment of the requirements for the degree of Master of Science in Biology.

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In the present study, we developed reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay targeting the 5'untranslated gene for the detection of CSFV. The RT-LAMP assay is very simple and rapid, wherein the amplification can be obtained in 60 min under isothermal conditions at 65℃ by employing a set of six specific primer mixtures. A positive amplification was visualized with the naked eye by using Hydroxynaphthol blue (HNB) dye; indicated by a color change from violet to sky blue and had a ladder-like appearance by agarose gel electrophoresis. The sensitivity of this assay was 100 copy numbers. No cross-reactivity related to Japanese encephalitis (JE) virus and Porcine reproductive and respiratory syndrome (PRRS) virus was found. Our results clearly demonstrated that the developed HNB RT-LAMP assay visualized with naked eye is an extremely rapid, cost-effective, sensitive, and specific method for the detection of CSFV RNA. In addition, the FTA technology was investigated for its use for storage, transport, collection, and subsequent molecular analysis of CSFV RNA, facilitating epidemiological investigations in the field.

Department of Biology Graduate School, Silpakorn University Academic Year 2010
Student's signature ……..
Thesis Advisors' signature 1……2. ……

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คำสำคัญ: เชื้อไวรัสอหิวาต์สุกร/ วิธี RT-LAMP/ สี Hydroxynaphthol blue

กนกวรรณ วงษ์สวัสดิ์ การตรวจกรดนิวคลีอิกของเชื้อไวรัสโรคอหิวาต์สุกรโดย Reverse Transcription Loop - Mediated Isothermal Amplification (RT-LAMP). อาจารย์ที่ปรึกษาวิทยานิพนธ์: อ. ดร. จันทร์ดี ระแบบเลิศ และ ศ. ดร. พญ. ธารารัชต์ ธารากุล. 72 หน้า.

ในการศึกษาครั้งนี้ผู้วิจัยได้พัฒนาวิธีตรวจหาเชื้อไวรัสอหิวาต์สุกรด้วยเทคนิค reverse transcription loop-mediated isothermal amplification (RT-LAMP) จากบริเวณ 5'untranslated gene ซึ่งเป็นวิธีที่ง่ายและรวดเร็ว กล่าวคือ สามารถเพิ่มปริมาณ gene ได้ในเวลา 60 นาที ภายใต้อุณหภูมิ 65 องศาเซลเซียส เพียงอุณหภูมิเดียว โดยใช้ primer ที่จำเพาะเจาะจง จำนวน 6 เส้น ปริมาณ gene ที่เพิ่มจำนวนอ่านผลด้วยตาเปล่าโดยดูการเปลี่ยนสี Hydroxynaphthol blue (HNB) จากสีม่วงเป็นสีฟ้าน้ำเงิน และอ่านผลด้วยวิธี Agarose gel electrophoresis จะปรากฏรูปร่างคล้ายขั้นบันได ค่าความไวของวิธีนี้ที่วัดได้คือจำนวน 100 copy ไม่สามารถตรวจวัดเชื้อไวรัสไข้สมองอักเสบ (JEV) และเชื้อไวรัสก่อโรคระบบทางเดินหายใจและระบบสืบพันธุ์ในสุกร (PRRSV) จากผลการทดลองแสดงให้เห็นชัดเจนว่าวิธี HNB RT-LAMP อ่านผลด้วยตาเปล่า วิธีนี้มีประโยชน์สำหรับการตรวจเชื้อ CSFV ในสุกร ที่ว่องไวและรวดเร็ว นอกจากนี้ ผู้วิจัยได้ศึกษาการใช้ประโยชน์จากเทคโนโลยี FTA สำหรับการเก็บ ขนส่ง สะสม และการวิเคราะห์ด้านอณูโมเลกุลของ RNA เชื้อ CSFV ซึ่งมีความสะดวกสบายในการศึกษาทางด้านระบาดวิทยาในภาคสนาม

ภาควิชาชีววิทยา บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร ปีการศึกษา 2553

ลายมือชื่อนักศึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1. .....2. .....

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Kanokwan Wongsawat

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CHAPTER I

INTRODUCTION

Classical swine fever (CSF) is a highly contagious disease affecting swine, resulting in severe economic losses (12). Classical swine fever virus (CSFV), the causative agent of CSF is a member of the genus *Pestivirus* within the family *Flaviviridae* (30). CSFV is a small, enveloped virus with a 12.5 kb, positive single-stranded RNA genome containing a single, large open reading frame (ORF) flanked by two highly conserved untranslated regions (UTR) at the 5' and 3' ends. The 5' UTR functions as an internal ribosomal entry site for translation initiation of the pre-polyprotein and genome replication (56). The host range of CSFV is narrow; this virus is restricted to its natural hosts, domestic pigs and feral pigs. The control of CSF is based on stamping out policies and/or on vaccination. However, failed immunization against CSF has been reported, mainly due to interference from maternal antibodies and other infections (25, 59).

In Thailand, CSF was first reported in 1950 at Bangkhen area in Bangkok. It was declared a notifiable disease in 1954 up to date; it has gradually become enzootic (53). The disease is generally subjected to statutory control, involving slaughter of affected pig herds and restrictions on movements of pigs and pig products from affected regions (30). Although eradicated from many countries, CSF continues to cause serious problems in different parts of the world (43). Rapid and accurate diagnosis is the key factor for the control of CSF. The diagnosis based on clinical signs is often difficult, because clinical signs are rather variable and may be mistaken for other febrile and/or haemorrhagic diseases of pigs, essentially African swine fever (31). Rapid, accurate, and pre-clinical laboratory diagnosis of CSFV is therefore a matter of urgency in order to prevent and control the epidemics. Current laboratory diagnoses of CSFV rely on virus isolation, serological methods, detection of antigen and nucleic acid amplification (8, 20, 49, 67). The PCR-base procedures are generally considered to be the most sensitive *in vitro* method for detecting CSFV infection.

However, these techniques require centralized laboratory facilities and clinical specimen submissions.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method developed by Notomi *et al*. (37). The benefits of LAMP compared with other nucleic acid amplification techniques is easy operation, no need for special equipments, superior sensitivity and speed, low contamination risk, and suitability for high-throughput DNA detection. Additionally, LAMP products can be observed by the naked eye when a white precipitate of magnesium pyrophosphate is present in the reaction mixture (32). However, this detection is limited when the turbidity of reaction is low. In gel electrophoresis, LAMP products stained with ethidium bromide was used (44). Unfortunately, the product detection system with ethidium bromide has several limitations, such as generation of hazardous waste and sensitivity less than that of SYBR Green. Therefore, many investigators have developed RT-LAMP to be visualized by naked eye and with SYBR Green for DNA detection (24).

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) was used as tool for amplification and has emerged as a powerful RNA amplification tool due to its simplicity, speed, specificity and cost-effectiveness. This technique is being used increasingly for rapid detection and typing of emerging viruses such as Severe acute respiratory syndrome coronavirus (22), Japanese encephalitis virus (42), Pseudorabies virus (15) and Classical swine fever virus (4). Rapid and cost-effective RT-LAMP assays for the pre-clinical detection of CSFV visualized directly with the naked eye by addition of SYBR Green have been described (4, 65-66). SYBR Green is an intercalating agent binding with double-stranded DNA; thus a disadvantage of SYBR Green is that it is equally incorporated into every amplicon. Should unspecific sequences be amplified, the measured signal would correspond to both non-specific and specific products (18). Then, Tomita *et al*. (60) developed a simple colorimetric assay for the detection of the RT-LAMP reaction by adding calcein, a fluorescence metal indicator. The result showed that detection limit of the LAMP assay using calcein was 10 times lower than those using SYBR Green. In addition, the brightness of calcein fluorescence was significantly weaker than that of SYBR green fluorescence. It has been reported that Hydroxynaphthol blue (HNB) was used as a

colorimetric indicator for the titration of calcium ion and magnesium ion. In addition, the sensitivity of LAMP assay using HNB was equivalent to that of the assay using SYBR Green (19). Therefore, this colorimetric assay is suitable not only for laboratory research but also for clinical diagnoses of many infectious diseases.

Flinders Technology Associates (FTA) Classic Card is a treated filter paper for the collection, storage, and transportation of blood samples or other biological samples for subsequent DNA/RNA analysis. Nucleic acids are recovered by punching the impregnated area and eluting with classical reagents. Advantages of FTA technology have been demonstrated for the molecular characterization of RNA from foot and mouth disease virus, rabies virus (33, 45) but have not been well documented for CSFV.

Overall, the purpose of our study was to develop RT-LAMP assay using HNB dye and its application with using FTA cards for the collection, storage, and transport of CSFV.

CHAPTER II

LITERATURE REVIEW

1. Classical swine fever virus (CSFV)

CSFV belongs together with viruses in the Bovine viral diarrhea virus (BVDV) species and viruses in the Border disease virus (BDV) species, to the genus *Pestivirus*, in the family *Flaviviridae* (52). CSFV is an enveloped virus with a diameter of 40–60 nm. The single-stranded RNA genome has a size of 12.5 kb. It has positive polarity with one open reading frame (ORF) flanked by two nontranslated regions (NTRs). The 5'NTR functions as an internal ribosomal entry site (IRES) for cap-independent translation initiation of the large ORF that code for a polyprotein of about 3,900 amino acids. The polyprotein is co- and post-translationally processed by viral and cellular proteases. From 11 viral proteins, four of them constitute the structure of the particle: namely three envelope glycoproteins (E^{rns}, E1, and E2) and one core (C) protein. The remaining seven nonstructural proteins are N^{pro}, p7, NS2-3, NS4, and NS5. The main target for neutralizing antibodies is the viral envelope glycoprotein E2. To a lesser extent, the host immune system produces neutralizing antibodies to E^{rns}. This viral envelope glycoprotein occurs as a disulfide-bonded homodimer in the virus particle, and is also secreted by CSFV-infected cells. E^{rns} was shown to be a potent ribonuclease specific for uridine. N^{pro} has an autoproteolytic activity that achieves cleavage from the downstream nucleocapsid protein. *In vitro*, it has been shown that N^{pro} interferes with the induction of interferon-α/β, and *in vivo* CSFV with a deletion in the N^{pro} gene was able to infect pigs but had lost its pathogenicity, as shown in Figure 1 (56).

2. Clinical signs

Historically, different levels of virulence have been reported from peracute, acute, chronic or prenatal forms of CSF. The virulence of a strain is difficult to establish as the same isolate can induce different signs depending on the age

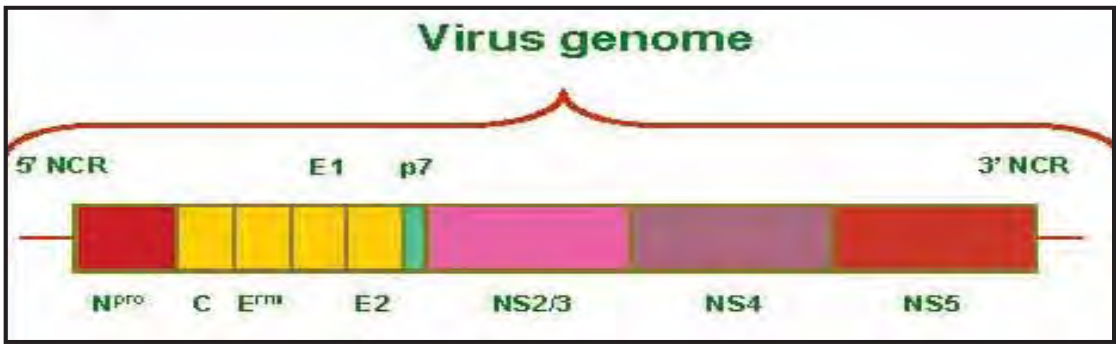


Figure 1 Organization of the approximately 12.5 kb CSFV RNA genome: 5'NCR, N-terminal proteinase (N^{pro}), Core (C), Envelop (E^{rns}, E1, E2), Protein(p7), Non-structural proteins (NS2/3, NS4, NS5) and 3'NCR

(e.g. younger age animals are more susceptible), breed, health status and immune status of the inoculated pigs (31).

Piglets develop more evident clinical signs than the adults. The constant symptom is the hyperthermia (17), usually up to 40°C, but in adults it can be lower (39.5°C). The first usual signs in acute form are anorexia, lethargy, conjunctivitis, respiratory signs, and constipation followed by diarrhea. During a chronic course of the disease, the issue is generally fatal. After displaying at first similar clinical signs as in an acute form, the pigs survive for two to three months but normally not more. They display non-specific signs as fever, diarrhea, wasting, anorexia, and disorders.

In pregnant sows, CSFV is able to cross the placenta of and infect the fetuses during all the stages of pregnancy. Depending on the virulence of the strain and the time of gestation, the infection can result in abortion and stillbirths in early pregnancy and can lead to the birth of persistently viraemic piglets if infection occurs during the first 50-70 days of gestation. These piglets seem normal at birth but rapidly waste or display congenital tremor (61). These animals shed a lot of virus for several months and are very dangerous reservoirs and sources of infection.

In adult boars, experimental infection with the CSFV virus has no evident effects on libido and ejaculative parameters of adult boars (63). The clinical course was mild in the boars with an increase in body temperature, but never above 39.9°C and a transient anorexia. The libido remained good, and the quality of semen collected

in from three boars was always in the range of the minimum requirements for sperm that is used for artificial insemination. In another experiment carried out by Floegel *et al*. (16), four young boars were infected with a CSF field virus strain and semen was collected at least every other day after infection. The course of CSF infection was mild but clinically detectable during the second week of infection. CSF virus was isolated from semen of two animals during the pyretic phase and from the epididymis but not from the testes. Since CSF virus shedding via semen could be detected, it was concluded that the disease may also be transmitted by artificial insemination. Insemination boars may thus be of special epidemiological relevance for the dissemination of the CSF virus as clinical symptoms are mild.

3. Pathogenesis

CSFV is known to be immunosuppressive (57). However, neutralizing antibodies appear usually after one to two weeks post infection in recovering pigs. CSFV grows in cell culture without any cytopathogenic effect, preventing the antiviral effect of INFα and apoptosis (51). Since CSFV is noncytopathic *in vitro*, it has been suspected that the serious lesions seen *in vivo* were linked to immunopathological damages. The usual entry site is the oronasal route; the first site of virus replication are the tonsils. Then the virus spread to the regional lymph nodes, before reaching, via the peripheral blood, the bone marrow, visceral lymph nodes and lymphoid structures linked to the small intestine, and spleen. The spread of the virus within the pig is usually completed in less than 6 days during infection, severe changes occur in the bone marrow and in the circulating white cell population, suggesting an indirect cytopathic effect (CPE) induced in non infected cells either by a soluble factor, or by cell to cell contact (58). The pathogenic mechanism therein would involve indirect virus-host interactions, probably originating from the site of primary infection, rather than a direct effect of the virus or viral protein (57). The interactions between both viruses and the monocyte-macrophage system result in the release of mediator molecules, which are important for the further progression of the disease. The changes in the haemostatic balance are thought to be caused by pro-inflammatory and antiviral factors, inducing the thrombocytopenia and the mechanisms of the hemorrhages, which are characteristic in the infection (28). The

production of inflammatory cytokines by infected endothelial cells could play a role in the immuno-suppression, as well facilitating virus dissemination by attracting monocytic cells (1).

4. RNA isolation

RNA isolation is one of the most often underestimated critical steps when RT-PCR is used for mass sample screening. Two points in the isolation process have to be carefully considered: the treatment and handling of the samples prior to RNA isolation and the storage of the isolated RNA (2). Since the first step in the procedure is lysis of the cells by adding a strong denaturant (such as SDS, phenol) that inactivates the enzymes including the RNases. It is typically added prior to and after the isolation, when RNA integrity is at risk. Pretreatment of the samples and buffers for optimal performance of the commercially available kits are optimized for the kind of samples used (cell culture supernatant, serum, tissue, blood), and efficient RNA isolation can only be achieved if the right kits are used for the right samples. Different kits are recommended by the manufacturers for RNA extraction from different samples, such as serum, plasma, and cell culture supernatants, or from blood or from tissues. Two technologies are in use for nucleic acid isolation for RT-PCR: the liquid-based and the silica- or glass-based methods.

The liquid-based methods are enhancements of the original nucleic acid isolation using guanidinium thiocyanate/acid phenol: chloroform (6, 7). Ready-to-use reagents are commercially available under different brand names (TRIZOL®, TRI Reagent®, Stratagene RNA Isolation Kit®, etc.), allowing single-step disruption/separation procedures. The tissue or cell sample is disrupted and homogenized in the ready-to-use reagent, chloroform is mixed with the lysate, and then the mixture is separated into three phases by centrifugation. The RNA is then precipitated from the aqueous phase with isopropanol. This extraction method produces high yields of intact RNA molecules. The drawbacks are that skilled personnel are needed and that the extraction cannot be automated. Therefore, it is mostly used for RNA extraction from a few or single samples and more often from “difficult” samples (mainly rotten or degraded tissue).

Silica- or glass-based matrices or filters selectively absorb nucleic acids in the presence of chaotropic salts (3), which immediately inactivate the RNAses. After washing off the remaining components of the lysate using a high-salt buffer, the bound RNA is eluted with water. In many commercially available kits, the glass or silicon filter or fleece is housed in a spin column or in a 96-well plate. Solutions are driven through the filter by centrifugation or under vacuum. Some kits also include a proteinase K or DNAse incubation for removal of proteins and DNA, respectively. Although the yield of RNA is lower than that obtained with guanidinium thiocyanate/acid phenol: chloroform extraction, the silica-based kits can be adapted to be used in nucleic acid extraction robots; thus, allowing a full automation of CSFV diagnosis. In addition, no hazardous reagent is needed.

5. Standard RT-PCR

After RT-PCR, reaction mixture containing the amplicons is subjected to electrophoresis in agarose gels. Double-stranded DNA is visualized under ultraviolet (UV) light after incubation with a fluorescent intercalating stain, generally with ethidium bromide. By including a DNA marker, the size of the amplicon can be estimated and eventual false amplicons and primer–dimers become visible.

The standard RT-PCR protocols for diagnosis of CSF have been used in many laboratories such as the protocol amplifies 5'untranslated region (62), E2 glycoprotein gene (26), NS5B non-structural protein gene (9).

6. RT-LAMP

Loop-mediated isothermal amplification (LAMP) method developed by Notomi *et al.* (37) is a simple, rapid, and powerful gene amplification technique for early detection of microbial diseases. Since then, a one-step single-tube, reverse transcription loop mediated isothermal amplification (RT-LAMP) assays for rapid detection and typing of emerging viruses have been developed and evaluated, viz., Severe acute respiratory syndrome coronavirus (22), Human influenza A virus (46), Japanese encephalitis virus (42), Taura syndrome virus (27), H9 avian influenza virus (5) and Classical swine fever virus (4).

6.1 The principle of RT-LAMP primers design

RT-LAMP uses a set of two specially designed inner primers and two outer primers (described in detail below), which recognize six distinct regions of the target gene: the F3c, F2c and F1c regions at the 3' side, and the B1, B2 and B3 regions at the 5' side (Figure 2). A set of four primers are known as: FIP; Forward Inner Primer consisted of the F2 region (at 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at 5' end. BIP; Backward Inner Primer consisted of the B2 region (at 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at 5' end. F3; Forward Outer Primer consisted of the F3 region that is complementary to the F3c region. B3; Backward Outer Primer consisted of the B3 region that is complementary to the B3c region. Further, two loop primers viz. forward loop primer (FLP) and backward loop primers (BLP) are designed to accelerate and increase sensitivity of the amplification by binding to additional sites that are not accessed by internal primers. The FLP and BLP primers are composed of the sequences that are complementary to the sequence between F1 and F2 and B1 and B2 regions respectively (34, 37).

The size and sequence of the primers were considered so that their melting temperature (Tm) is between 60-65°C: the optimal temperature for *Bst* DNA polymerase. The F1c and B1c *Tm* values should be slightly higher than those of F2 and B2 to form the looped out structure. The *Tm* values of the outer primers F3 and B3 have to be lower than those of F2 and B2 to assure that the inner primers start their annealing and synthesis earlier than the outer primers. Additionally, the concentrations of the inner primers are higher than the concentrations of the outer primers. Furthermore, it is critical for LAMP to form a stem-loop DNA from a dumb-bell structure. The various sizes of loop between F2c and F1c and between B2c and B1c should be examined. The best results are given when loops of 40 nucleotides or longer are used. The size of the target DNA is an important factor on which the LAMP efficiency depends, because the rate limiting step for amplification is strand displacement DNA synthesis. Various target sizes should be tested and the best results are obtained with 130-200 bp DNA (37).

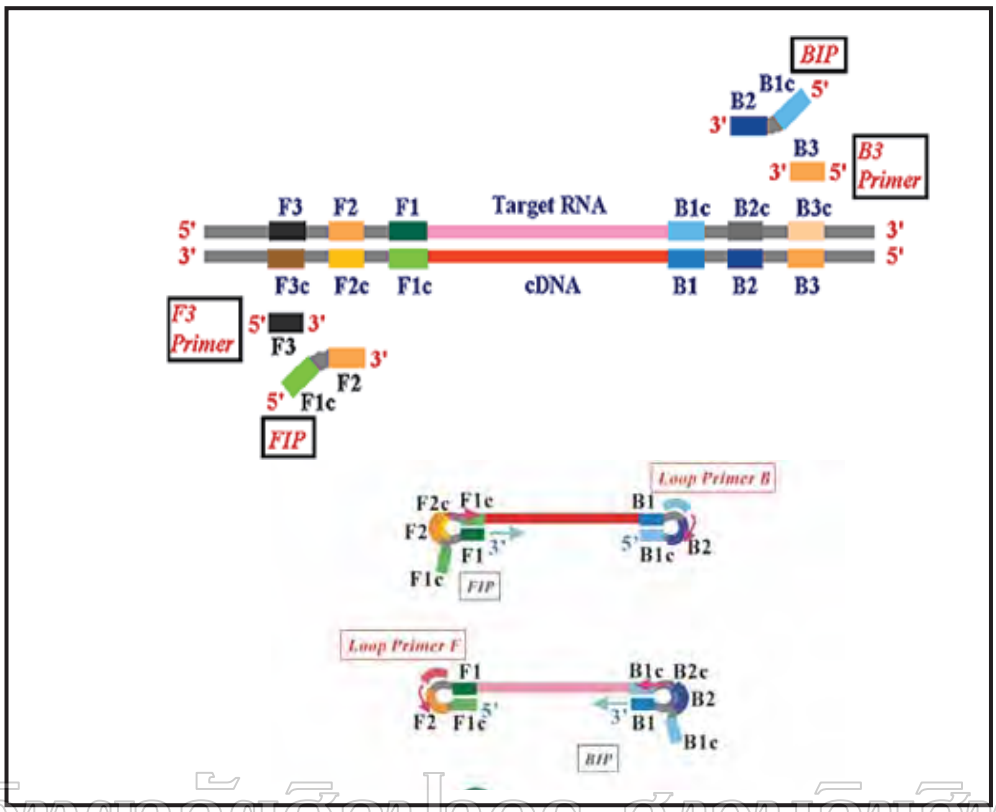


Figure 2 Six types of RT-LAMP primers are shown: FIP, BIP, F3, B3, FLP and BLP primers (13)

6.2 The principle of RT-LAMP method

RT-LAMP method could synthesize cDNA from the template RNA and amplify them in a single step. The reaction is carried out in a single tube by mixing primers, reverse-transcriptase, and DNA polymerase at a constant temperature (63-65°C) for 60 min. Moreover, simple incubators, such as a water bath or block heater are sufficient for the target amplification. When RNA template and the reagents are incubated at a constant temperature, the following reaction steps proceed (step 1-12): Process of reverse transcription (step 1-3), steps for starting structure formation (step 4-7), and LAMP cycling amplification steps (step 8-12). All steps are shown in Figure 3-10. (13).

6.2.1 Process of reverse transcription (step 1-3)

Step 1: Use RNA extraction kit to extract RNA from the samples, and then prepare the sample solution. Mix the sample solution and the reaction solution and incubate them at a constant temperature between 60-65°C. As shown in the following Figure 3, BIP anneals to the template RNA, and with the activity of reverse transcriptase, cDNA is synthesized.

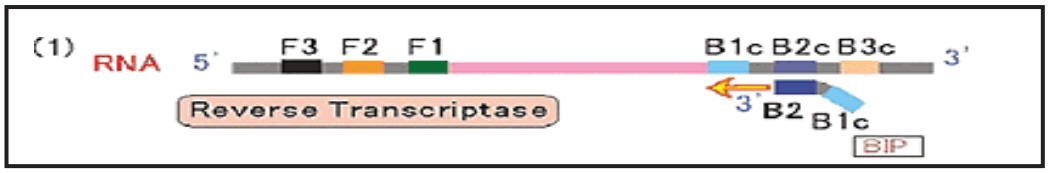


Figure 3 The principle of RT-LAMP method is shown in step 1.

Step 2: B3 primer anneals to the region outside of the BIP, with the activity of reverse transcriptase, a new cDNA is being synthesized, while concurrently releasing the cDNA strand previously formed by the BIP (Figure 4).

Figure 4 The principle of RT-LAMP method is shown in step 2.

Step 3: From step (2), the single stranded cDNA synthesized from BIP is released. The FIP, then, anneals to this single stranded cDNA (Figure 5).

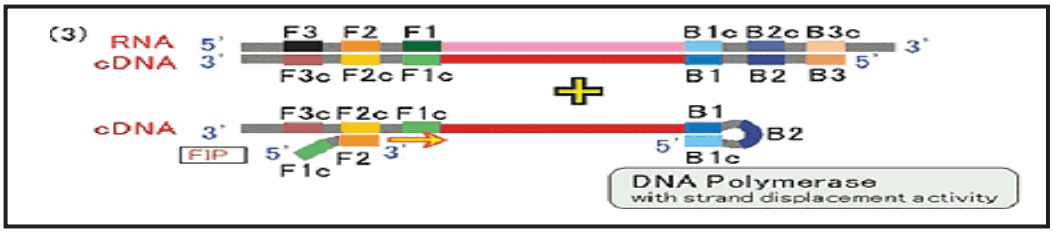


Figure 5 The principle of RT-LAMP method is shown in step 3.

6.2.2 Steps for starting structure formation (step 4-7)

Step 4: From reverse transcription step (3), through the activity of the DNA polymerase with strand displacement activity, the 3' end of F2 region in FIP becomes the starting point to synthesize complementary DNA strand (Figure 6).

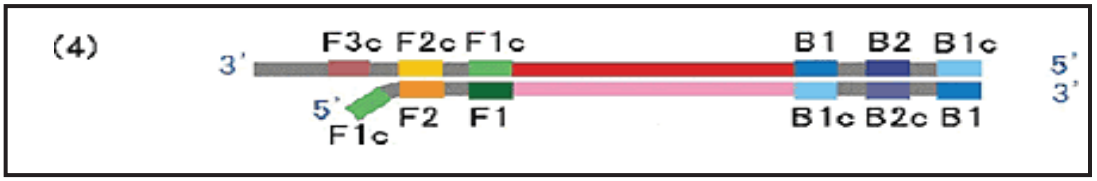


Figure 6 The principle of RT-LAMP method is shown in step 4.

Step 5: F3 primer anneals to the region outside of FIP, and its 3' end becomes the starting point to synthesize while concurrently releasing the DNA strand previously formed by FIP (Figure 7).

Figure 7 The principle of RT-LAMP method is shown in step 5.

Step 6: The DNA strand synthesized by F3 primer together with the template DNA strand forms a double stranded DNA (Figure 8).

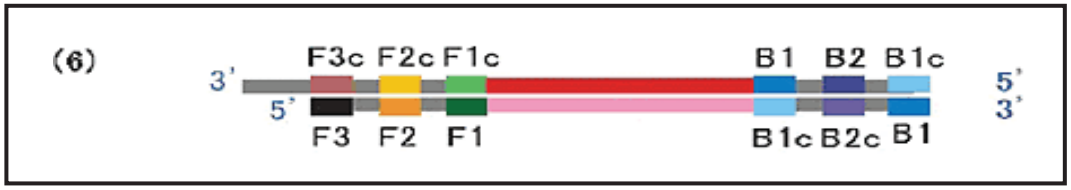


Figure 8 The principle of RT-LAMP method is shown in step 6.

Step 7: Since the FIP linked DNA strand, which was released in step (5), contains complementary sequences at both ends, it self-anneals and forms a

dumbbell-like structure. This structure (7) becomes the starting structure of the LAMP cycling amplification (Figure 9).

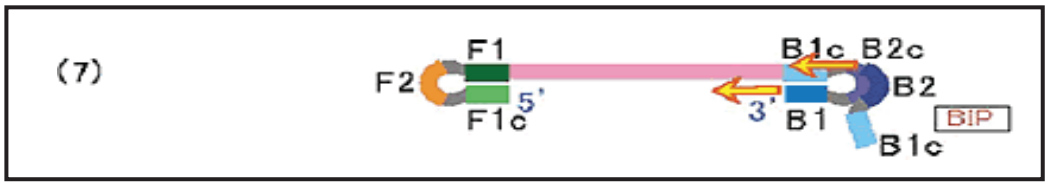


Figure 9 The principle of RT-LAMP method is shown in step 7.

6.2.3 LAMP cycling amplification steps (step 8-12)

As shown in Figure 10, **Step 8:** The dumbbell-like DNA structure (7) is quickly converted into a stem-loop DNA by self-primed DNA synthesis, which unfolds the loop at 5' end to extend DNA synthesis. The BIP anneals to the single stranded region in the stem-loop DNA to start DNA synthesis in step (8) while releasing the previously synthesized strand.

Step 9: This released single strand forms a stem-loop structure at the 3' end because of complementary F1c and F1 regions. Then, starting from the 3' end of the F1 region, DNA synthesis starts using self-structure as a template, and releases BIP-linked complementary strand. Structure (9) is formed.

Step 10: The released BIP-linked single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively. This structure is the turn-over structure of structure (7).

Step 11: Similar to step (7), structure (10) proceeds self-primed DNA synthesis starting from the 3' end of the F1 region. Furthermore, the FIP anneals to the F2c region and starts synthesizing DNA strand. This FIP-linked DNA strand is released by the strand displacement of self-primed DNA synthesis. Accordingly, similar to step (7), (8) and (10), step (10) and (11) proceeds and structure (7) is once again being formed.

Step 12: With the structure produced in step (9) or step (12), the FIP or BIP anneals to the single stranded F2c region or B2c region, and DNA synthesis continues by releasing double stranded DNA. As a result of this process,

various sized structures consisting alternately inverted repeats of the target sequence on the same strand are formed.

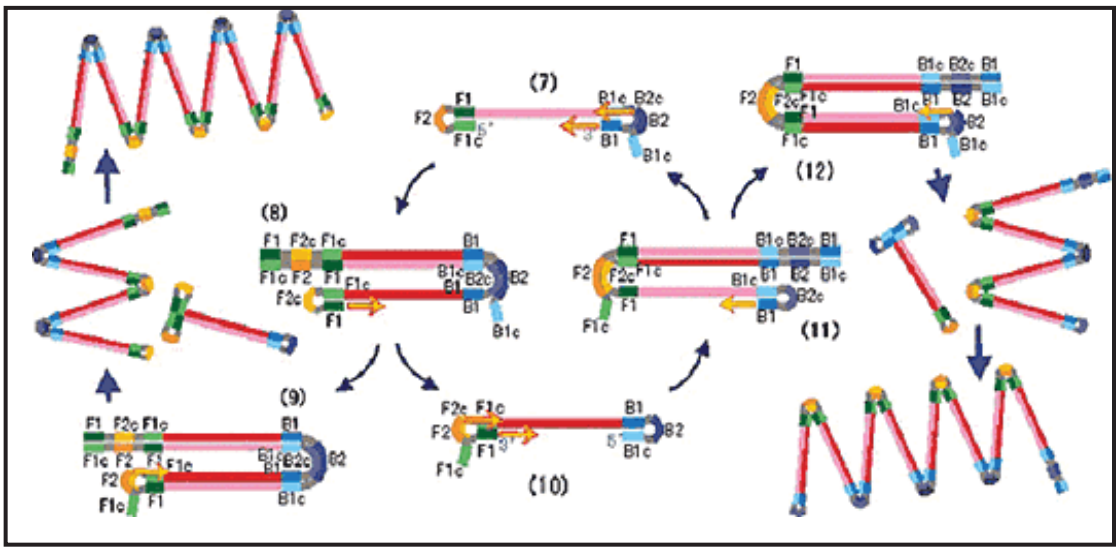


Figure 10 The principle of RT-LAMP method is shown in step 8-12.

For the loop primers (either loop primer B or loop primer F), the containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure (step 7), provide an increased number of starting points for DNA synthesis for the this method. Thus the RT-LAMP reaction was accelerated by the addition of two primers under isothermal conditions.

6.3 Monitoring of RT-LAMP amplification

Several methods could be used to monitor positive reactions. The most common is agarose gel electrophoresis. The gel is subsequently stained with an intercalating agent such as ethidium bromide. Under UV illumination, the gel shows many bands of different sizes, forming ladder-like structures which are the various length stem-loop products of the positive RT-LAMP reaction (37). In order to facilitate the field application of the RT-LAMP assay, the monitoring of RT-LAMP amplification was also carried out with visualization by the naked eye. During the RT-LAMP amplification, the reaction yields a large amount of the by-product

pyrophosphate ion, which reacts with magnesium ions in the reaction to form white magnesium pyrophosphate precipitate. This precipitant production correlates with the amount of the DNA synthesized. As a result, real-time monitoring of the RT-LAMP reaction can be achieved by the naked eye or by real-time measurement of turbidity (32). However, this detection used an expensive real-time turbidimeter. The use of expensive equipment decreases the versatility of RT-LAMP and greatly limits the wide use of this procedure, especially in developing countries.

Detection of turbidity by the naked eye is the simplest and most cost-efficient method for judging a positive or negative LAMP reaction, although this method requires some skill for assessing the result. For better visibility of the reaction result, a DNA fluorescent intercalating dye such as SYBR green (4, 36, 38), Picogreen (15, 11), Propidium iodide (21) was added to the solution after the reaction was completed. When the RT-LAMP reaction was positive, a color change was observed under UV light or normal light. A disadvantage of the intercalating agent is that it is equally incorporated into every amplicon. Should unspecific sequences be amplified, the signal measured would correspond to both non-specific and specific products (18).

The RT-LAMP reaction results in large amounts of pyrophosphate ion by-product; these ions react with magnesium ions to form the insoluble product magnesium pyrophosphate. Since magnesium ion concentration decreases as the RT-LAMP reaction progresses, the RT-LAMP reaction can be quantified by measuring the magnesium ions concentration in the reaction solution.

On the basis of this phenomenon, Tomita *et al*. (60) developed a simple colorimetric assay for the detection of the RT-LAMP reaction by adding calcein, a fluorescence metal indicator, to the pre-reaction solution. This result implied that the detection sensitivity of the LAMP assay using calcein was 10 times lower than using HNB and SYBR Green. In addition, the brightness of calcein fluorescence was significantly weaker than that of SYBR green fluorescence. Goto *et al*. (19) reported a simpler colorimetric assay for the detection of the LAMP reaction by using another metal ion indicator, namely, Hydroxynaphthol blue (HNB).

HNB is a metal indicator for calcium and a colorimetric reagent for alkaline earth metal ions. HNB could be a novel indicator for the LAMP reaction by

monitoring the change in the magnesium ions concentration since the large fragment of *Bst* DNA polymerase synthesizes DNA under alkaline conditions (pH 8.8 at 25°C). When the LAMP reaction is positive, a color change was observed. The color of HNB changes depending on the pH of the solution and is induced by the chelation of magnesium ions by dNTPs. The LAMP assay using HNB was superior to the other assays because (*i*) cost-effectiveness; (*ii*) high-throughput DNA and RNA detection. (*iii*) the detection sensitivity was equivalent to that of the assay using SYBR green; and (*iv*) the positive/negative result of the LAMP reaction could be easily judged by the naked eye under normal light. In this study, the 120 μM HNB was added into the reaction mixture after amplification. The positive of the RT-LAMP reaction, a color changes from violet to sky blue. The absence of amplification, the color will not change.

6.4 Advantages of RT-LAMP method

RT-LAMP method facilitates the detection RNA of pathogenic organisms. RT-LAMP method has several advantages as the followed: (*i*) RT-LAMP method could synthesize cDNA from template RNA, and amplify them in a one step. The reaction is carried out in a single tube by mixing primers, reverse-transcriptase and DNA polymerase at a constant temperature (63-65°C) for 60 min. Thus, this method is performed a highly simple and rapid because of it does not require thermal cycling. Only a water bath or heating block is needed. It dose not require special reagents and sophisticated temperature control device to which the total cost can be reduced; (*ii*) the amplification specificity is extremely high because the RT-LAMP reaction requires a set of four oligonucleotide primers that recognize six distinct regions on the target DNA; (*iii*) In comparison to the conventional RT-PCR, RT-LAMP method is shorter in detection time and demonstrates 10 to 100 fold more sensitivity with a detection limit 0.01 to 10 PFU of virus, viz., West Nile, SARS coronavirus, Chikungunya, Japanese encephalitis. The advantages and disadvantages of RT-LAMP are compared to RT-PCR as shown in Table 1; (*iv*) the RT-LAMP reaction can be accelerated by using two specially designed loop primers; and (*v*) the monitoring of RT-LAMP product can be used several methods, as described above. Therefore, the RT-LAMP method using HNB dye is low-cost and provides a simple,

rapid, sensitive and specific. It has potential use for a rapid laboratory diagnosis and surveillance of an infectious disease pathogen in the field (19, 22, 34, 37, 39-42)

Table 1 Comparison of the advantages and disadvantages of RT-LAMP and RT-PCR

	RT-LAMP	RT-PCR	Reference
Thermal cycler	not required	required	(37)
Reaction time	30 to 60 min	over 60 min	(37)
Sophisticated equipments	not required	required	(37)
Visualization by the naked eye	yes	no	(32)

7. FTA Classical Card

FTA Classic Card is a treated filter paper for the collection, storage, and transportation of blood samples, or other biological samples for subsequent DNA/RNA analyses. The FTA Card system is impregnated with a chemotropic agent that denatures infectious micro-organisms. Proprietary chemicals impregnated into the paper lyse cells, denature proteins, and fix and preserve DNA and RNA within the fiber matrix. Hence the samples are no longer considered infectious. This allows the storage and transport of samples without specific biohazard precautions. The main advantage of the FTA Card system is the long-term stability of nucleic acids at room temperature and the subsequent easy storage of fixed specimens for further molecular testing. Nucleic acids can be recovered by punching the impregnated area and eluting with classical reagents. Advantages of FTA technology have been demonstrated for human DNA processing (10, 64), for wildlife DNA samples (55), and recently for the molecular characterization of DNA and RNA viral pathogens from plant tissues (35), RNA from foot and mouth disease virus, rabies virus (33, 45). The detection of the viral RNA was here performed directly with the samples applied to the FTA Card without the RNA extraction step described above to investigate its potential application in the field.

CHAPTER III

MATERIALS AND METHODS

1. CSFV-specific primers designing

Nucleic acid sequences of different CSFV were obtained from Gen Bank, National Center of Biotechnology Information (NCBI), USA. and aligned with using the CLUSTALW multiple sequence alignment programs (54). The conserved fragment was chosen to be the target region, which was used to design the CSFV primers for RT-LAMP and RT-PCR by the Primer Explorer V4 software program (14) and Primer3 Input (version 0.4.0) program (47), respectively. All oligonucleotide primers were customary synthesized by the Bio Basic Inc. (East Markham, Ontario, Canada).

2. Viruses and vaccines

The CSFV Bangkhen strain, obtained from Department of Livestock Development, Ministry of Agriculture and Cooperatives, was used as the viral nucleic acid/positive standard in the assay system employed in this study. The other virus included Japanese encephalitis virus (JEV), cultured at Department of Biology, Faculty of Science, Silpakorn University. Hog-cholera tissue culture live vaccine (LPC-PRK strain) was purchased from Formosa Biomedical Inc. (Taiwan) and provided as 10 vaccine doses per vial of which each dose contained tissue culture of Hog-cholera virus fluid at least $10^{3.5}$ RID_{50}. Porcine reproductive and respiratory syndrome virus (PRRSV) modified live vaccine was purchased from Boehringer IngelheimVetmedica, Inc., Missouri, USA. All viruses and vaccines were identified by conventional RT-PCR.

3. RNA extraction

The spleen from a swine infected with CSFV Bangkhen strain was harvested and homogenized with PBS pH 7.4. The cell pellet was centrifuged at 400*g*

for 10 min at 4°C. Total RNA was extracted from the suspension with QIAmp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instruction. After elution, the RNA samples were stored at -80°C until required.

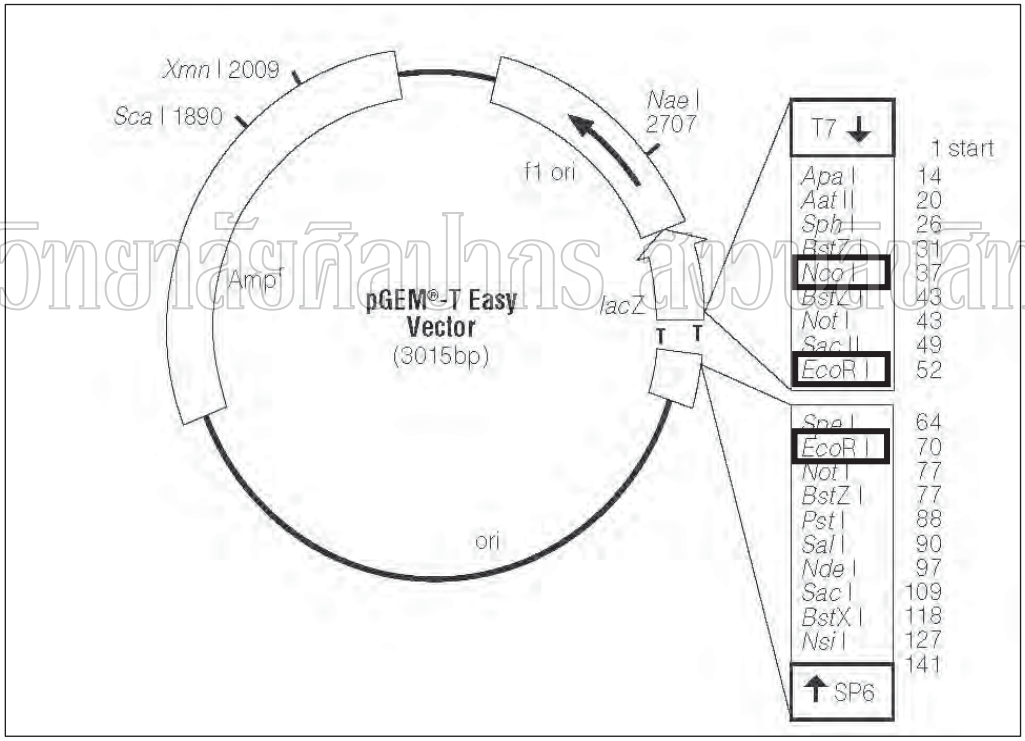
4. RT-PCR

RT-PCR product was generated and checked with the positive standard of viruses and vaccine. One-step RT-PCR was performed by employing the two primer pairs targeting the 5'UTR gene of CSFV (CSU1 [GAGGTTAGTTCATTC TCGTATGC] and CSU2 [ATCGTATACCGGTTCCTCCAC]). The amplification was carried out in a 50 µl total reaction volume by using a QIAGEN OneStep RT-PCR kit (Qiagen, Germany) with 20µM of CSU1 and CSU2 primers and 20 µl of RNA template, according to the manufacturer's protocol. The thermal profile of RT-PCR was 50°C for 30 min and 95°C for 15 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min and a final extension cycle at 72°C for 10 min. After amplification, the PCR products were stored overnight at 2-8°C or at -20°C for longer-term storage until required. In addition, the PCR products could be analyzed by electrophoresis using 1.0% of agarose gel (Sigma-Aldrich, Missouri, USA.) in Tris acetate-EDTA (TAE) buffer, followed by staining with ethidium bromide and visualization on a UV transilluminator. The 100 bp DNA ladder (Geneaid, Taiwan) was used as molecular weight marker.

5. Recombinant plasmid construction

The targeted region of the 5'UTR gene from total RNA was amplified by QIAGEN OneStep RT-PCR kit using forward (CSU1) and backward (CSU2) primers as described above. The 445-bp amplicon was purified using a QIAquick Gel Extraction kit (Qiagen, Germany) and then cloned into the pGEM-T easy vector system I (Promega, Madison, WI, USA.), according to the manufacturer's specifications. The recombinant plasmid was transformed into *Escherichia coli* strain DH5α competent cells followed by blue-white colony selection. The white colonies were picked and inoculated into Luria-Bertani broth and incubated at 37°C overnight with horizontal shaking. Plasmid DNA was extracted from the culture with the QIAprep Spin Miniprep kit (Qiagen, Germany) as followed manufacture's

instructions and checked of DNA insertion by using a vector-specific restriction enzyme digestion (*EcoR*I and *Nco*I), as shown in Figure 11. The digested products were detected by gel electrophoresis, as described above and used the 100 bp DNA ladder and 1,000 bp DNA ladder as molecular weight marker. The concentration of the plasmid was determined by measuring the optical density (OD) at 260 nm with NanoDrop (NanoDrop, Wilmington, Delaware). The copy numbers/µl was determined by using the following formula: copies/µl = 6.023×10^{23} (copies/mol) x concentration of recombinant plasmids (g/µl) / average molecular weight of recombinant plasmids (g/mol). The plasmid was diluted to determine the sensitivity of RT-LAMP assays.



pGEM T-Easy vector

Figure11 pGEM T-easy vector-specific restriction enzyme digestion: *EcoR*I and *Nco*I (pGEM-T easy vector manual book; Promega, Madison, WI, USA.)

6. RT-LAMP assay

The RT-LAMP reaction was carried out in a total volume of 25 µl, with mixture of 1×Thermo buffer (New England Biolabs Inc., Beverly, MA, USA.) contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$ and 0.1% Triton X-100, 0.8 M betaine (Sigma-Aldrich, Missouri, USA.), 1.0 mM dNTP mix (Promega, Madison, WI, USA.), 8 U *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA.), 5 U AMV reverse transcriptase (Promega, Madison, WI, USA.), 0.2 µM each of the outer primers CSUF3 and CSUB3, 1.6 µM each of the inner primers CSUFIP and CSUBIP, 8 µM each of the loop primers ULF and ULB, 4.5 µl of template RNA. The mixture was incubated at 65°C for 60 min, and then heated at 80°C for 3 min to stop the reaction.

7. Monitoring of RT-LAMP amplification

(*i*) Agarose gel eletrophoresis analysis. After amplification, the 2 µl aliquots of RT-LAMP products were analyzed by gel electrophoresis, as described above and used the 100 bp DNA ladder as a molecular weight marker.

(*ii*) Visualization by the naked eye. In order to facilitate the field application of the RT-LAMP assay, the monitoring of RT-LAMP amplification was also carried out with inspection by the naked eye. Following amplification, the tubes were inspected for white turbidity of magnesium pyrophosphate using the naked eye after a pulse spin to deposit the precipitate in the bottom of the tube.

In this study, the inspection for amplification was also performed through observations of the color change affter the addition of 120 µM Hydroxynaphthol blue (HNB) dye (Sigma-Aldrich, Missouri, USA.) to the tube. In the case of positive amplification, the original violet color of the dye would change into blue sky that can be judged under natural light. In case there is no amplification, the original violet color of the dye would remain. This change of color is permanent and, thus, can be kept for record purposes.

8. Sensitivity of RT-LAMP for CSFV

The sensitivity was determined by testing serial 10-fold dilutions of a cloned target that had previously been quantified through copy number

determinations. The template over a range of 10^{10} to 10 copy numbers was obtained. The RNase-free water was used as the negative control. Monitoring of RT-LAMP amplification is performed by analyses of Agarose gel electrophoresis and HNB dye, as described above. Monitoring of RT-PCR amplification is also performed by analyses of Agarose gel electrophoresis

9. Specificity of RT-LAMP for CSFV

For the specificity of RT-LAMP assay, cross-reactivity test was conducted by using different sources of RNA as templates which consisted of Hog-cholera tissue culture live vaccine, JE virus and modified live PRRS vaccine. The Hog-cholera live vaccine was rehydrated with sterilized saline and was also diluted with pig whole blood, which was collected initially and confirmed as CSFV-negative. These samples were performed in two methods. After amplification, monitoring of RT-LAMP product was performed by analyses of Agarose gel eletrophoresis and visual color change of HNB by the naked eye, as described above. The cross-reactions of the developed RT-LAMP with RNA of JEV and PRRSV were carried out.

Method 1: The samples were extracted with QIAmp viral RNA mini kit (Qiagen, Germany), according to the manufacturer's instruction. After elution, RNA suspension was used as template in the specificity test of this assay. RNA from Hog-cholera live vaccine was used as positive control. The RNase-free water was used as the negative control.

Method 2: The 120 µl of each sample suspensions and JE virus were applied onto the centre of each FTA Classic Card (Whatman International Limited, UK), allowing the collection and archiving of nucleic acids. The cards were air dried for 1 h at room temperature. Then, a 2.0-mm disc was cut from one matrix circle of each loaded card and purified with FTA Purification Reagent (Whatman International Limited, UK) by following standard procedures. Briefly, the discs were placed in a 1.5 ml microcentrifuge tube and 200 µl of FTA purifification reagent was added. It was incubated for 5 min at room temperature, after thorough mixing. The FTA reagent was removed and discarded using a pipette, and washing was repeated. Then, it was rinsed twice with 200 µl of TE buffer (5 min each). The TE buffer was removed and discarded using a pipette, and the discs were allowed to dry at room

temperature for an hour. The disc was used directly as the template in subsequent RT-LAMP assay. RNA from Hog-cholera live vaccine and healthy swine blood was used as positive and negative control, respectively.

In addition, we studied stability of the RNA on the card compared with the RNA suspension. The RNA suspension ranging from 1 to 4 µl compared with one to four discs of FTA Card were used directly as the template. After amplification, monitoring of RT-LAMP product was performed by analyses of Agarose gel eletrophoresis.

CHAPTER IV

RESULTS

1. Designing the CSFV-specific primers

In the present study, the primers were designed on the basis of highly conserved regions. The nucleic acid sequence of 33 CSFV were obtained from GenBank (accession numbers: AF091507, AF091661, AF092448, AF099102, AF326963, AF333000, AF352565, AF407339, AF531433, AY072924, AY259122, AY367767, AY382481, AY554397, AY568569, AY578688, AY646427, AY775178, AY805221, CQ867021, DQ127910, EU490425, EU497410, EU789580, EU857642, FJ265020, FJ529205, HCU45477, HCVPOLYP2, NC_002657, X87939, X96550, Z46258), and were aligned by CLUSTALW multiple sequence alignment program (54) to find a conserved fragment. The resulted as conserved fragment of 445 bp was found in the 5'untranslated gene at the positions 7 to 451, which showed highly conserved regions, as shown in Figure 12. The CSFV primers for RT-LAMP and RT-PCR were designed according to the conserved sequence.

The RT-LAMP specific primers included a set of six primers comprising of two outer, two inner, and two loop primers that recognized eight distinct regions on the target sequence, as shown in Figure 12. All oligonucleotide primers of RT-LAMP were designed with the Primer Explorer V4 software program (14). The primers were selected based on criteria described previously by Notomi *et al*. (37). These primers comprising of two outer primers were described as being forward outer primer (CSUF3) and backward outer primer (CSUB3). The inner primers were described as being forward inner primer (CSUFIP) and backward inner primer (CSUBIP). Furthermore, two loop primers, viz, forward loop primer (ULF) and backward loop primer (ULB), were designed to accelerate the amplification reaction. CSUFIP consisted of a complementary sequence of F1 and a sense sequence of F2. CSUBIP consisted of a complementary sequence of B1 and a sense sequence of B2. FIP and BIP were high-performance liquid chromatography-purified primers. The ULF and

ULB primers were composed of the sequences that were complementary to the sequence between the F1 and F2 and B1 and B2 regions, respectively. The details of the each primer with regard to their positions in the genomic sequences were shown in Table 2.

For the RT-PCR specific primers, a set of two primers comprising of forward primer (CSU1) and backward primer (CSU2) were designed by Primer3 Input (version 0.4.0) program (47), as shown in Figure 12. The details of the each primer with regard to their positions in the genomic sequences were shown in Table 2.

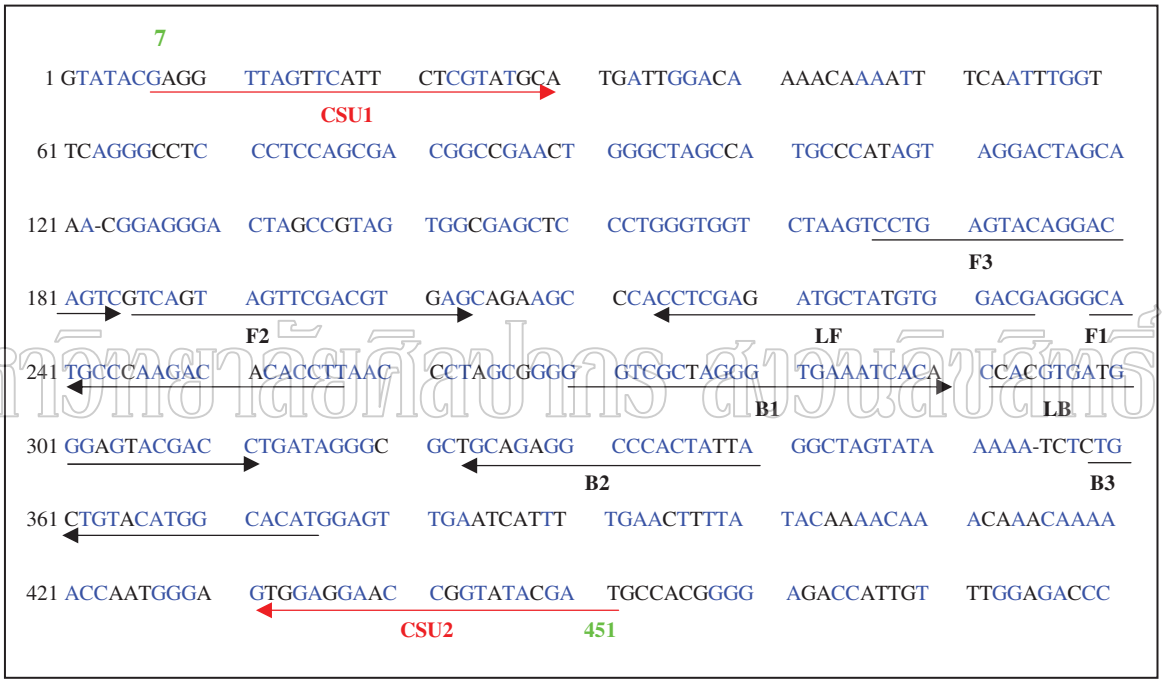


Figure 12 The nucleotide positions 7 to 451 on the viral genome was selected from the aligned sequences and six primers that recognized eight distinct regions on the target sequence (*i.e.* F3, F2, between the F1c and F2, B1, between the B1 and B2, B2 and B3 regions) for RT-LAMP and the positions forward primer (CSU1), backward primer (CSU2) for RT- PCR. The blue letters indicated conserved bases and the primer sites were indicated by the arrows. The sequence of the positive sense was shown.

Table 2 Details of RT-LAMP and RT-PCR primers designed for CSFV detection

Primers names	Genome positions	Length of oligonucleotides (bp)	Sequence (5' - 3')
A. RT-LAMP			
CSUF3	166-184	19	TCCTGAGTACAGGACAGTC
CSUB3	358-375	18	ATGTGCCATGTACAGCAG
CSUFIP (F1c + TTTT + F2)	238-257(F1c)	20	AAGGTGTGTCTTGGGCATGC
	185-203(F2)	19	GTCAGTAGTTCGACGTGAG
CSUBIP (B1c + TTTT + B2)	270-290(B1c)	21	GGTCGCTAGGGTGAAATCACA
	323-340(B2)	18	TAATAGTGGGCCTCTGCA
ULF	213-234	22	CGTCCACATAGCATCTCGAGGT
ULB	291-311	21	CCACGTGATGGGAGTACGACC
B. RT-PCR			
CSU1	7-29	23	GAGGTTAGTTCATTCTCGTATGC
CSU2	431-451	21	ATCGTATACCGGTTCCTCCAC

2. RT-PCR product

The RT-PCR product was generated by employing the two primer (forward primer: CSU1 and backward primer: CSU2) targeting the 5'UTR gene corresponding to genome positions 7 to 451 of CSFV. The target was amplified by using one step RT-PCR; reverse transcription and PCR were carried out sequentially in the same tube. After amplification, the PCR products could be analyzed by electrophoresis using 1.0% of agarose gel in TAE buffer, followed by staining with ethidium bromide and visualization on a UV transilluminator. The 100 bp DNA ladder was used as the molecular weight marker. For the positive, the RT-PCR product size was 445 bp. In contract, the RNase-free water did not produce band, as shown in Figure 13.

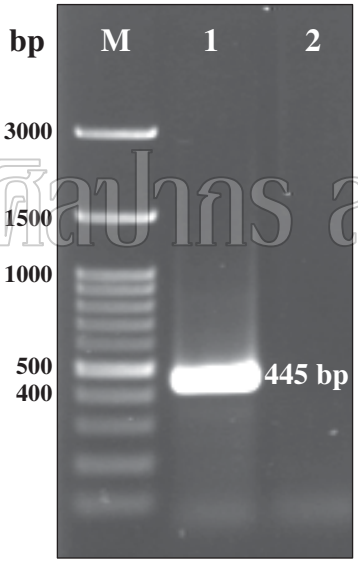


Figure 13 RT-PCR product of amplification as observed by Agarose gel analysis

Lane M : 100 bp DNA ladder

Lane 1 : Product of 5'UTR gene (445 bp)

Lane 2 : Product of RNase-free water (negative control)

3. Recombinant DNA construction

After having inserted a fragment of the 5'UTR gene into the pGEM-T easy vector, the recombinant DNA was transformed into *E.coli* strain DH5α. The white colonies were picked and screened by restriction enzyme digestion of *Nco*I or *EcoR*I.

The recombinant DNA was digested with *Nco*I (C▾CATG▴G) for one cut site. The DNA pattern showed one band at the size of approximately 3,460 bp (molecular weight of CSFV-5'UTR DNA and pGEM-T easy vector were 445 bp and 3,015 bp, respectively). The recombinant DNA was digested with *EcoR*I (G▾AATT▴C) for two cut sites. The DNA patterns showed two bands of approximately 450 bp and 3,000 bp (5'UTR DNA and pGEM-T easy vector, respectively). The sizes were determined by comparison to a molecular ladder which had bands of known sizes, as shown in Figure 14.

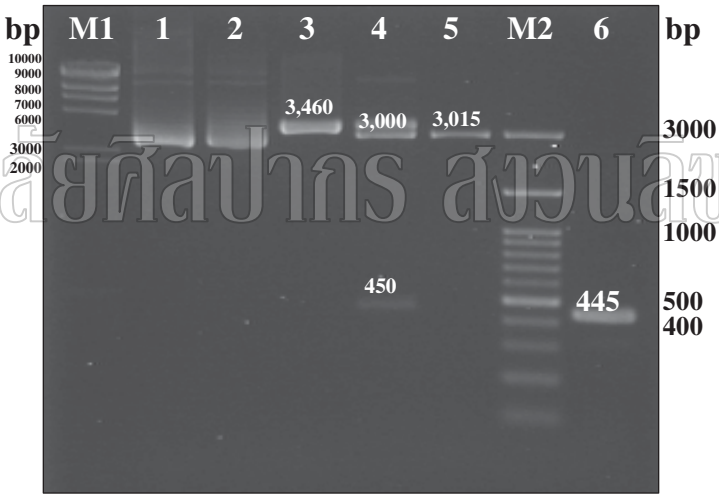


Figure 14 The size of recombinant plasmid compared with plasmid control after digested with restriction enzymes as observed from agarose gel electrophoresis.

Lane M1 : 1,000 bp DNA ladder

Lane 1-2 : Uncut recombinant DNA control

Lane 3 : *Nco*I restriction (~ 3,460 bp)

Lane 4 : *EcoR*I restriction (~ 450 and ~ 3,000 bp)

Lane 5 : pGEM-T easy vector (3,015 bp)

Lane M2 : 100 bp DNA ladder

Lane 6 : PCR product of 5'UTR gene amplification (445 bp)

4. Performance of RT-LAMP assay

The success of RT-LAMP assay amplification relies on the specificities of the primer sets for the one-step RT-LAMP assay system which was standardized for rapid detection of the CSFV. Amplification products of RT-LAMP were detected by agarose gel electrophoresis and visualization by the naked eye.

(i) Analyses of agarose gel electrophoresis

For positive amplification, the gel showed many bands of different sizes, forming ladder-like structures which were the various length stem-loop products. For the negative, the RNase-free water was used as the template. We did not observe band pattern on the gel, as shown in Figure 15.

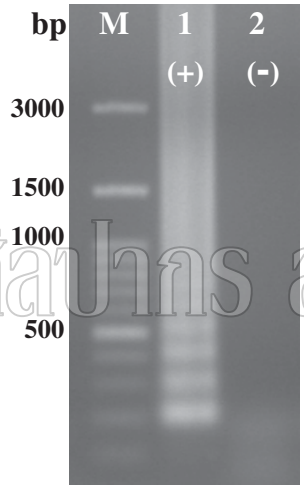


Figure 15 Agarose gel electrophoresis analysis of CSFV RT-LAMP assay products.

Lane M : 100 bp DNA ladder

Lane 1 : Positive amplification

Lane 2 : Negative amplification

(ii) Visualization by the naked eye

In order to facilitate the field application of RT-LAMP assay, monitoring of the amplification can also be accomplished with a naked eye inspection either in the form of turbidity or the color change reaction of Hydroxynaphthol blue (HNB) dye.

A. Visual turbidity

In visual inspection for the amplification products, the positive reaction could be observed by the presence of the white turbidity of magnesium pyrophosphate. The negative reaction showed clear solution in the tube, as shown in Figure 16.

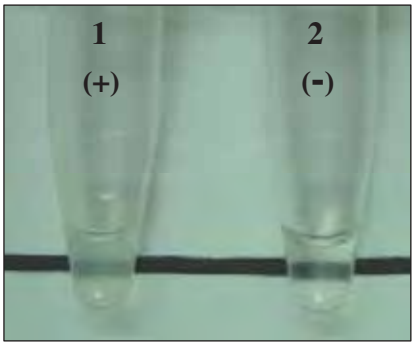


Figure 16 Monitoring of RT-LAMP products by visual inspection of white turbidity

Tube 1 : Positive amplification

Tube 2 : Negative amplification

B. Visual color change of HNB

Determining of RT-LAMP amplification was also carried out by naked-eye inspection, followed by addition the 120 μM of HNB dye to the amplified products. A positive amplification was indicated by a color change from violet to sky blue whereas RNase-free water did not show color change, as shown in Figure 17.

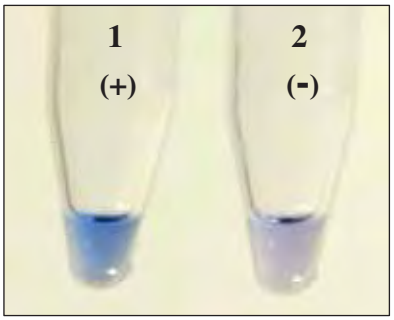


Figure 17 Monitoring of RT-LAMP products by visual color change of HNB dye

Tube 1 : Positive amplification

Tube 2 : Negative amplification

5. Sensitivity of RT-LAMP assay

The sensitivity of RT-LAMP assay for the detection of CSFV was determined with the serial dilutions of a recombinant CSFV-5'UTR inserted into plasmid. The ranging from 10^6 to 10 copy numbers was used as templates. The assay detected 100 copy numbers, showing the characteristic ladder-like pattern in the gel, as shown in Figure 18 and could be visualized directly with the naked eye by addition of hydroxynaphthol blue (HNB) dye, resulting in color changes from violet to sky blue, as shown in Figure 19. For the negative control, the RNase-free water did not show either ladder-like pattern in the gel (Figure 18) or color change of HNB dye (Figure 19). Monitoring of RT-PCR amplification was also performed by analyses of Agarose gel electrophoresis, as shown in Figure 20.

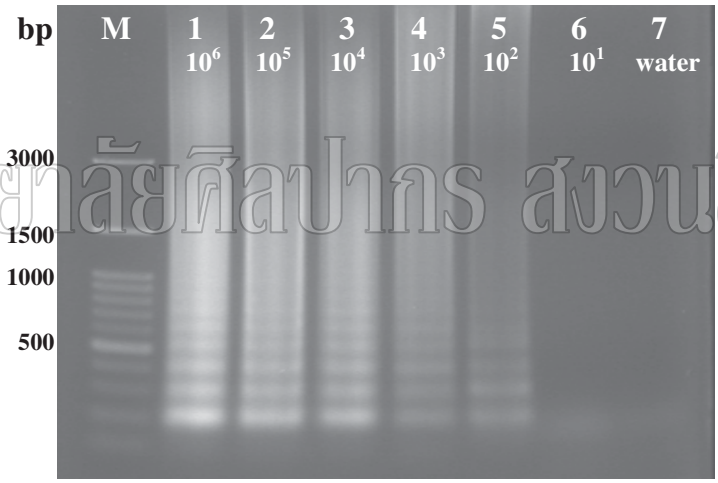


Figure 18 Sensitivity of LAMP was analyzed by Agarose gel electrophoresis.

Lane M : 100 bp DNA ladder
Lane 1 : Product of recombinant DNA 10^6 copies/tube
Lane 2 : Product of recombinant DNA 10^5 copies/tube
Lane 3 : Product of recombinant DNA 10^4 copies/tube
Lane 4 : Product of recombinant DNA 10^3 copies/tube
Lane 5 : Product of recombinant DNA 10^2 copies/tube
Lane 6 : Product of recombinant DNA 10^1 copies/tube
Lane 7 : Product of RNase-free water (negative control)

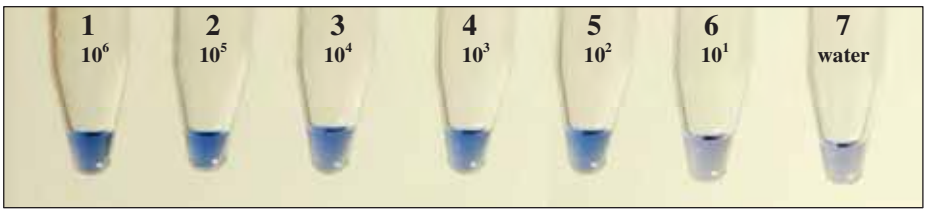


Figure 19 Sensitivity of LAMP was analyzed by visualization HNB dye

Tube 1 : Product of recombinant DNA 10^6 copies/tube

Tube 2 : Product of recombinant DNA 10^5 copies/tube

Tube 3 : Product of recombinant DNA 10^4 copies/tube

Tube 4 : Product of recombinant DNA 10^3 copies/tube

Tube 5 : Product of recombinant DNA 10^2 copies/tube

Tube 6 : Product of recombinant DNA 10^1 copies/tube

Tube 7 : Product of RNase-free water (negative control)

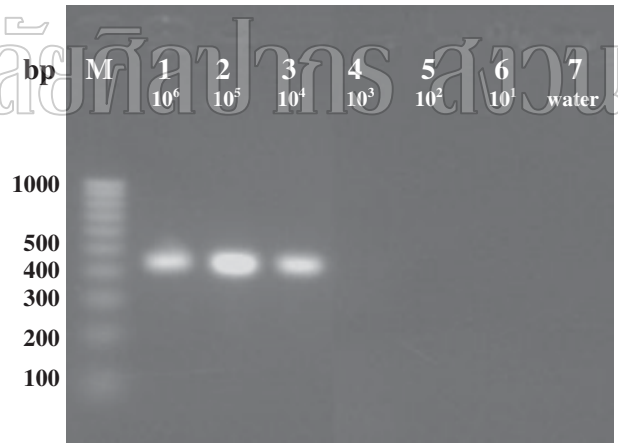


Figure 20 Sensitivity of PCR was analyzed by Agarose gel electrophoresis.

Lane M : 100 bp DNA ladder

Lane 1 : Product of recombinant DNA 10^6 copies/tube

Lane 2 : Product of recombinant DNA 10^5 copies/tube

Lane 3 : Product of recombinant DNA 10^4 copies/tube

Lane 4 : Product of recombinant DNA 10^3 copies/tube

Lane 5 : Product of recombinant DNA 10^2 copies/tube

Lane 6 : Product of recombinant DNA 10^1 copies/tube

Lane 7 : Product of RNase-free water (negative control)

6. Specificity of CSFV RT-LAMP assay

Analytical cross-reaction of CSFV RT-LAMP assay with other pig disease viruses consisted of JE virus and PRRS virus. Viral RNA was used as the template in this specificity test. Specificity was performed by two methods: first, by using RNA suspension, and second, by using RNA fixed on the FTA cards. Overall, the results showed that the expected RT-LAMP product was successfully amplified from either saline diluted or pig whole blood diluted Hog-cholera tissue culture live vaccine, revealing the characteristic ladder-like pattern in the gel and the color changes from violet to sky blue after the addition of HNB dye, as shown in Figure 21-22, respectively, but presenting negative results for JEV, PRRSV, and the negative control. This study suggested that the RT-LAMP assay has a high specificity for CSFV detection.

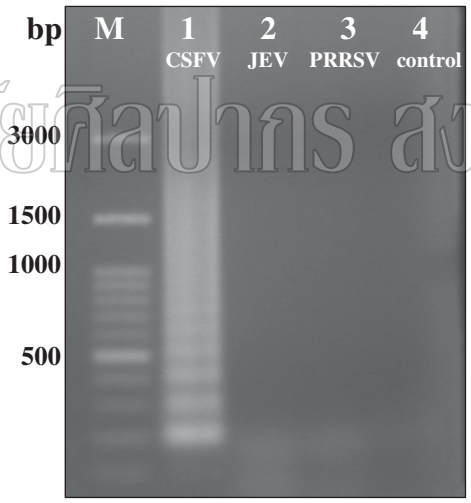


Figure 21 Specificity of RT-LAMP products visualized with Agarose gel electrophoresis

Lane M : 100 bp DNA ladder

Lane 1 : Positive amplification of CSFV

Lane 2 : Negative amplification of JEV

Lane 3 : Negative amplification of PRRSV

Lane 4 : Negative control

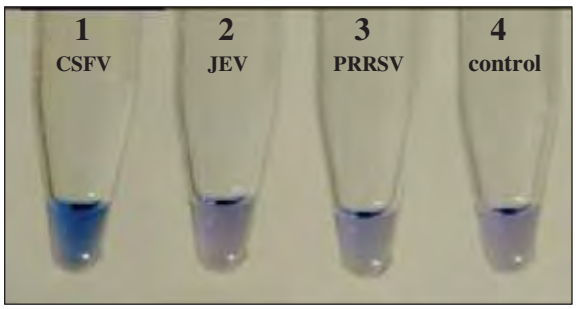


Figure 22 Specificity of RT-LAMP products visualized with HNB dye

Tube 1 : Positive amplification of CSFV

Tube 2 : Negative amplification of JEV

Tube 3 : Negative amplification of PRRSV

Tube 4 : Negative control

For the stability of viral CSF RNA on the card compared with RNA suspension. Monitoring of RT-LAMP product was performed by analyses of Agarose gel electrophoresis. The RT-LAMP assay can be performed directly with the samples applied to the FTA Classic Card without the RNA extraction step, as shown in Table 3.

Table 3 Stability of RNA extraction from RNA on the FTA card compared with that of the suspension

Disc / Volume	Stability of RNA	
	FTA Card	Suspension
1	-	-
2	-	-
3	+	+
4	+	+

CHAPTER V

DISCUSSION

Classical swine fever virus (CSFV), a member of the *Pestivirus* genus causes a highly contagious febrile disease worldwide. Outbreaks of CSF cause heavy losses in pig production and severely hamper the international trade in livestock (12). Previous studies have been reported that CSFV and PRRSV may cause disease with similar clinical symptoms (29). Additionally, CSFV co-infection with PRRSV played an important role in reproducing typical post weaning multisystemic wasting syndrome (50). Taxonomically, JEV belonged to the same *Flaviviridae* family as CSFV (4). Therefore, the development of a simple and rapid diagnostic tool that can detect CSFV and differentiate it from 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.

It is very important to find a conserved nucleic acid fragment to design specific RT-LAMP primers. In this study, the nucleic acid sequence of 33 CSFV in Gen Bank was analyzed with the CLUSTALW multiple sequence alignment programs (54). The most conserved of 445 bp was found in the 5' UTR gene which appeared highly conserved. The CSFV primers for RT-LAMP and RT-PCR targeting the conserved sequence of 5' UTR genes were designed successfully with the Primer Explorer V4 software program (14) and Primer3 Input (version 0.4.0) program (47), respectively.

The RT-LAMP assay is a simple diagnostic tool in which the reaction is carried out in a single tube by a mixing of the buffer, primers, reverse transcriptase, and DNA polymerase, and incubating the mixture at 65°C for 60 min. Besides, the higher amplification efficiency of the RT-LAMP reaction yields a large amount of a by-product, pyrophosphate ion, leading to white precipitate of magnesium pyrophosphate in the reaction mixture. Since the increase in the turbidity of the reaction mixture according to the production of precipitate correlates with the amount

of the DNA synthesized, monitoring of the RT-LAMP reaction can be achieved with the naked eye or in a real-time with a turbidimeter (32). Another useful feature was that RT-LAMP products could be directly observed by the addition of Hydroxynaphthol blue (HNB) dye to the amplified products. Therefore, this technique is effective due to the high specificity and amplification efficiency, and may facilitate the application of RT-LAMP, especially in the field.

The results revealed that RT-LAMP assay detected at least 100 copy numbers whereas that of RT-PCR was 10,000 copy numbers. The sensitivity of RT-LAMP to amplify the CSFV from recombinant DNA has been shown to be 100 fold superior to that of RT-PCR. Our data are in accordance with the sensitivity of other RT-LAMP methods for detection of SARS coronavirus, JEV, and PRRSV (22, 42, 48). Compared to RT-PCR, the RT-LAMP has the advantages of reaction simplicity and detection sensitivity.

Furthermore, the specificity of CSFV RT-LAMP did not show cross-reactivity with JEV and PRRSV, suggesting that this method is highly specific among the viral strains we used. The higher sensitivity and specificity of the RT-LAMP reaction are attributed to its continuous amplification under the isothermal conditions employing six primers that recognized eight distinct regions of the target.

The filter paper demonstrated previously by Smith and Burgoyne (55) to be eminently suitable for the collection of biological samples, including blood and saliva, from a wide range of wildlife species, could be easily used in the future for epidemiological surveys of bats and others mammals, by collecting micro-samples from living animals. Owing to the filter paper matrix of the FTA system impregnated with chemotropic agent that inactivated the microorganism, it would be a valuable tool for CSFV genome transport to the laboratory for carrying out epidemiological and genetic studies without the fear of biohazard (45). Additionally, FTA cards will be of immense value for sample transport from one country to another and within a country without the necessity of a cold chain. The advantage of using FTA cards for both the sender and the receiver is rapid and also safe (33). In this study, we investigated stability of the RNA on the card compared with the RNA suspension for RT-LAMP. The result showed that CSFV viral particles fixed on the FTA cards was equivalent to that of the RNA suspension. Our data are in contrast with the RNA

isolation method of FMDV that was superior to that of the direct use of the FTA cards (33). Disadvantage of RNA isolation from serum suggested that *(i)* samples should be transported at 4 ◦C or on ice for the extraction of the viral RNA from the blood to prevent RNA degradation; *(ii)* the RNA extraction is a time-consuming step when done manually; *(iii)* it becomes expensive when it is done with RNA extraction kits, *(iv)* the handling of RNA, especially in the case of non-eukaryotic RNA requires skilled manipulation and specific facilities such as a designated lab bench or room for an exclusive use of RNA work (23).

CHAPTER VI

CONCLUSION

In summary, the developed RT-LAMP assay is an extremely rapid, cost-effective, sensitive, and specific method for the detection of CSFV RNA. The method requires only simple conditions and less time to obtain a result using the HNB dye, compared with the traditional gel electrophoresis. Therefore, the assay is more suitable for use under field conditions for rapid diagnosis of CSFV, which would allow emergency control measures to be implemented to prevent spread of infection.

In conclusion, the FTA technology can be useful for the storage, transport, collection, and subsequent molecular analysis of viral classical swine fever RNA to facilitate epidemiological investigations in the field.

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APPENDIX

APPENDIX A

LIST OF ABBREVIATIONS

%	=	Percentage
× g	=	Acceleration gravity
°C	=	Degree Celsius
$(NH_4)_2SO_4$	=	Ammonium sulfate
A, T, U, G, C	=	Bases adenine, thymine, uracil, guanine and cytosine, respectively
Amp^r	=	Ampicillin resistance gene
AMV	=	Avian Myeloblastosis Virus
B3 primer	=	Backward outer primer
BDV	=	Border disease virus
BIP primer	=	Backward inner primer
BLP primer	=	Backward loop primer
bp	=	Base pair (s)
Bst	=	*Bacillus stearothermophilus*
BVDV	=	Bovine viral diarrhea virus
$CaCl_2$	=	Calcium chloride
cDNA	=	Complimentary DNA
CH_3COOK	=	Potassium acetate
CPE	=	Cytopathic effect
CSF	=	Classical swine fever
CSFV	=	Classical swine fever virus
dATP	=	Deoxyadenosine triphosphates
dCTP	=	Deoxycytosine triphosphates
dGTP	=	Deoxyguanosine triphosphates
dNTP	=	Deoxynucleotide triphosphates
DNA	=	Deoxyribonucleic acid
dTTP	=	Deoxythymidine triphosphates
DNAse	=	Deoxyribonuclease

E.coli	=	*Escherichia coli*
e.g.	=	Exampli gratia (for example)
EDTA	=	Ethylenediamine tetraacetic acid
et al.	=	Et alii (and others)
F3 primer	=	Forward outer primer
FIP primer	=	Forward inner primer
FLP primer	=	Forward loop primer
FTA	=	Flinders Technology Associates
g	=	Gram
HNB	=	Hydroxynaphthol blue
hr	=	Hour
i.e.	=	Id est- that is
INFα	=	Interferon alfa
IPTG	=	Isopropylthio- β -D-galactoside
IRES	=	Internal ribosomal entry site
JEV	=	Japanese encephalitis virus
kb	=	Kilobase
KCl	=	Potassium chloride
KOH	=	Potassium hydroxide
LAMP	=	Loop-mediated isothermal amplification
LB	=	Luria-Bertani (medium)
M	=	Molar
mg	=	Milligram
$MgCl_2$	=	Magnesium chloride
$MgSO_4$	=	Magnesium sulfate
min	=	Minute
ml	=	Millilitre
mm	=	Millimeter
mM	=	Millimolar
µg	=	Microgram
µl	=	Microlitre
MOPS	=	3-(N-Morpholino)propanesulfonic acid

NaCl	=	Sodium chloride
NCBI	=	National Center of Biotechnology Information
ng	=	Nanogram
NTR	=	Nontranslated region
OD	=	Optical density
ORF	=	Open reading frame
PBS	=	Phosphate-buffered saline
PCR	=	Polymerase chain reaction
PFU	=	Plaque forming unit
pH	=	Negative logarithm of hydrogen ion activity
PRRSV	=	Porcine reproductive and respiratory syndrome virus
RID_{50}.	=	Rabbit infectious doses
RNA	=	Ribonucleic acid
RNases	=	Ribonuclease
rpm	=	Revolutions per minute
RT-LAMP	=	Reverse transcriptase loop-mediated isothermal amplification
RT-PCR	=	Reverse transcription-polymerase chain reaction
RuCl	=	Ruthenium chloride
SDS	=	Sodium dodecyl sulfate
sec	=	Second
TAE	=	Tris acetate-EDTA
Tm	=	Melting Temperature
Tris-HCl	=	Tris-(hydroxylmethyl)-aminoethane hydrochloric acid
U	=	Unit of enzyme activity
UTR	=	Untranslated regions
UV	=	Ultraviolet
viz.	=	Videlicet
X-Gal	=	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

APPENDIX B

Method for RNA extraction: QIAmp viral RNA mini kit (Qiagen, Germany)

1. Pipet 560 µl of prepared Buffer AVL containing Carrier RNA into a 1.5 ml microcentrifuge tube.

2. Add 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL/Carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 sec.

3. Incubate at room temperature for 10 min.

4. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

5. Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 sec. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

6. Carefully apply 630 µl of the solution from step 5 to the QIAamp spin column (in a 2-ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6,000 x g for 1 min. Place the QIAamp spin column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

7. Carefully open the QIAamp spin column, and repeat step 6.

8. Carefully open the QIAamp spin column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6,000 x g for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

9. Carefully open the QIAamp spin column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g) for 3 min. Continue directly with step 10, or to eliminate any chance of possible Buffer AW2 carryover, perform step 9a, and then continue with step 10.

9a. (Optional): Place the QIAamp spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

10. Place the QIAamp spin column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Open the QIAamp spin column and add 60 µl of AVE buffer equilibrated to room temperature.

Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6,000 x g for 1 min. RNA samples were stored at -80°C until required.

Method for RT-PCR: QIAGEN OneStep RT-PCR kit (Qiagen, Germany)

1. Thaw template RNA, primer solutions, dNTP Mix, 5x QIAGEN OneStep RT-PCR Buffer, and sterile deionized water, and place them on ice.

2. Prepare a master mix according to: 10 µl of 5x QIAGEN one step RT-PCR buffer (contains 12.5 mM $MgCl_2$), 2 µl of 10 mM dNTP mix, 1.5 µl of 20µM of CSU1 and CSU2 primer, 2 µl of QIAGEN one step RT-PCR enzyme mix

3. Mix the master mix thoroughly and dispense appropriate volumes into PCR tubes.

4. Add 20 µl of RNA template and sterile deionized water to make a total volume of 50 µl into the individual PCR tubes.

5. Program the thermal cycler according to: Reverse transcription at 50°C for 30 min. Initial PCR activation step at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 10 min.

6. The PCR products could be stored overnight at 2–8°C or at –20°C for longer-term storage until required. In addition, the PCR products were analyzed by gel electrophoresis. The 100 bp DNA ladder was used as molecular weight marker.

Method for purification of PCR products: QIAquick gel extraction kit (Qiagen, Germany)

1. Excise the DNA fragment from the agarose gel with a clean, sharp and scalpel.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

5. Add 1 gel volume of isopropanol to the sample and mix.

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge at 11,000 x g for 1 min.

8. Discard flow-through and place QIAquick column back in the same collection tube.

9. (Optional): Add 500 µl of Buffer QG to QIAquick column and centrifuge at 11,000 x g for 1 min.

10. To wash, add 750 µl of Buffer PE to QIAquick column and centrifuge at 11,000 x g for 1 min.

11. Discard the flow-through. The column was centrifuged at 11,000 x g for an additional 1 min to eliminate residual of PE buffer.

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 30 µl of Buffer EB or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed let the column stand for 1 min, and then centrifuge for 1 min.

14. The eluted DNA was stored at –20 ºC until used.

Method for preparation of competent E. coli cells for transformation

1. A single colony of *E. coli* strain DH5α was inoculated into 5 ml of LB broth and incubated overnight at 37°C with shaking (approximately 250 rpm).

2. The 100 µl of the overnight culture was inoculated into 10 ml of LB broth (1% inoculums size) and incubated with shaking at 37°C for 2-3 h or until an OD value approximately 0.5 at wavelength of 600 nm was obtained.

3. Aliquot the culture into 1.5 ml microcentrifuge tube and placed on ice for 5 min.

4. The tube was centrifuged at 3,000 x g at 4°C for 15 min.

5. The supernatant was discarded and suspended the pellet in 4 ml of cold transformation buffer I (TFB I).

6. The tube was centrifuged at 3,000 x g at 4°C for 15 min.

7. The supernatant was discarded and suspended the pellet in 4 ml of cold transformation buffer II (TFB II).

8. The cells suspension was cooled on ice for 15 min. The 200 µl of cells suspension was aliquot into 1.5 ml microcentrifuge tube and immediately transferred to -80°C for storage.

Properties of pGEM-T Easy vector

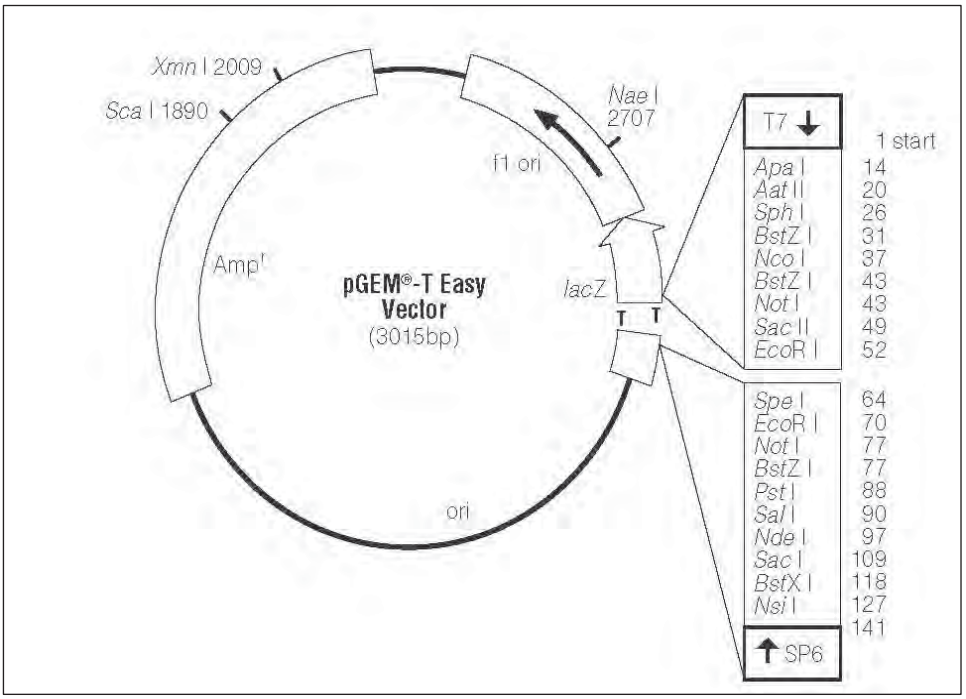
The pGEM-T easy vector (Promega, USA) was a commercial plasmid that was linear and contained multiple restriction sites within the multiple cloning regions. The vector was possesses 3' terminal thymidine (T) at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. The vector represents a convenient system for efficient cloning of PCR product because it contained *LacZ* gene encoding sequence of β-galactosidase within the cloning site allowing a recombinant clone to be identified by color screening on agar plate containing X-Gal. In the presence of DNA insertion, β-galactosidase can not function. Therefore, X-Gal will not be digested resulting in white bacterial colony formation. On the other hand, the plasmid without DNA insertion, X-gal will be digested by β-galactosidase resulting in blue colony formation. Additionally, the cloning site was flanked by the restriction site of many restriction enzymes, and the plasmid also contained ampicillin resistance gene (Amp^r) that will allow growing of the recombinant bacteria in selection media containing ampicillin. The pGEM-T easy vector manual book; Promega, USA showed physical map, cloning, and restriction enzyme digestion sites (Figure 23).

Method for ligation of DNA fragment

1. The ligation reaction was set up which consisted of 10 µl of 2X rapid ligation buffer, 1 µl of T4 DNA ligase, 1 µl of the vector, 5.5 µl of DNA inserts (ratios 8:1) and sterile deionized water to make a total volume of 20 µl.

2. Reaction was mixed by pipetting and was incubated overnight at 4°C.

To calculate the appropriate amount of DNA inserts (ng) into the ligation reaction, following equation: ng of insert = (ng of vector × kb size of insert)/kb size of vector × insert: vector molar ratio.



pGEM T-easy vector

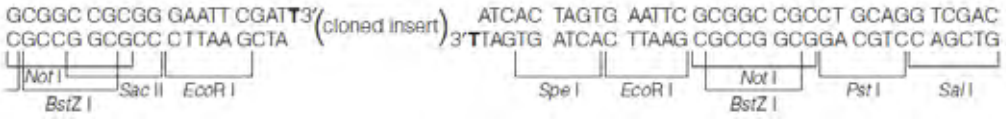


Figure 23 Physical map of the pGEM T-easy vector, cloning, and restriction enzyme digestion sites

Method for bacterial transformation by heat shock process

1. Remove the competent bacterial cells from – 70°C and thawed on ice

2. Transfer bacterial cells to sterile ice-cold 50 ml polypropylene tube.

3. Add 20 µl of ligation reaction, method as previously described to polypropylene tube.

4. The mixture was mixed and incubated on ice for 30 minutes.

5. Then, the tube was incubated at 42°C in water bath for 90 sec and immediately placed on ice for 2 min (heat shock process).

6. Tube of transformed cells was added with 800 µl of LB broth and incubated overnight at 37°C with shaking.

7. The overnight culture was centrifuged at 3,000 x g for 5 minutes, and then the supernatant was discarded approximately 900 µl.

8. The residual supernatant was resuspended the cells and was spread on LB agar plates contain ampicillin, X-Gal and IPTG.

9. The plates were incubated overnight at 37°C. Recombinant clones were identified by color screening on indicator plates (blue/white screening). The white colonies were selected.

Method for purification of plasmid: QIAprep spin miniprep kit (Qiagen, Germany)

1. A single bacterial white colony was picked from LB agar plate.

2. The colony was grown in 2 ml of LB broth contained ampicillin, and then incubated overnight at 37°C with shaking.

3. The bacterial culture was transferred into 1.5 ml microcentrifuge tube, centrifuged for 5 minutes at 11,000 x g, in order to collect the cells and discarded supernatant.

4. This process was repeated using the same microcentrifuge tube until transferred the entire culture.

5. A pellet bacterial cell was resuspended in 250 µl of P1 buffer.

6. Add 250 µl of Buffer P2 and gently invert the tube 4–6 times to mix and incubated for 3 min at room temperature.

7. Add 350 µl of Buffer N3 and invert the tube immediately but gently 4–6 times. The solution should become cloudy.

8. The solution was centrifuged for 10 min at 11,000 x g.

9. Applied the supernatants from step 8 to the QIAprep Spin Column by decanting or pipetting.

10. Centrifuge for 1 min. Discard the flow-through.

11. Wash QIAprep Spin Column by adding 750 µl of Buffer PE and centrifuging for 1 min.

12. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.

13. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl or deionized water to the center of each QIAprep Spin Column, let stand for 1 min, and centrifuge for 1 min.

14. The eluted DNA was stored at -20°C until used.

Method for analysis of DNA insertion

To checked of DNA insertion by using a vector-specific restriction enzyme digestion, *EcoR*I and *Nco*I as below.

1. Restriction enzyme *EcoR*I, prepare a reaction mixture according to: 1 µl of 10X buffer *EcoR*I, 0.5 µl of restriction enzyme *EcoR*I, 3 µl of plasmid DNA and sterile deionized water to make a total volume of 10 µl.

2. Restriction enzyme *Nco*I prepare a reaction mixture according to: 1 µl of 10X buffer 3, 0.5 µl of restriction enzyme *Nco*I, 3 µl of plasmid DNA and sterile deionized water to make a total volume of 10 µl.

3. Both reactions were incubated overnight at 37°C.

4. After digestion, the digested products were detected by agarose gel electrophoresis. The 100 bp DNA ladder and 1000 bp (Lamda DNA marker cut with both *EcoR*I and *Hind*III) were used as molecular weight marker.

Method for agarose gel electrophoresis

The amplification products and digested products were detected by electrophoresis using 1% (w/v) of agarose gel (Sigma-Aldrich, Missouri, USA.) were dissolved in Tris acetate–EDTA buffer (TAE) and was poured into an electrophoresis chamber set with comb inserted. After the gel was moulded, the well comb was pulled out and TAE buffer was added to electrophoresis chamber until the gel was submerged. The 5 µl of positive, negative products and marker comprising 100 bp DNA Ladder (Geneaid, Taiwan) and 1000 bp (Lamda DNA marker cut with both *EcoR*I and *Hind*III) were mixed with 1 µl of gel loading dye (Geneaid, Taiwan), and then were loaded into the gel. Electrophoresis was performed at constant voltage (90 volts) for 40 min. The gel was stained with 1 mg/ml of ethidium bromide solution for

10 minutes, followed by destaining in distilled water for 10 minutes. The gels were analyzed by an Ultra Lum Electronic U.V. Transilluminator (Ultra-Lum) and photographed using the Bio Image System (Syngene, Frederick, MD, USA).

Preparation of Reagents and Media

Media and reagents for bacterial culture

Luria-Bertani (LB) broth

Bacto-Tryptone	1.0	g
NaCl	0.5	g
Bacto-Yeast extract	0.5	g

Dissolve and adjust the volume to 100 ml with deionized water. Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches.

LB plate

Bacto-Tryptone	1.0	g
NaCl	0.5	g
Bacto-Yeast extract	0.5	g
Agarose	2.0	g

Dissolve and adjust the volume to 100 ml with deionized water. Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches left the media cool down to 55 °C at room temperature, poured the media on petridishes.

LB plate for Blue/White Screening

Bacto-Tryptone	1.0	g
NaCl	0.5	g
Bacto-Yeast extract	0.5	g
Agarose	2.0	g

Dissolve and adjust the volume to 100 ml with deionized water. Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches left the media cool down to 55 °C at room temperature, added 100 µg/ml of ampicillin, 0.5 mM of IPTG and 80 µg/ml of X-Gal, poured the media on petridishes.

Ampicillin (Amp): Stock of 100 mg/ml

Ampicillin	1.0	g
Sterilize-deionized water to	10	ml

Dispense the solution into 1 ml aliquoted and stored at -20 °C.

IPTG (Isopropylthio- β -D-galactoside): Stock of 1 M

Isopropylthio- β -D-galactoside	2.38	g
Deionized water	10	ml

Sterilized by filtration through a 0.2 μm disposable filter and dispense the solution into 1 ml aliquoted and stored in the dark at -20 °C.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) purchased from Promega, Madison, WI, USA. Concentration**:** 50 mg/ml in dimethylformamide.

Reagents for preparation of competent cells

Transformation buffer I (TFB I)

CH_3COOK	0.29	g
RuCl	1.21	g
$CaCl_2$. $6H_2O$	0.22	g
$MgCl_2$. $4H_2O$	0.99	g
Glycerol	15	ml

Dissolve in deionized water, adjust to pH 5.8 with 0.2 M acetic acid and adjust to 100 ml final volume with deionized water. Sterilized by filtration.

Transformation buffer II (TFB II)

MOPS	0.21	g
RuCl	0.12	g
$CaCl_2$. $6H_2O$	1.64	g
Glycerol	15	ml

Dissolve in deionized water, adjust to pH 6.5 with KOH and adjust to 100 ml final volume with deionized water. Sterilized by filtration.

Reagents for agarose gel electrophoresis

50X TAE buffer (stock solution)

Tris-base	242	g

Glacial acetic acid	57.1	ml
0.5 M EDTA (pH 8.0)	100	ml

Dissolve and adjust the volume to 1,000 ml with deionized water.

1X TAE buffer (working solution)

50X TAE buffer	20	ml

Dissolve and adjust the volume to 1,000 ml with deionized water.

1.0 % agarose gel

Agarose	1.0	g

Dissolve and adjust the volume to 100 ml with 1X TAE buffer.

Ethidium bromide solution: Stock of 10 mg/ml

Ethidium bromide	1.0	g
Deionized water	100	ml

Solution was stored in a dark bottle at 4 °C.

0.5 M EDTA (Ethylenediamine tetraacetic acid) pH 8.0

EDTA	18.61	g

Dissolve in deionized water, adjust to pH 8.0 with NaOH and adjust to 100 ml final volume with deionized water. Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches.

Reagents for RT-LAMP reaction

Deoxynucleotide triphosphates; dNTPs (Promega, Madison, WI, USA): Stock of 10 mM

100 mM dATP	10	µl
100 mM dCTP	10	µl
100 mM dGTP	10	µl
100 mM dTTP	10	µl

Sterilize-deionized water to 100 µl and stored at -20 °C

Magnesium sulphate ($MgSO_4$): Stock of 1 M

Magnesium sulphate	0.24	g

Dissolve and adjust the volume to 1.0 ml with deionized water. Sterilized by filtration.

CSFV-5'untranslated sequence alignment

The sequence alignment of 5' untranslated region of 33 CSFV (GenBank accession numbers: AF091507, AF091661, AF092448, AF099102, AF326963, AF333000, AF352565, AF407339, AF531433, AY072924, AY259122, AY367767, AY382481, AY554397, AY568569, AY578688, AY646427, AY775178, AY805221, CQ867021, DQ127910, EU490425, EU497410, EU789580, EU857642, FJ265020, FJ529205, HCU45477, HCVPOLYP2, NC_002657, X87939, X96550, Z46258) were shown in Figure 24.

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AF091507  GTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATCAAAATTATAATTTGGT
AF091661  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATCAAAATTTCAATTTGGT
AF092448  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAAACAAAATTTCAATTTGGT
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AF326963  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATCAAAATTTCAATTTGGT
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AF352565  GTATACGAGGTTAGTTCATTCTCGTGTACATGATTGGACAAATCAAAATCACTATTTGGT
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AF531433  GTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATCAAAATTATAATTTGGT
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AY259122  GTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATCAAAATTATAATTTGGT
AY367767  GTATACGAGGTTAGTTCATTCTCGTGTACAATATTGGACAAACCAAAATTCCGATTTGGC
AY382481  GTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATCAAAATTATAATTTGGT
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AY568569  GTATACGAGATTAGCTCGCCCTCGTGTACAATATTGGACAAAT-AAAATTCCGATTTGGC
AY578688  GTATACGAGGTTAGTTCATTCTCGTATACAGGATCGGACAAATCAAA-TTTCAATTTGGT
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AY775178  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAAACAAAATTTCAATTTGGT
AY805221  GTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATCAAAATTATAATTTGGT
CQ867021  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATCAAAATTTCAATTTGGT
DQ127910  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAAACAAAATTTCAATTTGGT
EU490425  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATTAAAATTTCAATTTGGA
EU497410  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAAACAAAATTTCAATTTGGT
EU789580  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATTAAAATTTCAATTTGGA
EU857642  GTATACGAGGTTAGCTCGTTCTCGTATATATGACTGGACAAATCAAAATTATAATTTGGT
FJ265020   GTATACGAGGTTAGCTCTTTCTCGTATACGACATTGGATACAC-TAAATTTCGATTTGGT
FJ529205   GTATACGAGGTTAGTTCATTCTCGTGTACAATATTGGACAAAC-AAAATTCCGATTTGGC
HCU45477  GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATCAAAATTTCAATTTGGT
HCVPOLYP2GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGAAAAATTAAAATTTCAATTTGGT
NC_002657 GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATCAAAATTTCAATTTGGT
X87939    GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATTAAAATTTCAATTTGGA
X96550    GTATACGAGGTTAGTTCATTCTCGTATGCATGATTGGACAAATCAAAATTCCAATTTGGT
Z46258    GTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATCAAAATTATAATTTGGT
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Figure 24 CSFV- 5'UTR sequence alignment. (-) indicates no consensus. (*) indicates single, fully conserved residue.

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AF091507 TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
AF091661  TCAGGGCCCCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
AF092448  TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
AF099102  TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
AF326963  TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
AF333000  TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
AF352565  TTAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
AF407339  TCAGGGCCTCCCTCCAGCGACGGCTGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
AF531433 TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
AY072924 CTAGGGTACCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
AY259122 TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
AY367767 CTAGGGCACCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
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AY554397 CTAGGGCACCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
AY568569 TTAGGGCACCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
AY578688 TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
AY646427  TCAGGGCATCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCTCATAGTAGGACTAGCA
AY775178 TCAGGGCCCCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
AY805221 TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
CQ867021  TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
DQ127910 TCAGGGCCCCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
EU490425  TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
EU497410  TCAGGGCCCCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
EU789580  TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCGCAGTAGGACTAGCA
EU857642  TCAGGGCTCCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
FJ265020  CTAGGGCACCCCTCCAGCGACGGCCGAAATGGGCTAGCCATGCCCATAGTAGGACTAGCA
FJ529205  TTAGGGCACCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
HCU45477 TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
HCVPOLYP2TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
NC_002657 TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
X87939     TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCACAGTAGGACTAGCA
X96550     TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCAATGCCCACAGTAGGACTAGCA
Z46258     TCAGGGCCTCCCTCCAGCGACGGCCGAACTGGGCTAGCCATGCCCATAGTAGGACTAGCA
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Figure 24 CSFV- 5'UTR sequence alignment. (-) indicates no consensus. (*) indicates single, fully conserved residue (cont.).

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AF091507 AAACGGAGGGACTAGCCATAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AF091661 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AF092448 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AF099102 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AF326963 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AF333000 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AF352565 AA-CGGAGGGACTAGCCATAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AF407339 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AF531433 AAACGGAGGGACTAGCCATAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
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AY259122 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AY367767 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AY382481 AAACGGAGGGACTAGCCATAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AY554397 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AY568569 AA-TGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AY578688 AA-CGGAGGGACTAGCCGTAGTGGTGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AY646427 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AY775178 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
AY805221 AAACGGAGGGACTAGCCATAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
CQ867021 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
DQ127910 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
EU490425 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
EU497410 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
EU789580 AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
EU857642 AAACGGAGGGACTAGCCATAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
FJ265020  AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
FJ529205  GA-TGGAGGGACTAACCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
HCU45477AA-CGGAGGGACTAGCCGTAGTGGCGAGCCCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
HCVPOLYP2AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
NC_002657AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
X87939    AA-CGGAGGGACTAGCCGTAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
X96550    AA-CGGAGGGACTAGCCGTAGTGGCGAGCCCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
Z46258    AAACGGAGGGACTAGCCATAGTGGCGAGCTCCCTGGGTGGTCTAAGTCCTGAGTACAGGA
                _ *__********** _ ** _ ******_ ****_ ****************************
```

Figure 24 CSFV- 5'UTR sequence alignment. (-) indicates no consensus. (*) indicates single, fully conserved residue (cont.).

```
AF091507 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
AF091661 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
AF092448 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AF099102 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
AF326963 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AF333000 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AF352565 CAGTCGTCAGTAGTTCGACGTGAGCAGGAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AF407339 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AF531433 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
AY072924 CAGTCGTCAGTAGTTCGACGTGAGCAGGAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AY259122 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
AY367767 CAGTCGTCAATAGTTCGACGTGAGCAGGAGCCCACCTCGAGATGCTAAGTGGACGAGGGC
AY382481 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
AY554397 CAGTCGTCAGTAGTTCGACGTGAGCATGAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AY568569 CAGTCGTCAGTAGTTCGACGTGAGCAGGAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AY578688 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
AY646427 CAGTCGTCAGTAGTTCGACGTAAGCGGAAGCTCACCTCGAGATGCTACGTGGACGAGGGC
AY775178 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
AY805221 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
CQ867021 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
DQ127910 CAGTCATCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
EU490425 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
EU497410 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
EU789580 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGATATGCTATGTGGACGAGGGC
EU857642 CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
FJ265020  CAGTCGTCAGTAGTTCGACGTGAGCACCAGCCCACCTCGAGATGCTACGTGGACGAGGGC
FJ529205  CAGTCGTCAGTAGTTCGACGTGAGCAGGAGCTCACCTCGAGATGCTATGTGGACGAGGGC
HCU45477CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
HCVPOLYP2CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
NC_002657CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTATGTGGACGAGGGC
X87939    CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGATATGCTATGTGGACGAGGGC
X96550    CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
Z46258    CAGTCGTCAGTAGTTCGACGTGAGCAGAAGCCCACCTCGAGATGCTACGTGGACGAGGGC
          ***** _*** _*********** _*** ___ *** _********* _******* _************
```


Figure 24 CSFV- 5'UTR sequence alignment. (-) indicates no consensus. (*) indicates single, fully conserved residue (cont.).

```
AF091507 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACGCCACGTGAT
AF091661 ATGCCCAAGACACACCTTAACTCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AF092448 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AF099102 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AF326963 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AF333000 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AF352565 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AF407339 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AF531433 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AY072924 ATGCCCAAGACACACCTTAACCCTGGCGGGGGTCGCCAGGGTGAAATCACACCATGTGAT
AY259122 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AY367767 ATGCCCAAGACACACCTTAACCCTGGCAGGGGTCGCCAGGGTGAAATCACACCATGTGAT
AY382481 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AY554397 ATGCCCAAGACACACCTTAACCCTGGCGGGGGTCGCCAGGGTGAAATCACACCATGTGAT
AY568569 ATGCCCAAGACACACCTTAACCCTGGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AY578688 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AY646427 ATGCCCAAGACACACCTTAACCCTGGCAGGGGTCGCCAGGGTGAAATCACACTATGTGAG
AY775178 ATGCCCAAGACGCACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
AY805221 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
CQ867021 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
DQ127910 ATGCCCAAGACGCACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
EU490425 ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
EU497410 ATGCCCAAGACGCACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
EU789580  ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
EU857642 ATGCCCAAGACGCACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
FJ265020  ATGCCCAAGACACACCTTAACCCTGGCGGGGGTCGCTAGGGTGAAATCACACTATGTGAT
FJ529205  ATGCCCAAGACACACCTTAACCCTGGCGGGGGTCGCCAGGGTGAAATCACACCATGTGAT
HCU45477ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
HCVPOLYP2ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
NC_002657ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
X87939    ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
X96550    ATGCCCAAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
Z46258    ATGCC-AAGACACACCTTAACCCTAGCGGGGGTCGCTAGGGTGAAATCACACCACGTGAT
          *****_***** _********** _** _** _********* _************** _* _* _****_
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Figure 24 CSFV- 5'UTR sequence alignment. (-) indicates no consensus. (*) indicates single, fully conserved residue (cont.).

```
AF091507 GGGAGTACGACCTGATAGGGCGCCGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AF091661 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AF092448 GGGAGCACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AF099102 GGGAGTACGACCTGATAGGGTGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AF326963 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AF333000 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AF352565 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AF407339 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AF531433 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AY072924 GGGGGTACGACCTGATAGGGTGCTGCAGAGGCCCACTAACAGGCTAGTATAAAAA-TCTC
AY259122 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AY367767 GGGGGTACGACCTGATAGGGTGCTGCAGAGGCCCACTAACAGGCTAGTATAAAAA-TCTC
AY382481 GGGAGTACGACCTGATAGGGCGCTGCGGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AY554397 GGGGGTACGACCTGATAGGGTGCTGCAGAGGCCCACTAACAGGCTAGTATAAAAA-TTTT
AY568569 GGGGGTACGACCTGATAGGGTGCTGCAGAGGCCCACTAACAGGCTAGTATAAAAA-TCTC
AY578688 GGGAGTACGACCTGATAGGGTGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AY646427 GGGGGTACGACCTGATAGGGTGCTGCAGGGGCCCACTATTAGGCTAGTATAAAAA-ACTC
AY775178 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
AY805221 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
CQ867021 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
DQ127910 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
EU490425 GGGAGTACGACCTGATAGGGTGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
EU497410 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
EU789580 GGGAGTACGACCTGATAGGGTGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
EU857642 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
FJ265020 GGGGGTACGACCTGATAGGGTGCTGCAGAGGCCCACTAGCAGGCTAGTATAAAAAATCTC
FJ529205 GGGGGTACGACCTGATAGGGTGCTGCAGAGGCCCACTAACAGGCTAGTATAAAAAATCTC
HCU45477 GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
HCVPOLYP2 GGGAGTACGACCTGATAGGGTGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
NC_002657GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
X87939    GGGAGTACGACCTGATAGGGTGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
X96550    GGGAGTACGACCTGATAGGGTGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
Z46258    GGGAGTACGACCTGATAGGGCGCTGCAGAGGCCCACTATTAGGCTAGTATAAAAA-TCTC
          *** -* -*************** -** -** -* -********** -- *************** --- * -
```


Figure 24 CSFV- 5'UTR sequence alignment. (-) indicates no consensus. (*) indicates single, fully conserved residue (cont.).

```
AF091507  TGCTGTACATGGCACATGGAGTTGAATCGCTTTGAACTTTTATACAAAACAAACAAACAA
AF091661  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAATAAACAA
AF092448  TGCTGTTCATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAGAACAAACAAACAA
AF099102  TGCTGTACATGGCACATGGAGTTGAATCGTTCTGAACTTTTATACAAAACAAACAAACAA
AF326963  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
AF333000  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
AF352565  TGCTGTACATGGCACATGGAGTTGACTCGTTTTGAACTTTTATACAAAACAAACAAGCAA
AF407339  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
AF531433  TGCTGTACATGGCACATGGAGTTGAATCACTTTGAACTTTTATACAAAACAAACAAACAA
AY072924  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTATTATACAAAACAAACAAACAA
AY259122  TGCTGTACATGGCACATGGAGTTGAATCACTTTGAACTTTTATACAAAACAAACAAACAA
AY367767  TGCTGTACATGGCACATGGAGTTGAATCACTTTGAACTATTATACAAAACAAACAAACAA
AY382481  TGCTGTACATGGCACATGGAGTTGAATCACTTTGAACTTTTATACAAAACAAACAAACAA
AY554397  TGTTGTACATGGCACATGGAGTTGAATCATTTTGAACTATTATACAAAACAAACAAACAA
AY568569  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTATTATACAAAACAAACAAACAA
AY578688  TGCTGTACATGGCACATGGAGTTGAATCGTTCTGAACTTTTATACAAAACAAACAAGCAA
AY646427  TGCTGTACATGGCACATGGAGTTGAATCACTTTGAACTTTTATACAAAACAAACAAACAA
AY775178  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
AY805221  TGCTGTACATGGCACATGGAGTTGAATCACTTTGAACTTTTATACAAAACAAACAAACAA
CQ867021  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
DQ127910  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
EU490425  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACGAACAA
EU497410  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
EU789580  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACGGAACAAACAAACAA
EU857642  TGCTGTTCATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
FJ265020  TGCTGTACATGGCACATGGAGTTGAAACATTTTGAATTATTATACAAAACAAACAAACAA
FJ529205  TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTATTATACAAAACAAACAAACAA
HCU45477  TGCTGTACATGGCACATGGAGTTGAATCACTTTGAACTTTTATACAAAACAAACAAACAA
HCVPOLYP2 TGCTGTACATGGCACATGGAGTTGAATTATTTTGAACTTTTATACAAAACAAACAAACAA
NC_002657 TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
X87939    TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
X96550    TGCTGTACATGGCACATGGAGTTGAATCATTTTGAACTTTTATACAAAACAAACAAACAA
Z46258    TGCTGTACATGGCACATGGAGTTGAATCACTTTGAACTTTTATACAAAACAAACAAACAA
          **_ ***_ ********************* _____  * _ **** _*_******* __ ****** __ * _***
```


Figure 24 CSFV- 5'UTR sequence alignment. (-) indicates no consensus. (*) indicates single, fully conserved residue (cont.).

```
AF091507AAACCAATGGGAGTGGAGGAACCGGTGTACGATGCCACGGGGAGACCATTGTTCGGAGAC
AF091661AAACCAATGGGAGTGGAGGAACCGGTATACGATGCCACAGGGAGACCATTGTTTGGAGAC
AF092448 AAACCAATGGGAGTGGAGGAACCGGTATACGATGCCACGGGGAGGCCATTGTTTGGAGAC
AF099102AAACCAATGGGAGTAGAGGAACCGGTATACGATGTCACGGGGAGACCATTGTTTGGAGAC
AF326963AAACCAATGGGAGTGGAGGAACCGGTATACGATGCCACGGGGAGGCCATTGTTTGGAGAC
AF333000 AAACCAATGGGAGTGGAGGAACCGGTATACGATGCCACGGGGAGACCATTGTTTGGAGAC
AF352565AAACCAGTGGGAGTGGAGGAGCCGGTGTATGATGCCACGGGGAGACCGTTGTTTGGAGAC
AF407339AAACCAGTGGGAGTGGAGGAACCGGTATACGATGCCACGGGGAGACCATTGTTTGGAGAC
AF531433AAACCAATGGGAGTGGAGGAACCGGTGTACGATGCCACGGGGAGACCATTGTTCGGAGAC
AY072924AAACCAATGGGAGTGGAGGAACCGGTGTATGACATCGAGGGGAGACCACTATTTGGGGAC
AY259122AAACCAATGGGAGTGGAGGAACCAGTGTACGATGCCACGGGGAGACCATTGTTCGGAGAC
AY367767AAACCGATGGGAGTGGAGGAACCGGTGTATGACATCGCAGGGAGACCATTATTTGGGGAC
AY382481 AAACCAATGGGAGTGGAGGAACCGGTGTACGATGCCACGGGGAGACCATTGTCCGGAGAC
AY554397AAACCAATGGGAGTGGAGGAACCGGTGTATGACATCGCAGGGAGACCACTATTTGGGGAC
AY568569AAACCAATGGGAGTGGAGGAACCGGTGTATGACATCGCAGGGAGACCATTATTTGGGGAC
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DQ127910AAACCAATGGGAGTGGAGGAACCGGTATACGATGTCACGGGGAGACCATTGTTTGGAGAC
EU490425AAACCAATGGGAGTGGAGGAACCGGTATACGATGCCACGGGGAAACCATTGTTTGGAGAC
EU497410 AAACCAATGGGAGTGGAGGAACCGGTATACGATGTCACGGGGAGACCATTGTTTGGAGAC
EU789580AAACCAAAGGGAGTGGAGGAACCGGTATACGATGCCACGGGGAAACCATTGTTTGGAGAC
EU857642AAACCAATGGGAGTGGAGGAACCGGTGTATGATGCCACGGGGAGACCATTGTTTGGAGAC
FJ265020 AAACCAGTGGGAGTGGAGGAACCGGTGTATGACGCCGCGGGGAGACCACTATTTGGGGAC
FJ529205 AAACCAATGGGAGTGGAGGAACCGGTGTATGACATCGCAGGGAGACCATTATTTGGGGAC
HCU45477AAACCAATGGGAGTGGAGGAACCGGTGTACGATGCCACGGGGAGACCATTGTTCGGAGAC
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Z46258  AAACCAATGGGAGTGGAGGAACCGGTGTACGATGCCACGGGGAGACCGTTGTTCGGAGAC
        ***** ___  *** _ *__ **_ ** _**_** _** _**  _______  ****  __ ** ____  *__ ** _** _
```


Figure 24 CSFV- 5'UTR sequence alignment. (-) indicates no consensus. (*) indicates single, fully conserved residue (cont.).

BIOGRAPHY

Name	Miss Kanokwan Wongsawat
Home Address	13/4 Moo 5, Jedrew, Ban Paew, Sumut Sakhon, 74120.
Office and Position	Teacher Omnoisophonchanupathom School 1/13 Moo 4, Om Noi, Krathum Baen, Sumut Sakhon, 74130.
Education Background	
2001	Bachelor of Science, Department of Biology, Faculty of Science, Nakhon Pathom Rajabhat University
2002	Graduate Diploma of Profession Degree, Major: Education, Faculty of Education, Nakhon Pathom Rajabhat University
2011	Master of Science, Department of Biology, Faculty of Science, Graduate School, Silpakorn University
Working Experience	
1999	Trainee Student: 1) Department of Medical Sciences, Ministry of Public Health 2) Sumut Sakhon Coastal Fisheries Research and Development Center
2001	Student teachers: The Demonstration School of Nakhon Pathom Rajabhat University
2002-2008	Teacher: Kusolwittaya School