UPPSALA
UNIVERSITET

Development and evaluation of loop mediated isothermal amplification assays for the detection of Classical swine fever virus and African swine fever virus

Chowdry Vinay Kumar

Degree project in applied biotechnology, Master of Science (2 years),
2013 Examensarbete i tillämpad bioteknik 30 hp till masterexamen, 2013
Biology Education Centre, Uppsala University, and Department of Virology,
Immunobiology and Parasitology (VIP), National Veterinary Institute (SVA)
Supervisor: Dr. Lihong Liu

TABLE OF CONTENTS

ABSTRACT

Loop-mediated isothermal amplification (LAMP) assays were developed for the detection of Classical swine fever (CSFV) and African swine fever (ASFV). Four primers specific to six distinct regions were selected, based on the alignment of genomic sequences of CSFV and ASFV available in GenBank. The assays were carried out in a Biometra T3000 thermocycler at a constant temperature of 63°C. The products showed a ladder–like pattern on a 1.5% agarose gel. The specificity of ASFV was confirmed by restriction digestion with SmaI. A duplex LAMP assay was developed aiming to investigate simultaneously if a sample is positive for CSFV or ASFV. The performance of the LAMP assays was compared with real-time PCR using 26 CSFV isolates and 14 ASFV isolates, respectively. On average, the single-plex LAMP assay was 10- to 100- fold less sensitive compared to real-time PCR method, while the duplex LAMP assay was 10- fold further less sensitive than single-plex LAMP assays. In summary, the assays provide a simple, rapid and reliable tool for the detection of CSFV and ASFV with acceptable sensitivity. The assays can also be used as a simple-to-use field diagnostic tool which can help in field detection of CSFV and ASFV.

1. INTRODUCTION

1.1 Classical swine fever

Classical swine fever (CSF) is a highly contagious viral disease caused by a pestivirus, classical swine fever virus (CSFV). It tops the list of the Office International des Epizooties (OIE) List A diseases. Both wild and domestic pigs can be infected. CSFV is present in Eastern Europe, Southeast Asia, Central America and South America. At present CSFV is eradicated from domestic pigs in Western Europe but still remains common in some populations of wild boar and therefore the farms around these areas are at a greater risk of re-infection (*Edwards et al., 2000*).

In the early 19th century, initial clinical outbreaks were reported *(Fuchs, 1968)* and characterized as viral in nature by 1903 *(Wise, 1981).* Historically depending upon the virus virulence infection was categorized into per-acute, acute, chronic, or prenatal forms of CSF. Same isolates can induce different signs depending on pig's age, breeding, health and immune status and hence strain virulence characterization is quite difficult (*Leforban et al., 1990a; Wensvoort, 1989*).

CSFV is a small, enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Pestivirus* of the family *Flaviviridae* (*Becher et al., 1999*). It is non-cytopathogenic in cell culture with few exceptions. Its genome size is about 12.5-16.5 kb and encodes a single polyprotein (*Meyers et al., 1989*).

Mature proteins are produced by co- and post-translational process of the single polyprotein with the help of virus and proteases of the host cells (*Rumenapf et al., 1993*). Structural proteins are comparatively well studied and characterized compared to non-structural proteins. Fully formed virions are released through exocytosis without harming the host cells but the process of replication and packaging of the virion particles are not well characterized (*Rumenapf et al., 1993*).

CSFV is antigenically and genetically diverse (*Vanderhallen et al., 1999*). Through monoclonal antibodies its antigenic diversity can be established. Genetic diversity could be determined by genome sequencing (*Edwards et al., 1991*).

CSFV is categorized into three major genetic groups (*Lowings et al., 1996*) with further

categorization into sub-groups; 1.1, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3, 3.4 (*Paton et al., 2000c*).

There have been findings about the relation between genotype and geographic region based on phylogenetic analysis (*Bartak and Greiser-Wilke, 2000; Stadejek et al., 1997; Vilcek et al., 1996*).

Depending on the clinical signs, major targets for the virus replication include endothelial cells, lymphorecticular cells, macrophages but the pathological findings vary *(Moennig and Plagemann, 1992; Van Oirschot, 1999*). Due to thrombosis and/or endothelial damage, hemorrhagic diathesis petechial bleedings and postnatal lesions are caused. Other consistent features include Bronchopneumonia and Substantial thrombocytopenia. Severe loss of B-lymphocytes in the blood and lymphoid tissues is an indication of fatal acute infection, which is regarded as the final stage of infection (*Susa et al., 1992*).

1.2 African swine fever

African swine fever (ASF) is caused by a large, enveloped DNA virus (ASFV), which is the only member of the family *Asfaviridae* genus *Asfivirus (Murphy et al., 1995)*. It is a highly contagious hemorrhagic disease in pigs. The infection causes a wide range of clinical signs and lesions that are quite similar to those of CSFV infection. It is also one among the Office International des Epizooties (OIE) List A diseases. Presently it has affected quite a number of animals in African countries, Mediterranean island and Sardinia (Italy) *(De Tray, 1957; Heuschele and Coggins, 1965).*

The natural reservoirs of ASF virus under natural conditions include both wild and domestic pigs. Unapparent infection with ASFV is common in wart hogs and bush pigs and both species act as reservoir hosts in Africa (*De Tray, 1957; Heuschele and coggins, 1965).* Apparently soft ticks have also been shown to be both a reservoir and a vector of ASFV especially *Ornithodorus moubata* and *O. erraticus*. The primary site for replication of ASF virus is in the cells of the mononuclear phagocytic system. The prolonged sustenance of the virus as an enzootic disease in Africa, allowed for selection of varying virulence of the virus. No distinct antigenic types have been identified but different genotypes have been differentiated through restriction enzyme digestion of the genome from different parts of the world.

ASFV is a complex, icosahedral, deoxivirus with characters that are common to both the iridovirus and poxivirus families *(Murphy et al., 1995).* The virion is composed of number of concentric structures with an external hexagonal membrane through the cell membrane by the process of budding *(Carrascosa et al., 1984).*

ASFV genome is a double stranded linear DNA with a size of around 170-190 kb, depending on the virus strain *(Blasco et al., 1989, Tabares et al., 1980),* with terminal inverted repeats (*Sogo et al., 1984*), a conserved central region of about 125 kb, and variable ends. The complete DNA sequence of the BA71v strain of ASFV is composed of 170,101 nucleotides, with 151 open reading frames encoding five multigene families *(Yanez et al., 1995).* Around 28 intracellular structural proteins and more than 100 virus induced proteins have been identified *(Tabares et al., 1980).* ASFV is very resistant to different pH and freeze/thaw cycles.
(Plowright and Parker, 1967).

The mortality rate and incubation period of preacute, acute, subacute and chronic forms (rarely seen in out breaks) occur depending on the virus virulence *(Manso Ribeiro et al., 1963).* In acute diseases, incubation period is around 5-7 days followed by high fever up to 42°C and death in 7-10 days. Clinical signs include loss of appetite, depression, respiratory distress, vomiting, bleeding nose, diarrhea, hyperemia of the skin near ear, abdomen & legs and sometimes first event that occur is abortion if the pig is pregnant or if there is an outbreak. The virus virulence varies depending on the genotype of the virus. Acute diseases are caused due to the infection of virulent type. During this period, all the body fluids and tissue contain large amounts of virus from the time of onset till the death of the animal *(Gomez-Villamandos et al., 1995; Mebus et al., 1983; Arias et al., 1986).*

Transmission of the virus from the pigs infected with less virulent type can only occur one month after the infection. The transmission is only possible through the blood *(Carrasco et al., 1996; Minguez et al., 1988).* The primary route of infection of ASFV is through the upper respiratory tract. The virus replication occurs in the tonsil and lymph nodes and soon it enters the tissue and blood stream and their concentration increases gradually leading to severe damage to blood clotting mechanism causing hemorrhagic lesions especially in lymph nodes, kidneys (almost invariably as petechiae), and heart (*Arias et al., 1986; Moulton and Coggins, 1968).*

1.3 LAMP (**Loop-mediated isothermal amplification**)

"LAMP" is a simple, rapid, specific and cost-effective nucleic acid amplification technique developed by Eiken Chemicak Co., Ltd.

1.3.1 Special features

This approach of nucleic acid amplification uses strand displacement method and thus does not require an extra step to denature double stranded into a single stranded form (*Tsugunori et al., 2000*). It makes use of four different primers which are specifically designed to recognize six distinct regions on the target gene and hence is able to specifically amplify the target gene. Since it does not requires any sophisticated instruments and special reagents, the method is quite cost effective The amplification efficiency of this method is quite high since it can amplify DNA up to $10^9 - 10^{10}$ times in just 15-60 min at around 60-65°C incubation temperature. This technique can also be made use for amplification for RNA templates by following the same procedure as with DNA templates by just adding reverse transcriptase enzyme to the reaction mixture.

1.3.2 LAMP primers

LAMP primers are shown in Table 1 and the six distinctive regions of the target gene on the basis of which they are designed are shown in the Figure 1 (F3c, F2c and F1c regions at 3' end & B1, B2 and B3 region at 5' end).

Table 1: *Four different primers required for a LAMP assay*

Primer name	Type
FIP	Forward inner (F1c+F2)
F3	Forward outer
BIP	Backward inner(B1c+B2)
B3	Backward outer

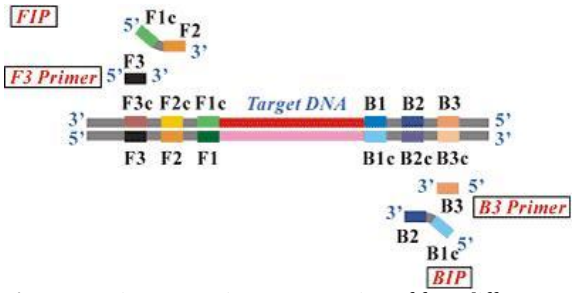


Figure 1: *Diagrammatic representation of four different primers and their complimentary regions (Tsugunori et al., 2000)*

The four different primers that are used in a

Proper primer design is very important to have an effective, efficient and sensitive LAMP assay and can be designed using software named Primer explorer which is special software used to

design LAMP primers. The main parameters which are considered while designing LAMP primers are discussed in Table 2.

Table 2: *Different parameter and there considerations during the designing of the LAMP assay primers*

Parameters	Considerations
Distance between primer regions	Distance between 5' end of F2 and B2: 120-180 bp Distance between F2 and F3 as well as B2 and B3: 0-20 bp
Tm value	60-65^{o}C in GC rich region and normal; 55-60^{o}C in AT rich region
GC content	50-60% in GC rich and normal region; 40-50% IN at RICH REGION
Secondary structure	Primers should not easily form secondary structures. 3' end should not be AT rich and complimentary to other primers/

1.3.3 LAMP Amplification Pathway

The chemical pathway for nucleic acid amplification in LAMP is quite complex (Figure 2). DNA amplification is accomplished through the use of Bst DNA polymerase which exhibits strand displacing activity. Because Bst is capable of displacing double stranded DNA during polymerization, a thermal denaturation step is not required. This strand displacing activity allows the LAMP reaction to proceed under isothermal conditions in contrast to PCR which requires a thermal denaturation step to produce single stranded DNA for primer binding.

The key feature of LAMP is the generation of artificial stem loop structures flanking the target sequence. The stem loop structures allow for self-priming as well as to provide a single-stranded region for inner primers and loop primers to bind and initiate DNA polymerization.

The reaction steps can be divided in into phase 1, phase 2 and phase 3.

<u>Phase 1: Generation of the artificial stem loop</u>

Phase 1 can be further divided into 4 steps. In step 1, forward inner primer (FIP) binds to the target sequence and initiates polymerization. In step 2, forward outer primer (F3) binds to the product and through strand displacement displaces a structure with a single artificial stem loop at the target region. In step 3, backward inner primer (BIP) binds to the product and initiates DNA polymerization. In step 4, backward outer primer (B3) binds to the product and displaces the product with two artificial stem loops flanking the target sequence.

<u>Phase 2: The main amplification cycle</u>

This phase consists of steps 5, 6 and 7. In step 5, one of the terminal loops self primes and

generates double-stranded DNA along with the length of the target. In step 6, FIP binds to the single stranded loop of the stem loop and initiates DNA polymerization with strand displacement. In step 7, a terminal stem loop primes and displaces the product.

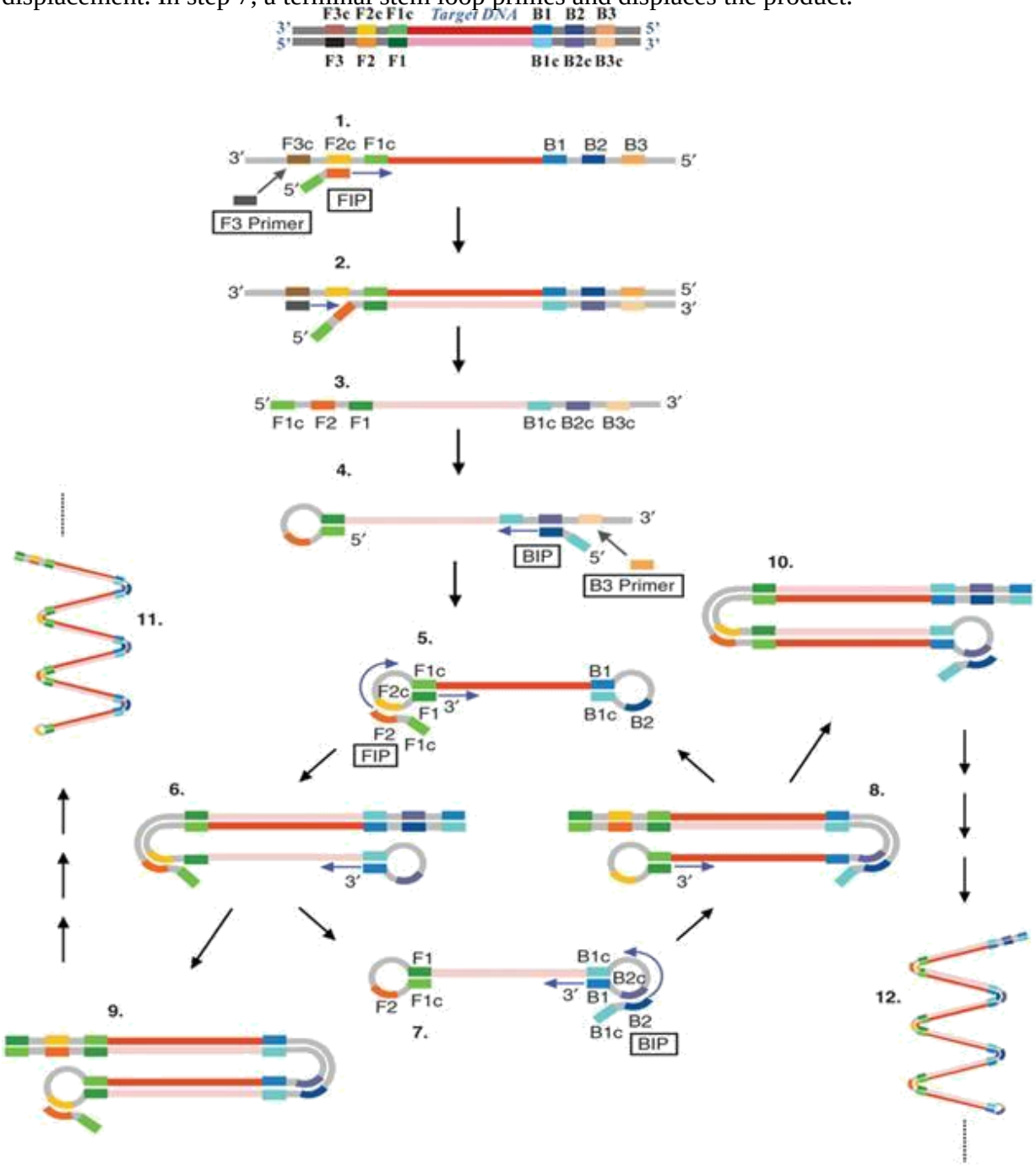


Figure 2: *Schematic representation of LAMP reaction mechanism (Tsugunori et al., 2000)*

Now, this product can continue with many subsequent amplification steps including self priming,

a terminal stem loop and priming by inner primer binding to single stranded loops.

Phase 3: Cycling of amplification step

This phase consists of steps 8, 9 and 10 which proceed exactly as steps 5, 6 and 7 with the displacement of the products and the regeneration of the original double stem loop structures.

This central amplification cycle repeats continuously throughout the LAMP reaction and generates many copies of the products.

The final product consists of stem loop DNAs with various stem lengths and multiple loops produced due to annealing of alternatively inverted repeats of the target sequence in the same strand

To initiate the DNA synthesis from the original unamplified DNA in LAMP assay, four primers are used, which recognizes six distinct sequences, unlike with conventional PCR. This ensures the high specificity for target amplification and also has high target selectivity when compared with other systems.

2. AIM OF THE PROJECT

The project aims to;

1. develop a Loop mediated isothermal amplification (LAMP) assays for the detection of Classical swine fever virus (CSFV) and African swine fever virus (ASFV).
2. develop a duplex LAMP assay to investigate simultaneously if a sample is positive for CSFV or ASFV
3. compare the performance of the LAMP assays (single-plex and duplex) with real-time PCR.

3. MATERIALS AND METHODS

3.1 Viruses and clinical samples:

Twenty-six CSFV and 14 ASFV virus samples were obtained and more details of these isolates are summarized in Table 5 and Table 6. Isolate Argentina with genotype 1.1 was used to evaluate CSFV single-plex and duplex LAMP assays. For ASFV single-plex and duplex LAMP assays, Lerida 1975 was used.

3.2 Design of primers for LAMP assays:

The primers specific to CSFV were designed based on sequences available in GenBank using Primer Explorer version 4 (http://primerexplorer.jp/elamp4.0.0/index.html). The set included two outer primers (F3 and B3) and two inner primers (FIP and BIP). The ASFV primers were taken from a publication (*James H. E. et al., 2010*). Further details of the primers are summarized in Table 3 and Table 4.

Table 3: *Primers used for CSFV LAMP assay*

Primer name	Type	Sequence (5'-3')
FIP	Forward inner (F1c+ F2)	GTCTTGGGCATGCCCTCGTCTTTTCGTCAGTAGTTCGACGTGAG
F3	Forward outer	GGGTGGTCTAAGTCCTGAGT
BIP	Backward inner(B1c+B2)	ACACCTTAACCCTAGCGGGGGTTTTCAGCACCCTATCAGGTCGTA
B3	Backward outer	AGCCTAATAGTGGGCCTCTG

Table 4: *Primers used for ASFV LAMP assay**

Primer name	Type	Sequence (5'-3')
FIP	Forward inner (F1c+F2)	GCAACGTAGCCCCCGAACTGGAAATGCTTCGCYTCCAACA
F3	Forward outer	GGCGCAAAATTTTAGCCGG
BIP	Backward inner(B1c+B2)	ATCACCATGGCGACATGTCGTGGATAGAGGTGGGAGGAGC
B3	Backward outer	GCCGAAGCTTCCTATGCC

* *James H. E. et al., 2010*

3.3 Initial optimization the LAMP assays

Experiments were performed to optimize the LAMP assays to get the best LAMP results. For CSFV assay, incubation time, Betaine concentrations and amplification temperature were

optimized. For ASFV assay, along with incubation time, Betaine concentrations and amplification temperature, $MgSO_4$ concentration was also tested. The reactions were incubated on a Biometra T3000 thermocycler at a single temperature and the products were visualized on a 1.5% agarose gel. The optimized CSFV LAMP assay in a total reaction volume of 25 µl consisted of 2.5 µl of 10 × Thermo buffer (NEB), 3.3 µl of 2.5 mM dNTPs, 0.5 µl 10 µM of F3 and B3 primers, 0.5 µl of 100 µM of FIP and BIP primers, 2 µl of 5 M Betaine, 2 µl of *Bst* DNA polymerase (8 U/µl), 6 µl of Cloned AMV-RT (1.5 U/µl) and the rest with water to make up the reaction volume. The optimized ASFV LAMP assay in a total reaction volume of 25 µl consisted of 2.5 µl of 10 × Thermo buffer (NEB), 3.3 µl of 2.5 mM dNTPs, 0.5 µl 10 µM of F3 and B3 primers, 100 µM of FIP and BIP primers, 5 µl of 5 M Betaine, 3 µl of 50 mM $MgSO_4$, 2 µl of *Bst* DNA polymerase (8 U/µl) and the rest with water to make up the reaction volume. The reaction mixtures were incubated at 63°C for 60 min. After the reaction was completed, the LAMP products were visualized on a 1.5% agarose gel.

3.4 Restriction enzyme digestion to conform LAMP results

Restriction enzyme digestion was performed on LAMP products to confirm that correct region of the genome has been amplified. Restriction enzymes were chosen to cut specifically a single site within the target region. PstI was only specific to CSFV and SmaI was only specific to ASFV. The selection of restriction enzyme was done using the tool NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/). Products of CSFV and ASFV LAMP assays were digested with PstI and with SmaI separately. The digestion reaction in a total volume of 10 µl was 5 µl LAMP products, 1 µl of respective enzyme, 1 µl of 10 × NEB buffer 4 and 3 µl with water. The incubation was done for 60 min at 37°C for reaction with restriction enzyme PstI and 25°C for reaction with restriction enzyme SmaI. After incubation, the digested products were visualized on a 2% agarose gel.

3.5 Optimization of the duplex LAMP assay

After optimizing individual LAMP assays, a duplex LAMP assay was optimized. The optimized duplex LAMP assay in a total reaction volume of 25 µl consisted 2.5 µl of 10 × Thermo buffer (NEB), 3.3 µl of 2.5 mM dNTPs, 0.5 µl 10 µM of F3 and B3 primers (both for CSFV and ASFV), 100 µM of FIP and BIP (both for CSFV and ASFV), 4 µl of 5 M Betaine, 2 µl of *Bst*

DNA polymerase (8 U/µl), 6 µl of Cloned AMV-RT (1.5 U/µl) and the rest with water to make up the reaction volume. The reaction mixture was incubated at 63°C for 60 min; the products were then visualized on a 1.5% agarose gel.

3.6 Evaluation of the LAMP assay

The specificity of the assay was tested by performing LAMP reaction with all the different ASFV and CSFV isolates mentioned in Table 5 and Table 6. Serial dilutions of all the samples were made and LAMP reaction was performed to check how far the LAMP assays were sensitive.

3.7 Real-time PCR

CSFV and ASFV real-time PCR assays were performed using AgPath-ID One-step master mix (Applied Biosystems). The reaction was performed in a total volume of 25 µl 3 µl of template. The reaction mixture consisted of 12.5 µl of 2 × RT-PCR buffer, 1 µl of 10 µM Forward primer, 1 µl of 10 µM Reverse primer, 0.5 µl of 10 µM Probe, 1 µl of 25 × RT-PCR enzyme mix and the rest with nuclease free water to make up the reaction volume. Cycling steps for the reaction was 45°C /10min, 95°C /10min, 45 cycles of 95°C /15sec and 60°C /45 sec.

4. RESULTS

4.1 Optimization of LAMP assay

When the products were analyzed on a 1.5% agarose gel, unlike a typical ladder-like pattern from LAMP reaction, a thick smeared band was observed, which may indicate that the products were not separated by electrophoresis (Figure 3 and Figure 4). To better the visualization of the results, the LAMP products were diluted 10, 15, 20, 30 and 50 times. The 10-fold dilution was found to be optimal, which gave a clear laddering pattern for both CSFV and ASFV LAMP assays.

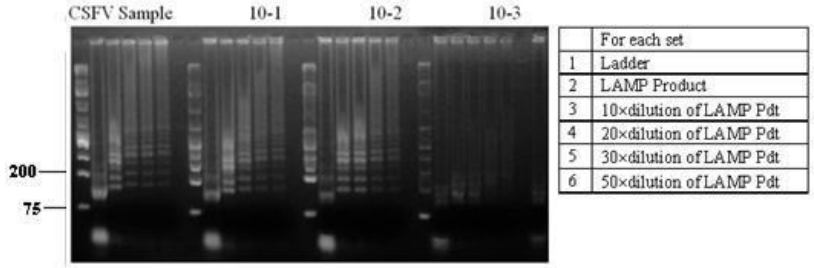


Figure 3: *Optimization of CSFV LAMP assay: The above gel picture is divided into 4 sets in the respective order: CSFV original sample, 10^{-1} dilution, 10^{-2} dilution and 10^{-3} dilution. The sensitivity of the assay was till 10^{-3} dilution but it was strong till 10^{-2} dilution. So for further experiment 10^{-2} dilution samples was used. A LAMP reaction with water as the template was used as a negative control and was loaded in the last well of the gel.*

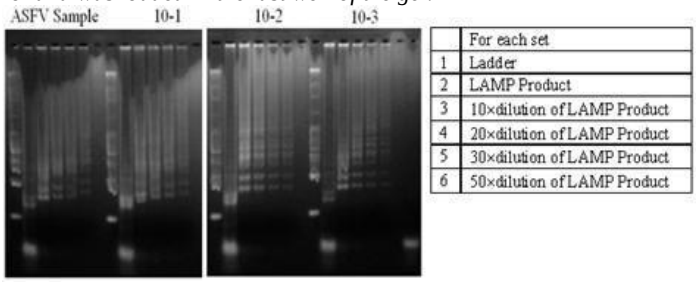
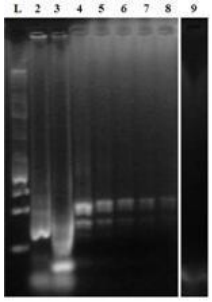


Figure 4: *Optimization of ASFV LAMP assay: The above gel picture is divided into 4 sets in the respective order: ASFV original sample, 10^{-1} dilution, 10^{-2} dilution and 10^{-3} dilution. The sensitivity of the assay was strong till 10^{-3} dilution. So for further experiment 10^{-3} dilution samples was used. A LAMP reaction with water as the template was used as a negative control and was loaded in the last well of the gel.*

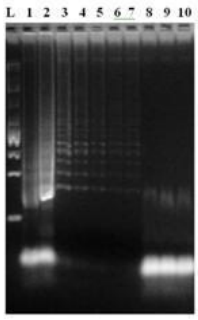
4.2 Restriction enzyme digestion

The LAMP amplicons were designed to contain unique sequences that are recognized by SmaI for ASFV and PstI for CSFV. When ASFV products were digested by SmaI, a single band was observed. By contrast, CSFV products were not digested as a ladder like pattern remained (Figure 5). Even though PstI site was included in one of the CSFV primer, no digestion was

observed. It may be due to some changes in the nucleotide sequence recognized by PstI during the reaction which might have interfered with the digestion process. ASFV products were not digested by PstI (Figure 6).

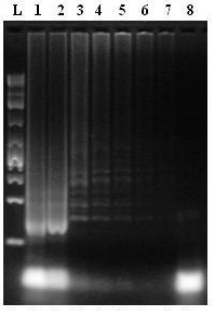


L	Ladder
2	**ASFV_5_10^{-3} dilution_63°C_1**_LAMP_Pdt
3	ASFV_5_10^{-3} dilution_63°C_1_25°C_digested
4	ASFV_5_10^{-3} dilution_63°C_1_25°C_ digested _20× dilution
5	ASFV_5_10^{-3} dilution_63°C_1_25°C_ digested _50× dilution
6	ASFV_5_10^{-3} dilution_63°C_1_25°C_ digested _100× dilution
7	ASFV_5_10^{-3} dilution_63°C_1_25°C_ digested _150× dilution
8	ASFV_5_10^{-3} dilution_63°C_1_25°C_ digested _200× dilution
9	Water_63°C

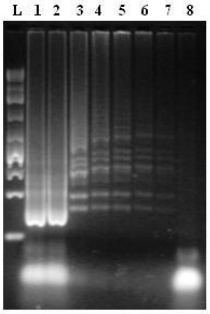


L	Ladder
1	**CSFV_512_10^{-2} dilution_63°C_1**_LAMP_Pdt
2	CSFV_512_10^{-2} dilution_63°C_1_25°C_digested
3	CSFV_512_10^{-2} dilution_63°C_1_25°C_digested_10× dilution
4	CSFV_512_10^{-2} dilution_63°C_1_25°C_digested_20× dilution
5	CSFV_512_10^{-2} dilution_63°C_1_25°C_digested_30× dilution
6	CSFV_512_10^{-2} dilution_63°C_1_25°C_digested_50× dilution
7	CSFV_512_10^{-2} dilution_63°C_1_25°C_digested_100× dilution
8	Water_63°C
9	Water_63°C
10	Water_63°C

Figure 5: *Restriction enzyme digestion of ASFV and CSFV by SmaI: Gel picture showing the results for SmaI digestion of CSFV and ASF LAMP products. There is a clear thick band for ASFV and a clear laddering pattern for CSFV confirming a specific and successful LAMP amplification*



L	Ladder
1	**CSFV_512_10^{-2} dilution_63°C**_LAMP_Pdt
2	CSFV_512_10^{-2} dilution_63°C_37°C_Pst1_digested
3	CSFV_512_10^{-2} dilution_63°C_37°C _Pst1_digested _10× dilution
4	CSFV_512_10^{-2} dilution_63°C_37°C _Pst1_digested _20× dilution
5	CSFV_512_10^{-2} dilution_63°C_37°C _Pst1_digested _30× dilution
6	CSFV_512_10^{-2} dilution_63°C_37°C _Pst1_digested _50× dilution
7	CSFV_512_10^{-2} dilution_63°C_37°C _Pst1_digested _100× dilution
8	Water_63°C

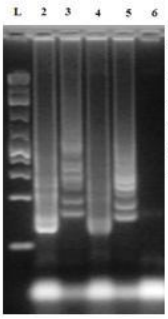


L	Ladder
1	**ASFV_10^{-3} dilution_63°C**_LAMP_Pdt
2	ASFV_10^{-3} dilution_63°C_37°C_Pst1_digested
3	ASFV_10^{-3} dilution_63°C_37°C _Pst1_digested _10× dilution
4	ASFV_10^{-3} dilution_63°C_37°C _Pst1_digested _20× dilution
5	ASFV_10^{-3} dilution_63°C_37°C _Pst1_digested _30× dilution
6	ASFV_10^{-3} dilution_63°C_37°C _Pst1_digested _50× dilution
7	ASFV_10^{-3} dilution_63°C_37°C _Pst1_digested _100× dilution
8	Water_63°C

Figure 6: *Restriction enzyme digestion of CSFV and ASFV by PstI: Gel picture showing the results for PstI digestion of CSFV and ASF LAMP products. In principal there should be a thick single band for CSFV but unfortunately the system did not work. Four to five trials were made with different conditions, but still observed the same result.*

4.3 duplex LAMP assay

When the reaction products were analyzed on a 1.5% agarose gel, a thick smeared band was seen. So the LAMP products were diluted 10 times and loaded onto the gel along with their respective LAMP products, which produced a clear laddering pattern (Figure 7). This indicated a successful LAMP amplification. The duplex assay was developed for the detection of unknown samples, to investigate whether CSFV/ASFV infections are present in the samples. To differentiate CSFV and ASFV, restriction enzyme digestion was performed.



L	Ladder
2	ASFV Duplex LAMP Pdt
3	ASFV Duplex LAMP Pdt × 10 dilution
4	CSFV Duplex LAMP Pdt
5	CSFV Duplex LAMP Pdt × 10 dilution
6	Water

Figure 7: *Duplex LAMP assay: Gel picture showing successful amplification by optimized duplex LAMP assay*

4.4 Diagnostic sensitivity of the LAMP assays compared to real-time PCR

Table 5 and Table 6 summarize the ASFV and CSFV LAMP assays and their respective real-time PCR results for all ASFV and CSFV isolates.

For ASFV, out of 14 samples, single-plex LAMP assay for 3 samples had same sensitivity, 5 samples were 10-fold less sensitive and 6 samples were 100-fold less sensitive than real-time PCR. With respect to duplex LAMP assay, 12 samples had same sensitivity and 2 samples were 10-fold less sensitive than single-plex LAMP assay.

For CSFV, out of 26 samples, single-plex LAMP assay for 8 samples had same sensitivity, 10 samples were 10-fold less sensitive and 8 samples were 100-fold less sensitive than real-time PCR. With respect to duplex LAMP assay, 9 samples had same sensitivity and 12 samples were 10-fold less sensitive and 5 samples were 100-fold less sensitive than single-plex LAMP assay.

LAMP assay was on average 10- to 100- fold less sensitive than the real-time PCR and duplex LAMP assay was on average 10-fold less sensitive than single-plex LAMP assay.

Table 5: *List of ASFV samples for the study along with real-time PCR and LAMP results*

	Isolate	Isolation country	real-time PCR		LAMP (positive)	
			Dilution	C_t	Singleplex	Duplex
1	Mozambique 1964	Mozambique	10^{-2}	35.73	10^{-2}	10^{-2}
2	Angola 1972	Angola	10^{-4}	36.35	10^{-3}	10^{-2}
3	Chalaswa 1983	Malawi	10^{-3}	33.35	10^{-2}	10^{-2}
4	Cape Verde 1997	Cape Verde	10^{-2}	35.65	Original	Original
5	Hoima 2003	Uganda	10^{-2}	31.70	10^{-1}	10^{-1}
6	Kenya 2006	Kenya	10^{-2}	37.09	Negative	Negative
7	Kenya 2007	Kenya	10^{-3}	39.50	10^{-2}	10^{-2}
8	Burkina Faso 2007	Burkina Faso	10^{-4}	37.87	10^{-2}	10^{-2}
9	Pontevedra 1970	Spain	10^{-5}	38.72	10^{-3}	10^{-3}
10	Badajoz 1971	Spain	10^{-5}	34.92	10^{-5}	10^{-5}
11	Lerida 1975	Spain	10^{-5}	38.39	10^{-3}	10^{-3}
12	Lisbon 60	Portugal	10^{-3}	34.52	10^{-3}	10^{-3}
13	Sardinia 1988	Italy	10^{-3}	36.28	10^{-3}	10^{-3}
14	Port-au-Prince 81	Haiti	10^{-6}	38.65	10^{-5}	10^{-4}

Table 6: *List of CSFV samples for the study along with real-time PCR and LAMP results*

	Original Name	Genotype	Country	real-time PCR		LAMP (positive)	
				Dilution	C_t	Singleplex	Duplex
1	31240/96	1.1	Slovak Republic	10^{-2}	35.92	10^{-1}	Original
2	Romania I 01	1.1	Romania	10^{-3}	40.08	10^{-2}	10^{-1}
3	Alfort 187	1.1	France	10^{-3}	42.52	10^{-3}	10^{-1}
4	Isolate Argentina	1.1	Argentina	10^{-3}	38.05	10^{-2}	10^{-2}
5	742/Ru	1.1	Russia	10^{-2}	36.54	Original	Original
6	7/3	1.2	Poland	10^{-4}	41.34	10^{-1}	Original
7	No.3/ brescia	1.2	Romania	10^{-3}	38.93	10^{-1}	Original
8	JIK/Ru	1.2	Russia	10^{-1}	39.43	10^{-1}	Original
9	VRI 4167	1.3	Malaysia	10^{-2}	37.45	10^{-2}	10^{-2}
10	94-14901 / 02-94	1.3	Costa Rica	10^{-1}	40.74	10^{-1}	Negative
11	97-7446/ #4	1.3	Honduras	10^{-1}	37.59	Negative	Negative
12	Guatemala HC / #4409	1.3	Guatemala	10^{-1}	37.96	Negative	Negative
13	907/1	2.1	Germany	10^{-2}	40.22	10^{-2}	Original
14	VRI 2277	2.1	Malaysia	10^{-2}	36.99	10^{-2}	10^{-1}
15	2000/8	2.1	Great Britain	10^{-2}	35.89	10^{-1}	Original
16	V2/97	2.1	Germany	10^{-3}	39.50	10^{-1}	Original
17	4905 I 97/03	2.1	Italy	10^{-4}	41.72	10^{-2}	Original
18	V 273/89	2.2	Germany	10^{-4}	37.89	10^{-3}	10^{-3}
19	P40/07/87	2.2	Singapore	10^{-3}	38.89	10^{-1}	Original
20	VA 531	2.2	Italy	10^{-3}	38.88	10^{-2}	10^{-1}
21	2213/97	2.2	Czech Republic	10^{-3}	37.47	10^{-3}	Original
22	2699/Osterode	2.3	Germany	10^{-3}	36.62	10^{-1}	Original
23	Pomi / 2004; “Isolate 4”	2.3*Rostock	Romania/ Satu Mare	10^{-2}	39.95	10^{-2}	Original
24	30853	2.3	Israel	10^{-3}	39.73	10^{-2}	10^{-2}
25	591/02	2.3*Uelzen	Bulgaria	10^{-2}	37.35	Original	Original
26	M7 19928/60; Viro 1416/2	2.3*Slovakia	Hungary /Pest	10^{-1}	32.53	Original	Original

5. DISCUSSION

The study describes the development and evaluation of single-plex and duplex LAMP assays to detect CSFV and ASFV. For gel electrophoresis the products had to be diluted to get a clear ladder-pattern. This could be due to the presence of high amount of LAMP products or due to the interference of high Betaine concentration, buffer used in LAMP reaction etc. When diluted these effects were minimized leading to a clear ladder-pattern.

The optimized duplex LAMP assay was able to amplify ASFV and CSFV correctly without cross-reaction. The duplex format would provide a useful and convenient tool for simultaneous detection of CSFV/ASFV in a sample as both viruses gave similar clinical signs and differentiating diagnosis is indeed.

By restriction enzyme digestion, it was thought to differentiate CSFV and ASFV. However, even though PstI site was included in one of the CSFV primer, no digestion was observed. It may be due to some changes in the nucleotide sequence recognized by PstI during the reaction which might have interfered with the digestion process. Even when the reaction was performed according to the manufacturer's specified conditions, still the system did not work and therefore the issue is still open for further investigations.

The performance of the LAMP assays was compared with real-time PCR for the detection of CSFV and ASFV. We found that LAMP assay was 10 to 100 fold less sensitive when compared to real-time PCR. We also observed that some original samples gave a negative result in LAMP assay but were positive in real-time PCR. This may be due to the concentration of the viral RNA/DNA template in the original samples. The concentration could be at the detection limit or may be lower than the detection limit of LAMP assay, but higher than that of the highly sensitive real-time PCR. We observed that the duplex LAMP assay was about 10 fold less sensitive than single-plex LAMP assay. This could be due to the presence of additional primers, which might interfere with specific amplification of targets.

LAMP assay has high analytical sensitivity but a small contamination can readily cause false positive results. LAMP assay is useful for less equipped laboratories due to its isothermal nature. It could also be used as a simple-to-use field diagnostic tool which can help in field detection of CSFV and ASFV and can also compliment for the detection of other swine diseases.

In conclusion, the results showed that a decent and reliable LAMP assay was developed for the detection of CSFV and ASFV.

6. ACKNOWLEDGEMENTS

I am heartily thankful to Dr. Lihong Liu for his excellent supervision from initial stage to the final stage of the project and evaluation of the report that enabled me to understand the subject and to develop scientific writing skills.

I thank Balaje Vijayaraghavan, Rajiv Harimoorthy, Harindranath Cholleti for helping me out with the planning and supporting me from the start till the end of the project. I would also like to thank them for their valuable suggestions and discussions during the lab work and also and for helping me keep my enthusiasm going on.

I thank Dr. Muhammad Munir, Dr Anne-Lee and Jay Lin for their encouragement, suggestions and discussions. I also would like to thank Prof. Sandor Belak, Prof. Michael Berg, Dr. Claudia Baule, Dr. Hongyan Xia and Dr. Mikhayil Hakhverdyan.

Last but not least, I thank my parents and my friends for their complete and total support and encouragement throughout my life.

7. REFERENCES

Arias ML, Escribano JM, Rueda A, Sánchez-Vizcaíno JM. 1986. La Peste Porcina Africana. Med Vet 3:333–350.

Blasco R, Aguero M, Almendral JM, Viñuela E. 1989. Variable and constant regions in African swine fever virus DNA. Virology 168:330–338.

Bartak P, Greiser-Wilke I. 2000. Genetic typing of classical swine fever virus isolates from the territory of the Czech republic. Vet Microbiol 77:59–70.

Becher P, Orlich M, Kosmidou A, Konig M, Baroth M, Thiel HJ. 1999. Genetic diversity of pestiviruses: Identification of novel groups and implications for classification. Virology 262:64–71.

Carrasco L, de Lara FC, Martin de las Mulas J, Gomez-Villamandos JC, Hervas J, Wilkinson PJ, Sierra MA. 1996. Virus association with lymphocytes in acute African swine fever. Vet Res 27:305–312.

Carrascosa JL, Carazo JM, Carrascosa AL, Garcia N, Santisteban A,Viñuela E. 1984. General morphology and capsid fine structure of African swine fever virus particles. Virology 132:160–172.

De Boer CV. 1967. Studies to determine neutralizing antibody in sera from animals recovered from African swine fever and laboratory animals inoculated with African virus with adjuvants. Arch Gesamte Virusforsch 20:164–179.

Edwards S, Fukusho A, Lefevre PC, Lipowski A, Pejsak Z, Roehe P, Westergaard J. 2000. Classical swine fever: The global situation. Vet Microbiol 73:103–119.

Edwards S, Moennig V, Wensvoort G. 1991. The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. Vet Microbiol 29:101–108.

Fuchs F. 1968. Schweinepest. In Handbuch der virusinfektionen bei Tieren, Vol 3. H Rohrer, ed. Jena: Gustav Fischer.

Gomez-Villamandos JC, Hervas J, Mendez A, Carrasco L, Martin de las Mulas J, Villeda CJ, Wilkinson PJ, Sierra MA. 1995. Experimental African swine fever: Apoptosis of lymphocytes and virus replication in other cells. J Gen Virol 76:2399–2405.

James HE, Ebert K, McGonigle R, Reid SM, Boonham N, Tomlinson JA, Hutchings GH, Denyer M, Oura CA, Dukes JP, King DP. Detection of African swine fever virus by loop-mediated isothermal amplification. J Virol Methods. 2010 Mar;164(1-2):68-74.

Leforban Y, Edwards S, Ibata G, Vannier P. 1990a. A blocking ELISA to differentiate hog cholera virus antibodies in pig sera from those due to other pestiviruses. Ann Rech Vet 21:119–129.

Lowings P, Ibata G, Needham J, Paton D. 1996. Classical swine fever virus diversity and evolution. J Gen Virol 77:1311–1321.

Meyers G, Rumenapf T, Thiel HJ. 1989. Molecular cloning and nucleotide sequence of the genome of hog cholera virus. Virology 171:555–567.

Mebus CA, McVicar JW, Dardiri AH. 1983. Comparison of the pathology of high and low virulence African swine fever infections. In African Swine Fever. PJ Wilkinson, ed. Proc CEC/FAO Research seminar, Sardinia, September, 1981. Commission of the European Communities Publication EUR 8466 EN, pp. 183–194.

Minguez I, Rueda A, Dominguez J, Sánchez-Vizcaíno JM. 1988. Double labeling immunohistological study of African swine fever virus-infected spleen and lymph nodes. Vet Pathol 25:193–198.

Moulton J, Coggins L. 1968. Comparison of lesions in acute and chronic African swine fever. Cornell Vet 58:364–388

Moenning, V., Plagemann, g.W., 1992. The pestiviruses. Adv. Virus Res. 41, 53-98

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martinelli GP, Mayo MA, Summers MD. 1995. Virus taxonomy, 6th report of the International Committee on Taxonomy of Viruses. Arch Virol Supplement 10.

Paton DJ, McGoldrick A, Greiser-Wilke I, Parchariyanon S, Song JY, Liou PP, Stadejek T, Lowings JP, Bjorklund H, Belak S. 2000c. Genetic typing of classical swine fever virus. Vet Microbiol 73:137–157.

Plowright W. 1981. African swine fever. In Infectious Diseases of Wild Mammals, 2nd edition. JW Davis, LH Karstand, DO Trainer, eds. Ames: Iowa State University Press, pp. 178–190.

Plowright W, Parker J. 1967. The stability of African swine fever virus with particular reference to heat and pH inactivation. Arch Gesamte Virusforsch 21:383–402.

Rumenapf T, Unger G, Strauss JH, Thiel HJ. 1993. Processing of the envelope glycoproteins of pestiviruses. J Virol 67:3288–3294.

Susa, M., Konig, M., Saalmuller, A., Reddehase, M.J., Thiel, H.J., 1992. Pathogenesis of classical swine fever: b-lymphocyte deficiency caused by hog cholera virus. J. Virol. 66, 1171-1175.

Stadejek T, Vilcek S, Lowings JP, Ballagi-Pordany A, Paton DJ, Belak S. 1997. Genetic heterogeneity of classical swine fever virus in Central Europe. Virus Res 52:195–204.

Tabares E, Marcotegui MA, Fernandez M, Sanchez-Botija C. 1980. Proteins specified by African swine fever virus I. Analysis of viral structural proteins and antigenic properties. Arch Virol 66:107–117.

Tsugunori Notomi, Hiroto Okayama, Harumi Masubuchi, Toshihiro Yonelawa, Kaiko Watanabe, Nobuyuki Amino and Tetsu Hase 2000. Loop-mediated isothermal amplification of DNA. Nucleic acid research, Vol 28, No 12:e63

Van Oirschot, J.T., 1999. Hog cholera. In: Shaw, B.E., et al. (Eds), Diseases of swine, 8th edition. Iowa State University Press, Iowa, pp. 159-172.

Vanderhallen H, Mittelholzer C, Hofmann MA, Koenen F, Mittelhozer C. 1999. Classical swine fever virus is genetically stable in vitro and in vivo. Arch Virol 144:1669–1677.

Vilcek S, Stadejek T, Ballagi-Pordany A, Lowings JP, Paton DJ, Belak S. 1996. Genetic variability of classical swine fever virus. Virus Res 43:137–147.

Wensvoort G. 1989. Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies. J Gen Virol 70:2865–2876.

Wise GH. 1981. Hog cholera and its eradication. A review of the U.S. experience. Animal and Plant Health Inspection Service, United States Department of Agriculture, APHIS 91–55.

Yañez RJ, Rodriguez JM, Nogal ML, Yuste L, Enriquez C, Rodriguez JF, Viñuela E. 1995. Analysis of the complete nucleotide sequence of Africa swine fever virus. Virology 208:249–278.