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*Full Length Research Paper*

# Comparative detection of African swine fever virus by loop-mediated isothermal amplification assay and polymerase chain reaction in domestic pigs in Uganda

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**African swine fever (ASF) is a contagious viral disease, which can cause up to 100% mortality among domestic pigs. Pig production is growing rapidly in Uganda among East African countries and is not only a source of food but also an important income for many people living in the rural areas. Field diagnosis of ASF depends only on clinical signs and has to be confirmed in the laboratory since the clinical signs are not pathognomonic. Diagnostic techniques for ASF are focused on serological tests for detection of antigen and antibody, genomic DNA detection by polymerase chain reaction (PCR), and on virus isolation and localization in clinical samples. There have been many recent reports of ASF outbreaks in Uganda yet laboratory diagnosis is limited due to the high cost and expertise required. This work reports the evaluation and application of a loop-mediated isothermal amplification (LAMP) test for detecting African swine fever virus (ASFV) DNA based on the topoisomerase II gene. Thirty (30) tissue samples obtained from suspected ASF outbreaks were collected from different regions of Uganda. The tissue samples were found to have lesions consistent with ASF. One hundred and eighty eight (188) additional blood samples were obtained from the abattoir and field surveillance. Six primers targeting the topoisomerase II gene were used. The sensitivity and specificity of LAMP and OIE recommended diagnostic PCR were compared. The LAMP assay is rapid with results obtained within 1 h (45-60 min). The sensitivity of LAMP for the detection of ASFV was 100% (95% CI: 91.78-100) while the specificity was 44% (95% CI: 36.52-51.69). The Kappa statistic for level of agreement between PCR and LAMP test in the detection of ASFV was 23.7% (95% CI: 16.42-30.91). This Kappa value indicated a fair agreement between the two assays. No cross reaction was observed with *Porcine circovirus type 2* virus and *E. coli* isolated from pigs in Uganda. This is the first study evaluating and applying the LAMP assay in the detection of ASF in domestic pigs in Uganda. The LAMP assay was found to be more sensitive than PCR. Due to its simplicity, sensitivity and specificity, the LAMP assay has the potential for use in the diagnosis and routine surveillance of ASF in Uganda.**

**Key words:** African swine fever virus, loop-mediated isothermal amplification (LAMP), polymerase chain reaction (PCR), sensitivity, specificity, topoisomerase II gene.

## INTRODUCTION

The pig farming industry is one of the fastest growing livestock activities in the rural areas of Uganda and has become very attractive throughout the country as a means of increasing food, income and employment but has on several occasions been hampered by African swine fever (ASF) (Atuhaire et al., 2013). According to reports, Uganda has the largest and fastest growing pig production in Eastern Africa with the pig population standing at 3.2 million (Uganda Beaural of Statistics/The Ministry Of Agriculture, 2009). In 2011, Uganda had the highest per capita consumption of pork in sub-Saharan Africa (3.4 kg/person per year). ASF is a highly lethal haemorrhagic disease of domestic swine, with mortality rates approaching 100% (Costard et al., 2009). The causative agent, African swine fever virus (ASFV), is a unique and genetically complex DNA virus. It is the sole member of the Asfarviridae and the only known DNA arbovirus (Dixon et al., 2000). ASFV is a large icosahedral virus which contains a linear double stranded DNA genome (170 to 190 kbp). The virus is endemic in Africa and parts of southern Europe and presents a major economic problem for the development of pig industries in these countries. Depending on the infecting virus isolate, ASFV causes syndromes ranging from peracute to chronic.

Laboratory diagnosis is essential to establish a definitive diagnosis of ASF, provide relevant information about the time of infection and support successfully control and eradication programs (OIE, 2010). Virus isolation (VI) and the haemadsorption test (Malmquist and Hay, 1960) are specific and sensitive but also too laborious and time consuming to be employed for routine or rapid diagnosis in resource poor laboratories. Polymerase chain reaction (PCR) was described as a suitable rapid alternative to VI for the detection of ASFV (Steiger et al., 1992) and may be particularly useful for screening poor quality or degraded samples with non-recoverable virus (King et al., 2003). Several PCR and real-time PCR assays have been described for detection or genotype characterization of ASFV (Agüero et al., 2004; Giammarioli et al., 2008; King et al., 2003; McKillen et al., 2010; Zsak et al., 2005), as well as isothermal amplification assays (Hjertner et al., 2005; James et al., 2010) and Linear-After-The-Exponential PCR (LATE-PCR) assay (Ronish et al., 2011). Also *in-situ* hybridization (ISH) protocols to locate viral genetic material in tissues and cells have been developed (Oura et al., 1998).

In Uganda, diagnosis of ASF by field Veterinarians mainly relies on the clinical signs and post-mortem lesions though they are not pathognomonic for ASF. Laboratory diagnosis is mainly done only when farmers and Veterinary officers are seeking for help in cases of deaths of pigs at the National Disease Diagnostics and Epidemiology Centre, Entebbe and most recently at the College of Veterinary Medicine, Animal resources and Biosecurity (CoVAB), Makerere University by use of the OIE recommended diagnostic PCR. Routine surveillance of ASF is minimal if any (Rutebarika and Ademun, 2011). Serology has been limited to research only (Atuhaire et al., 2013; Björnheden, 2011; Gallardo et al., 2011; Tejlar, 2012). Some studies have shown no positive antibody response using the OIE-prescribed serological methods in any of the serum samples collected from ASF outbreaks in Uganda (Gallardo et al., 2011). Thus, the immune methods have low specificity and sensitivity. The conventional PCR method is sensitive, accurate but time consuming and requires expensive equipment. Therefore it does not meet the needs of detection in the field setting.

The loop-mediated isothermal amplification (LAMP) assay, since its development (Notomi et al., 2000) has gained popularity in the last decade in the diagnosis of many diseases as an easy to use alternative technique for DNA amplification under isothermal conditions especially in resource poor laboratories. LAMP has been found to be more sensitive and highly specific than PCR in many previous studies; moreover, results can be obtained in 1 h. Recently, a LAMP assay was developed for the detection of ASFV (James et al., 2010). This assay targets the topoisomerase II gene of ASFV and the detection format represents the first step towards developing a practical, simple-to-use and inexpensive molecular assay for ASF diagnosis in the field which is especially relevant to Africa where the disease is endemic in many countries (James et al., 2010).

In this study we report the application of a LAMP assay to the detection of African swine fever virus in suspected ASF outbreaks in Uganda. The study also aimed at using LAMP in establishing the extent ASFV might be circulating in the field. We have evaluated its sensitivity and specificity for the detection of ASFV based on the OIE recommended PCR.

## MATERIALS AND METHODS

### Samples collected

A total of 30 tissue samples (spleen, lymph nodes, tonsil, and kidney) were collected from domestic pigs after post-mortem in areas reporting suspected ASF outbreaks in Uganda between 2010 and 2013. The tissues were then transported in a cool box

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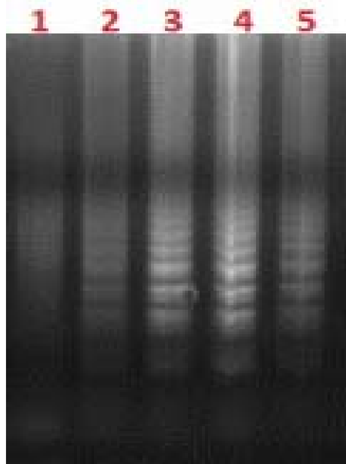


**Figure 1.** Determination of optimal temperature. 1.5% gel electrophoresis of LAMP products. Lanes 1, 2, 3, 4 and 5, reaction at 60, 63, 64, 65 and 66°C respectively.

containing cooling elements to the Molecular Biology Laboratory at the College of Veterinary Medicine, Animal resources and Biosecurity, Makerere University. Upon arrival, the tissue samples were stored at -80°C until required for DNA extraction. In order to determine the extent ASFV might be circulating in the field, 188 blood samples were collected from apparently healthy domestic pigs in Nalukolongo slaughterhouse, Kampala city.

#### Extraction of genomic DNA

Viral DNA was extracted directly from 200 µl aliquots of blood collected in EDTA tubes and from tissue samples by using a DNeasy Blood and tissue kit (Qiagen® USA).

#### Genomic amplification of viral DNA

A 278 bp region corresponding to the central portion of the p72 gene was amplified using the ASF diagnostic primer set recommended by the Office International des Epizooties (OIE) (Paris, France); primer 1 (5'-ATGGATACCGAGGGAATAGC-3') and primer 2 (5'-CTTACCGATGAAAATGATAC-3') (Wilkinson, 2000). Conditions for PCR assays were as previously described (Gallardo et al., 2009) with a modification in the annealing temperature from 50 to 55ºC. Amplification products were loaded on a 1.5% agarose gel and run against a 50 bp DNA ladder (BIORON®, Germany). Once sufficient electrophoretic separation was obtained, the products were visualized by UV irradiation and stained with ethidium bromide for gel imaging.

#### The LAMP assay

A one-step loop-mediated amplification (LAMP) assay targeting the topoisomerase II gene of ASFV was used on the field viruses using primers described recently (James et al., 2010) with modifications. The optimum LAMP reaction mixture (25 µl) contained 50 µM (each) of inner primers FIP and BIP, 5 µM (each) of outer primers F3 and B3, 20 µM of Loop primers, 0.6 mM each deoxynucleoside triphosphate, 0.4 M betaine, 1 × ThermoPol buffer (20 mM Tris-HCl, 10m M KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 3.2U of Bst DNA polymerase (Lucigen®, USA) and 2 µl of template DNA. The mixture was incubated at 65°C for 1 h and then heated at 80°C for 5 min to terminate amplification. The amplification products were viewed using three detection methods namely; 1.5% agarose gel, naked eye against a white background and under UV light after the addition of SYBR green dye.

#### Comparison of PCR and LAMP assay in ASFV DNA detection

A total of 218 test samples (blood n=188 tissue n=30) were subjected to the two assays. The negative controls constituted blood samples collected from domestic pigs in areas without outbreaks. DNA from *Porcine circovirus type 2* (PCV2) isolate, *E. coli* and *Trypanosoma brucei brucei* were selected for specificity testing.

#### Data analysis

PCR and LAMP data sets were analysed using the DAG-STAT software program for comparing diagnostic tests and determining the level of agreement between tests (Mckinnon, 2000).

## RESULTS

### Determination of optimal reaction time and temperature for LAMP assay

The optimal temperature and time for the LAMP reaction for the detection of ASFV were determined prior to testing the entire sample set. Amplicons were formed at 60, 63, 64, 65 and 66°C, but the clearest products were obtained at 65°C (Figure 1). No LAMP products were detected in the reaction mixture at 65°C within 30 min. LAMP products were detected after 45 min although well-formed bands could be detected after 60 min. Therefore, the optimal reaction temperature was 65°C for 60 min.

### The ASFV LAMP results after addition of SYBR 1 green

Negative and positive samples were selected and LAMP assay done at 65°C for 60 min. The LAMP reaction products were observed with a naked eye (Figure 2) and by use of a UV illuminator as shown in Figure 3.

### The ASFV LAMP assay results for the disease

A total of 188 field blood samples (collected during abattoir and field surveillance) were subjected to PCR assay and the LAMP assay. Representative results are shown in Figure 4. Tissue samples gave similar results.

### Comparison of OIE diagnostic PCR and LAMP assay in ASFV detection

A total of 218 samples were subjected to the two assays

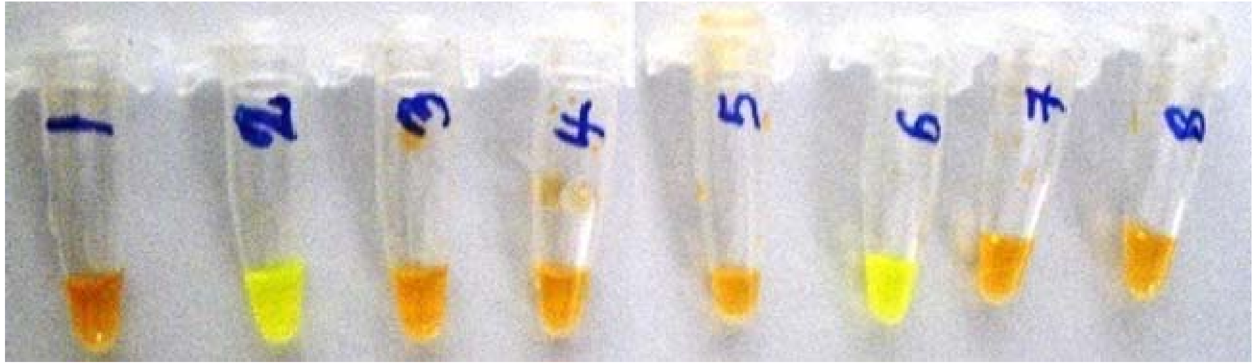


**Figure 2. Visualization of ASFV LAMP products with a naked eye on representative blood samples.** Tube 1 is negative control (nuclease free water), tube 2 is ASFV positive control, tubes 3, 4, 5, 7 and 8 are negative samples while tube 6 is a positive sample.

**Figure 3.** Visualization of ASFV LAMP products with UV illuminator on representative blood samples**.** Tubes 1 and 2 are negative controls, tubes 3, 4, 5 are negative samples, lanes 6-9 are positive samples and tube 10 is ASFV positive control.

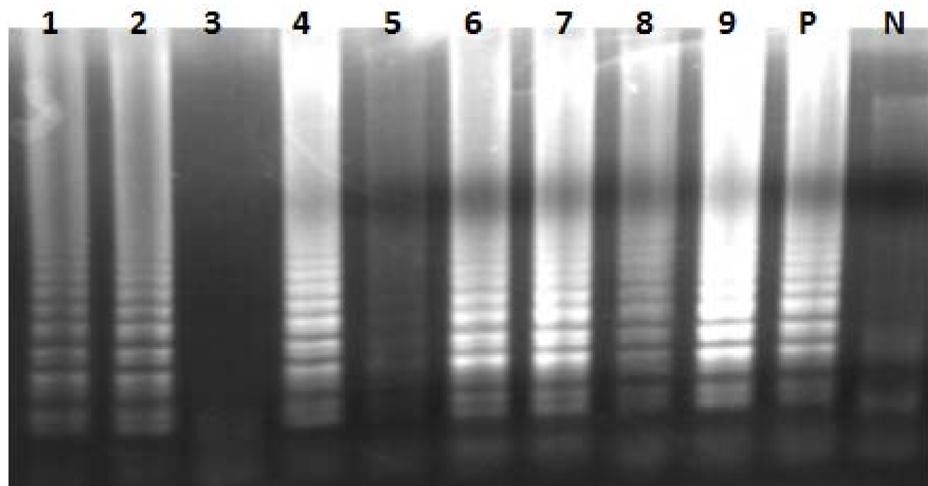


**Figure 4.** Application of ASFV LAMP assay on representative field blood samples. 1.5% agarose gel electrophoresis of LAMP products showing representative results. Lanes 1, 2, 4, 6, 7, 8, 9, are strong positives, lane 5 is a weak positive, lane 3 is a negative sample, lane P is a positive control (field isolate) and lane N is negative control (nuclease free water).

**Table 1.** Number of positive and negative sample types by both OIE PCR and LAMP assay in the detection of ASFV in domestic pigs in Uganda.

| Sample type | PCR | | LAMP | | |
|---|---|---|---|---|---|
| | Positive | Negative | Positive | Negative | Total |
| Tissue | 21 | 9 | 21 | 9 | 30 |
| Blood | 22 | 166 | 120 | 68 | 188 |
| Sub total | 43 | 175 | 141 | 77 | |
| Total | 218 | | 218 | | 218 |

**Table 2**. Comparison of PCR and LAMP in the detection of ASFV in domestic pigs in Uganda.

| LAMP (test) | PCR (OIE recommended) | | |
|---|---|---|---|
| | Positive | Negative | Total |
| Positive | 43 | 98 | 141 |
| Negative | 0 | 77 | 77 |
| Total | 43 | 175 | 218 |

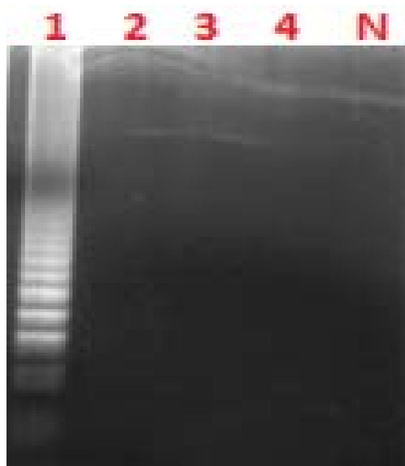


**Figure 5.** Specificity of the ASFV LAMP assay. 1.5% gel electrophoresis. Lane 1, The ASFV DNA (field isolate); lane 2, PCV2 DNA; lane 3, Porcine *E.coli* DNA; lane 4, *Trypanosoma brucei* DNA; Lane N, negative control (Nuclease free water).

out of which 188 were blood samples and 30 were tissue samples (Table 1).

Forty three samples (19.7%) tested positive with PCR while 141 (64.7%) tested positive with the LAMP assay. Forty three (43) samples were positive by both PCR and LAMP while no sample positive by PCR was negative by LAMP (Table 2).

Using LAMP as the test and the OIE diagnostic PCR as the criterion (reference test), the sensitivity of LAMP for the detection of ASFV was 100% (95% CI: 91.78-100) while the specificity was 44% (95% CI: 36.52-51.69). The positive predictive value of LAMP test was 30.5% (95% CI: 36.52-51.69). The negative predictive value of LAMP was 100% (95% CI: 95.32-100). The Kappa statistic for level of agreement between PCR and LAMP test in the detection of ASFV was 23.7% (95% CI: 16.42-30.91). This Kappa value indicated a fair agreement between the two assays.

### Specificity of LAMP assay for ASFV detection

For the DNA of *Porcine circovirus 2* and *E. coli* isolated from pigs in Uganda, *Trypanosoma brucei brucei* DNA and ASFV DNA (field strain) were subjected to the LAMP assay. The result of agarose gel electrophoresis indicated that only ASFV gave a positive reaction; a ladder-like pattern of bands (Figure 5).

## DISCUSSION

The aim of the current study was to evaluate and apply a recently developed ASFV LAMP assay (James et al., 2010) in the detection of ASFV in Uganda as a possible alternative to conventional OIE recommended PCR for future diagnostic purposes. The LAMP assay has been used previously to diagnose infections in humans and animals (Khan et al., 2012; Koizumi et al., 2012; Namangala et al., 2012; Njiru et al., 2011; Zhou et al., 2011). The LAMP assay relies on four specific primers and Bst DNA polymerase which has a helicase function. The target sequences can be amplified with high efficiency, rapidity, and specificity under isothermal conditions (Notomi et al., 2000). Addition of a pair of loop primers accelerates the reaction (Nagamine et al., 2002).

The optimal conditions for ASFV detection by LAMP were determined in this study to be 64-65°C for 45-60 min though amplification was observed as early as 45 min. The best results were obtained at 65°C for 60 min. These findings agree with a previous study by James et al. (2010) that found out an optimal reaction temperature of 64-66°C for 50 min. Since the time required for diagnosis is considered crucial for infections, the fact that results can be obtained within 45 min makes the LAMP assay a good choice for diagnosing ASF.

Furthermore, in this study, the LAMP assay amplify-cation was detected as fluorescence by the naked eye and with a UV illuminator on addition of SYBR 1 green due to the appearance of colour change indicating a positive result eliminating the need for gel electrophoresis and ethidium bromide staining suggesting that this assay can be applied in the field (Njiru et al., 2008).

However, the addition of SYBR 1 green to the reaction mixture was found to be very sensitive to contamination and could give false positive results. Reducing contamination and adding SYBR 1 green from a room different from the template addition and preparation room gave consistent results. A study by James et al. (2010) instead used gel electrophoresis in combination with lateral flow devices for visualisation of a positive LAMP reaction.

The results show that the ASF LAMP assay was highly sensitive for the detection of ASFV compared to the conventional OIE recommended diagnostic PCR. Previous studies have shown a higher sensitivity of the LAMP assay in other diseases than the conventional PCR (Nakao et al., 2010; Zhou et al., 2011) although LAMP and real-time PCR have been shown to have the same sensitivity in the detection of ASFV (James et al., 2010). A previous study established the analytical sensitivity of the ASFV LAMP assay as at least 330 genome copies (James et al., 2010). The sensitivity of LAMP was higher than PCR when the two techniques were applied on field samples obtained from domestic pigs. Twenty one tissues samples positive for ASFV with PCR were also positive with LAMP and nine tissues negative with the two tests. This shows that the two tests were in agreement in the confirmation of ASF outbreaks. However, the two tests gave differing results when compared in ASFV detection using blood samples. The specificity of LAMP was lower than PCR in this study. This finding is not surprising since the reference test (PCR) used for comparison is not the gold standard for detection of ASFV. The kappa statistic indicated a fair agreement between the two tests. A study on evaluation of LAMP and PCR on field samples for detection of *Staphylococcus aureus* in dairy cows suffering from mastitis indicating that LAMP was more sensitive than PCR (Tie et al., 2012).

In this study, no cross-reactivity was observed with PCV2, *E. coli* DNA isolated from pigs in Uganda or *T. brucei brucei* DNA suggesting a high specificity of the LAMP assay. These findings agree with a study (James et al., 2010) that found out that the ASF LAMP assay was specific since there was no cross reactivity with isolates of the classical swine fever virus. In addition, the specificity of LAMP assay was not affected by non-target genomic DNA in the reaction mixture since DNA was not extracted from cell cultures, which is a highly desirable trait in a diagnostic technique (Notomi et al., 2000). Previous studies show that the LAMP assay involves fewer steps than the PCR assay, and does not require expensive equipment to attain a high level of precision (Yamazaki et al., 2008).

In conclusion, this is the first study evaluating the LAMP assay in the detection of ASF in domestic pigs in Uganda. The assay was optimised and applied on field samples. The LAMP assay was found to be more sensitive than PCR in the detection of ASFV DNA on field samples. Therefore, the ASF LAMP could be an alter-native simple, rapid, specific, sensitive, practical, and visualized detection method which is suitable for detection of ASFV. Further studies are required to evaluate the LAMP assay using field samples directly without the need of first extracting DNA. This would further reduce on the diagnosis time for ASF compared to other molecular techniques. The OIE recommended PCR was used as a reference test in this study, therefore there is need to use an established gold standard for detection of ASFV in future evaluation studies of the LAMP assay in the field.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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