International Journal of Biotechnology and Food Science
Vol. 4(2), pp. 22-33, March 2016
ISSN: 2384-7344
Research Paper

Comparative detection of foot-and-mouth disease virus by reverse transcription loop-mediated isothermal amplification assay and real time polymerase chain reaction in Uganda

Hussein Kafeero Mukasa[1*] • Frank Norbert Mwiine[1] • David Kalenzi Atuhaire[2] • Sylvester Ochwo[1] • Ann Nanteza[1]

[1]College of Veterinary Medicine, Animal resources and Biosecurity, Makerere University, P. O. BOX 7062 Kampala, Uganda.
[2]Research and development Manager, Centre for Ticks and Tick-Borne Diseases, Private Bag A130, Lilongwe, Malawi.

*Corresponding Author's email: husseinmukasakafeero@gmail.com.

Accepted 21st February, 2016

Abstract. Foot-and-mouth disease (FMD) is a viral disease. FMD diagnosis in the field is based on clinical signs that are shared by other vesicular diseases, hence to confirm FMD a laboratory is needed. Laboratory diagnostic techniques including serology may fail to distinguish between vaccinated and new infection, virus isolation may take up to 4 days to yield results, while molecular techniques including PCR, which are accurate, sensitive, specific and rapid, are costly and require special training of the laboratory staff. These challenges limit laboratory diagnosis yet in Uganda FMD outbreaks are common since the disease is endemic. This work reports the comparative detection of Foot-and-mouth disease virus (FMDV) by reverse transcription-loop mediated isothermal amplification (RT-LAMP) and Real-Time polymerase chain reaction (rRT-PCR) in Uganda based on the 3D polymerase ($3D^{pol}$) gene. The rRT-PCR assay is considered as the gold standard. A total of 89 cattle samples that included epithelial tissues (16.9%) and oral swabs (83.1%) were collected from outbreak cases in Eastern Districts of Mbale and Budaka. These were applied to molecular assays of rRT-PCR and RT- LAMP using primers and probes targeting the $3D^{pol}$ gene. The diagnostic sensitivity and specificity of RT-LAMP as a screening test and rRT-PCR as the reference test was 94.44% (95% CI = 94.11 to 94.78%) and 98.59% (95% CI = 98.50 to 98.68%), respectively. The kappa value for diagnostic agreement between rRT-PCR and RT-LAMP was 93.0% (95% CI = 83.50 to 100%), showing a perfect agreement. In conclusion, the RT-LAMP assay had a very high sensitivity and specificity when compared to the reference test of rRT-PCR. It was also very rapid since it gave results in 45 to 60 min. Due to its simplicity, sensitivity and specificity, LAMP assay has the potential for use in routine surveillance of FMD in Uganda.

Keywords: Foot-and-mouth disease virus, real-time polymerase chain reaction, reverse transcription LAMP, sensitivity, specificity.

INTRODUCTION

Foot-and-mouth disease (FMD) is a viral disease adversely affecting cattle, pigs, sheep, and goats by reducing productivity and limitation of international trade (Rweyemamu, 1982) that caused serious economic losses (Syed and Graham, 2013). It is a highly contagious, trans-boundary, acute, vesicular disease of cloven-hoofed animals including those in the wild (Alexandersen and Mowat, 2005) that act as reservoirs of the virus for transmission to the domestic animals (Anderson et al., 1975). Foot-and-mouth disease (FMD) causal agent is called foot-and-mouth disease virus (FMDV). It is a small, non-enveloped, single stranded

RNA virus, 8.5 kb long with a positive polarity surrounded with icosahedral capsid symmetry, belonging to the genus *Aphthovirus* of the *Picornaviridae* family (Boothroyd et al., 1981). It has seven serotypes including A, O, C, Asia 1 and the Southern African territories (SAT) 1-3, of which all have occurred in most East African countries (Vosloo et al., 2002) except Asia 1 (Rweyemamu, 1982). Studies have shown that the predominant FMDV serotypes in Uganda are O and SAT-2 (Balinda et al., 2010). Other serotypes reported include SAT-1 and SAT-3 (Vosloo et al., 2002), but serotype C was last recorded in early 1971 (Vosloo et al., 2002).

The disease is characterized by short lasting fever, epithelial lesions on the tongue, dental pad and inner mouth area that lead to excessive salivation and drooling and lesions on the feet that cause lameness (Margo et al., 2013). The initial virus multiplication takes place in the pharynx epithelium and produce vesicles and lesions and then vesicles appear on the feet (Burrows et al., 1981a). Tissues in these areas are preferred specimens for diagnosis (Sutmoller, 1992).

The epidemiology of FMD in Africa is complicated by the widespread movement of animals, the wide host range of the virus involving wild and domestic animal reservoirs and the presence of multiple strains and sub-strains (Alexandersen and Mowat, 2005). Infection of cloven-hoofed animals can result in development of a carrier state in which case the virus can be found in the animals for more 28 days after infection (Alexandersen et al., 2002), and thus may influence the epidemiology of the disease and interfere with the diagnosis. The epidemiology of FMD in the wildlife populations has not been fully documented but it has been established that the African buffalo herds can harbor the infection for 24 years (Condy et al. 1985). In Uganda, molecular characterization of FMDV is not routine and therefore limited data on the epidemiology of FMD yet FMD outbreaks are frequent (Balinda et al., 2009) and difficult to control (Ayebazibwe et al. 2010).

The most common route of transmission of the virus is by direct contact between infected and naïve animals (Margo et al., 2013). Indirect contact also occurs by mechanical transfer via people, wild animals and birds, vehicles fomites and animal products e.g. milk or meat products (Alexandersen et al., 2002). The virus may also spread by inhalation of infectious droplets and droplet nuclei originating mainly from breath of infected animals (Seller and Gloster, 2008) that can be wind borne (Gloster et al., 2005). The FMDV is infective in small doses and produces an acute, systemic vesicular disease, which requires a differential diagnosis from other vesicular diseases (Sutmoller, 1992).

The virus has a short incubation period, and releases viral particles before the onset of clinical signs making FMDV highly contagious (Sanson et al., 2011). It is therefore absolutely necessary to detect FMD outbreaks as early as possible to initiate the appropriate control measures and prevent further spread among livestock (Sanson et al., 2011). Rapid identification of the causative agents is a key element in any control strategy (Anon, 2002). Following an outbreak, veterinary authorities enforce quarantines to restrict livestock and livestock product movement as the first control measure (Balinda et al., 2009). In addition the authorities impose a ban on slaughter in abattoirs for a period of six months. These measures are then followed by vaccination within and around the affected areas (Balinda et al., 2009).

In endemic areas, diagnosis may be based on clinical signs, virus isolation and antibody detection (OIE, 2009). However, clinical symptoms are not sufficient since other vesicular diseases such as swine vesicular virus disease may produce similar signs (Barnett and Cox, 1999). Similarly antibody detection may fail to distinguish between vaccinated and newly infected animals (Berger et al., 1990). Virus isolation may take up to 4 days to demonstrate the presence of the virus especially in low levels of viraemia and FMDV may fail to grow in a specific cell type (OIE, 2012). The cells of choice are bovine thyroid cells or ovine kidney cells (OIE, 2012). Molecular techniques including polymerase chain reaction (PCR) and its various modifications (Rodriguez et al., 1992), are being used. These are accurate, sensitive, specific and rapid (Longjam et al., 2011).

Currently in Uganda, the presence of FMDV in samples is detected by two methods. These include Enzyme Linked Immunosorbent Assay (ELISA) (Mwiine et al., 2010) and nucleic acid detection methods such as RT-PCR (Kasambula et al., 2012). Each of these has its own weaknesses. Enzyme-linked immunosorbent assay (ELISA) cannot reliably detect low density infections and yield positive results only with vesicular material but not with saliva, nasal swabs or serum (Longjam et al., 2011). Nucleic acid detection method using RT-PCR is time consuming and labor intensive and requires a centralized laboratory with sophisticated instrumentation as well as specialized training of the laboratory staff making it unsuitable for routine diagnosis of FMD (Reid et al. 2002).

Previously, a novel field deployable, rapid, simpler, single temperature, nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), has been developed for laboratory diagnosis of many infections (Notomi et al., 2000) including many viral infections like classical swine fever virus (Chen et al., 2009) and foot-and mouth disease (Hao-tai Chen et al., 2011). However, no study has been reported to evaluate the use of LAMP in the diagnosis of foot-and-mouth disease in Uganda. But it has been used for the timely diagnosis of malaria (Hopkins et al. 2013), African Swine fever Virus (Atuhaire et al., 2014) and Human African Trypanasomiasis (Matovu et al., 2010).

The aim of our study was to evaluate the potential of reverse transcription loop mediated isothermal amplification (RT-LAMP) in the detection of foot-and-mouth disease

Table 1. Districts, counties, villages and number of cattle sampled.

District	County	Village	Number of cattle sampled (%)
Mbale	Bungokho	Shibiniho	13 (16.9)
		Wangito	15 (14.6)
Budaka	Kamonkoli	Bunyoro	10 (11.2)
		Nyanza	39 (43.9)
		Sekulu	06 (6.7)
		Wibere	06 (6.7)
Total			89 (100.0)

virus in Uganda using real time PCR as the reference standard (Office International des Epizooties (OIE), 2008) However, the specific objectives of the study were to determine the sensitivity, specificity, optimal time and temperature at which results could be obtained by RT-LAMP assay.

MATERIALS AND METHODS

Study sites

The study was carried out between July 2014 to July 2015 on samples collected from Bungokho county Mbale district and Kamonkoli County in Budaka district during the foot-and-mouth disease outbreak in Uganda in 2014. Sampled villages and cattle (Table 1) were identified with the guidance of the District Veterinary Officers and the sub-county Veterinary Officers. Farmer consent was obtained orally before cattle sampling.

Study design

A cross-sectional study was carried out following reports of foot-and-mouth disease outbreaks in Mbale district, Bungokho County and in Budaka district, Kamonkoli County. Purposive sampling was done based on animals with clinical symptoms like oral lesions, history of infection but having healing lesions and any other asymptomatic cattle in the same farm/kraal or grazing with the symptomatic cattle as reported by the Sub-count Veterinary Officer and or the farmers. The inclusion criteria were cattle with clinical symptoms and the asymptomatic ones in the same farm while exclusion criteria were cattle in farms without any clinical signs or history of clinical signs. All farmers in the villages where sampling was done keep few cattle on average 3 to 4 animals per house hold and on zero grazing basis, transmission of the virus was assumed to be low between kraals/farms.

Sample size determination

The desired confidence interval for sensitivity estimates was 95% (width of 0.05). The sensitivity of FMDV RT-LAMP assay from previous studies (Hao-tai Chen et al., 2011) was in the range of 97% to 100%. Sample size at the required absolute precision level for sensitivity was calculated by applying Buderer's formula (Buderer, 1996). For sample size calculation, an estimate of specificity of 97% and a precision of 5% within the 95% confidence level was considered. In addition, a prevalence of 50% as recommended in outbreak cases was used (Buderer, 1996).

This gave a sample size of 89 cattle from which tissues and/or swabs which were subjected RT-LAMP assay as a screening test and real time RT-PCR as the gold standard (Office International des Epizooties (OIE), 2008) to determine the sensitivity, specificity, positive and negative predictive values of RT-LAMP.

Sample collection

Three separate purposive sampling were made in the Eastern districts of Mbale and Budaka in the counties of Bungokho and Kamonkoli respectively during the 2014/15 FMD outbreak in Uganda. Mbale samples were collected in July 2014 while in Budaka samples were collected in September the same year. Samples were collected from cattle with clinical signs, those which had healing lesions in the mouth, dental pad or on the feet and the asymptomatic animals in same kraals/ from the same farmer. At least one of the two types of samples were collected from each animal recruited in the study; epithelial tissues (ET) if present and oral swabs from the dental pad (OS) in case of asymptomatic cases. Exclusion criterion involved cattle from kraals with no any animal having clinical signs. These were taken as the non-cases.

After the identification of the animal as a case, epithelial tissues and/or swabs were collected in the crayon vials containing virus transport medium PBS/Glycerol, given a field identification number and kept in liquid nitrogen. The date of sample collection, district, county, sub-county, parish, GPS number, type of sample collected as well as the presence of clinical signs were all recorded in the field book. All samples were transported

Table 2. Total number of samples and sample type collected by district, county and village.

District	County	Village	Sample type		No. of samples (%)
			ET	OS	
Mbale	Bungokho	Wangito	2	13	15 (16.9%)
		Shibiniho	7	6	13 (14.6%)
Budaka	Kamonkoli	Bunyoro	3	7	10 (11.2%)
		Nyanza	3	36	39 (43.9%)
		Sekulu	0	6	6 (6.7%)
		Wibere	0	6	6 (6.7%)
Total			15	74	89 (100.0%)

Key: ET = Epithelial tissue, OS = Oral swabs.

to the virology laboratory, College of Veterinary Medicine Animal Resources and Bio security, Makerere University. The tissues and /or swabs were kept at -80°C pending further use.

A total of 89 cattle were used in this study from two sub-counties of Bungokho and Kamonkoli in two districts of Mbale and Budaka respectively in Eastern Uganda (Table 2). These included 28 (31.5%) from Bungokho and 61 (68.5%) from Kamonkoli. Two types of samples were collected which included epithelial tissues (16.9%) and oral swabs from dental pad (83.1%). The epithelial tissues were obtained from clinical cases and oral swabs from the asymptomatic animals sharing the same kraal/farm with the symptomatic ones.

RNA extraction

Total RNA was extracted from 140 µl original epithelial tissue and/or swab suspension using Qiagen® RNA extraction kit following the manufactures instructions.

cDNA synthesis

This was synthesized using the Invitrogen superscript First-Strand cDNA synthesis kit following the manufacturer's instructions. Briefly 2 µl of 10X RNA primer mix, 0.8 µl of 25X dNTPs, 2 µl of 10X RT buffer, 1 µl of RNase inhibitor, 3.2 µl of RNase free water and 1 µl of Superscript III Reverse Transcriptase to a 0.5 ml microcentrifuge tube to a total volume of 10 µl. The mixture was vortexed briefly to mix then placed on ice. Then 10 µl of RNA sample were dispensed to the reaction tube to make up the total reaction volume of 20 µl. The mixture was incubated in a thermal cycler at 42°C for 2 h followed by termination of the reaction at 80°C for 15 min. The mixture was chilled at 4°C for 30 min then transferred to ice and 1 µl of RNase H added followed by incubation at 37°C for 20 min to degrade the RNA template leaving only a single stranded DNA product. The cDNA was stored at -80°C until required for PCR and LAMP.

Real time reverse transcription – Polymerase chain reaction

In this study, the primers and probe previously described by Callahan et al. (2002) that detect the 3D RNA polymerase encoding gene were used. Forward Primer: 5'-ACT GGG TTT TAC AAA CCT GTGA-3' Reverse Primer: 5'-GCG AGT CCT GCC ACG GA-3' 3D Probe: (5'-FAM-TCC TTT GCA CGC CGT GGG AC-TAMRA-3'). This probe labeled with 6- (FAM) at the 5' end and the quencher tetramethyl rhodamine (TAMRA) at the 3'end in Real-time RT-PCR reaction detects the $3D^{pol}$ gene sequence in all the FMDV serotypes.

The real time RT-PCR reaction was based on one-step procedure combined with reverse transcription and Real-time assay. Therefore, real-time assay was carried out by Superscript III/Platinum Taq one-step rRT-PCR kit (Invitrogen). The composition of the 25 µl reaction/ Master Mix for the One-Step rRT-PCR included the following: 12.5 µl 2x- reaction buffer, 2.0 µl (10 pmol/µl) of each of the forward and reverse primer, 1.5 µl (1.5 µl) of the probe, 5.0 µl extracted RNA, 0.5 µl Superscript 111 RT/Platinum Taq mix, 1.5 µl of molecular grade water. The amplification was done at the following temperature cycle: Reverse transcription (one cycle), 48°C for 30 min, the initial denaturing (one cycle), 95°C for 10 min; then, 40 cycles consisting of 95°C for 15 s and 60°C for 1 min and 72°C for 30 s. Negative control (nuclease free water) and positive control (field isolate) were included in each run. PCR amplification was carried out in the thermal cycler Rotor- Gene Q (Qiagen®, Germany). The PCR amplification was carried out in the thermal cycler Rotor-Gene Q Qiagen®, Germany). The successfully amplified target gave an amplification curve and the cycle threshold, Ct at which the target amplicon was initially detected above the background fluorescent levels as determined by the instrument software noted. Each rRT-PCR was performed minimally in duplicate and the mean Ct value with standard deviation reported.

Table 3. Comparison of Real time PCR and RT-LAMP for FMD virus diagnosis on clinical samples /sample type.

Sample	Real time PCR		LAMP	
	Positive	Negative	Positive	Negative
Tissue	15	0	15	0
Swab	3	71	3	71
Sub Total	18	71	18	71
Total	89		89	

The LAMP reaction

The LAMP reaction was carried out using primers targeting the 3Dpol RNA polymerase encoding gene as previously described by Dukes et al. (2006) with minor modifications;

F3:5' CATGGACTATGGAACTGGGT3', B3:5'GGCCCTGGAAAGGCTCA3'
FIP:5'CACGGCGTGCAAAGGAGAGGATTTTACAAACC TGTGATGGCTTCG3'
BIP:
5'GGAGAAGTTGATCTCCGTGGCATTTTAAGAGACGC CGGTACTCG3'
FL: 5'TAGCCTCGAGGGTCTTCG3' BL: 5'GGACTCGCCGTCCACTCT3'

Briefly, the 25 µl of LAMP reaction mixture 1 µl each of FIP and BIP, 1 µl each of F3 and B3, 1 µl of LB and LF primers 1 µl of 8U *Bst* DNA polymerase large fragment (New England Biolabs, Inc.), 1 µl deoxynucleoside triphosphates, 5 µl of 4M betaine, 2.5 µl of BSt Thermopool buffer and 2 µl of cDNA template in 2727 model machine (Applied Biosystem). Negative control (nuclease free water) and positive control (field isolate) were included in each run. The reaction mixture was incubated at 65°C for 1 h and the heated at 80°C for 5 min to terminate the reaction (Notomi et al., 2000). The LAMP reaction was detected by addition of SYBR green, 2 µl of a 1:10 dilution per 25 µl reaction as previously described by Matovu et al. (2010). The mixture was gently mixed by simply tapping the bottom of the PCR tube as it was observed for colour change. A positive reaction turned green immediately while a negative reaction turned orange. For further verification, 2 µl the amplicons were electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized under UV light. Multiple size products gave a ladder-like pattern on the gels indicated a positive sample.

Data analysis

Every sample was tested twice by each of the methods (RT-LAMP and RT-PCR) and in case of a disagreement; the test was repeated for both assays to come up with the final result. Sensitivity and specificity of each test was then determined as percentages with 95% confidence intervals (CIs). The RT-LAMP test results were then compared to the reference test/gold standard (rRT-PCR) using Fisher's exact test. The sensitivities and specificities of each test compared to the gold standard were determined. Kappa values to assess the level of test agreement were also determined. All analyses were done at 95% CI using the Graph pad Software, Quick Calcs.

RESULTS

Comparison of real time PCR and RT- LAMP for FMD virus diagnosis

Out of the 89 epithelial tissues/ oral swab samples, 18 (20%) were positive by both real time PCR and LAMP (Table 3).

The results of the real time RT-PCR were assessed by Ct values and amplification curves (Figure 1). All the epithelial tissues collected (15/15)were positive (100%) by real time RT-PCR (Table 3) with Ct values ranging from 17.92 to 29.54 except one sample MBL/BUK/98 which gave a weak positive result with Ct value of 36. Only three of the 65 oral swabs (4.6%) were positive by real time RT-PCR (Table 3). The negative Ct value for any test and control sample considered Ct of ≥ 40 and was selected as the positive /negative cut-off Ct value. The LAMP assay positive samples included all the epithelial tissues 15/15 (100%) and three swabs (3/65) were positive (4.6%) by the same assay (Table 3). However, only two swab samples were positive by both assays. The results were consistent with both 2% agarose gel electrophoresis after staining with ethidium bromide, then visualized under U.V light (Figure 2), and with SYBR green staining then visualized with the naked eye (Figure 3). Similar results were obtained by using one step reaction using template RNA and reverse transcriptase in the ration 100:1 of BSt pol: RTase for both 2% agarose gel resolution of the amplicons (Figure 4) and visualization with SYBR green (Figure 5).

Comparison of the real time PCR reference standard and RT-LAMP assay in FMD virus detection

One sample; KAM/ BUD/65 was negative by real time

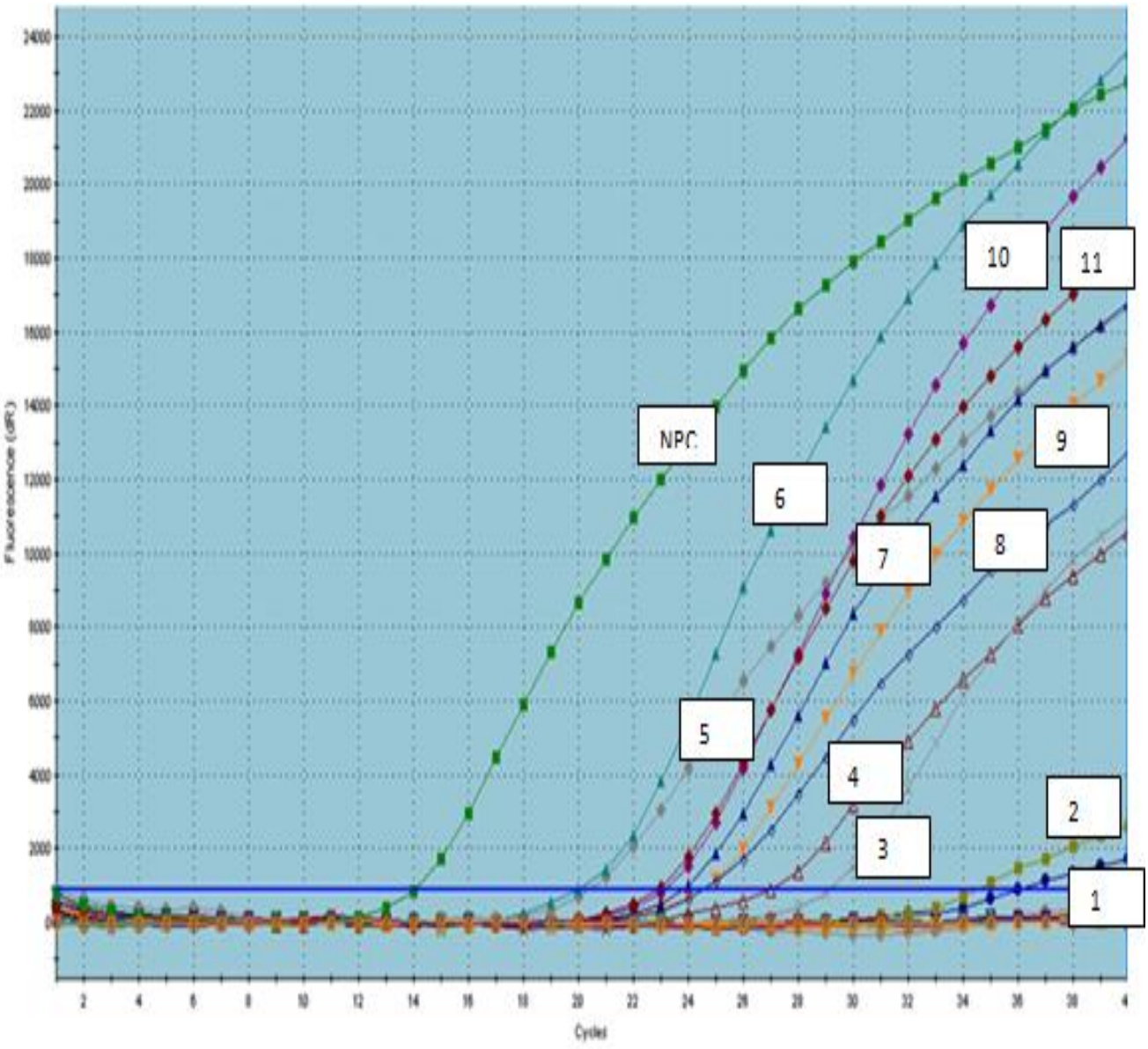

Figure 1. Real time representative amplification curves showing Ct values. NPC is the positive control (field isolate). Samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 are positive sample. Samples 1 and 2 are weak positive samples while the others are strong positive samples.

PCR but was detected as positive by LAMP and one sample; KAM/BUD/06 that was detected as positive by real time PCR but negative by LAMP assay (Table 4).

The RT-LAMP assay detected 17 samples (94.44%) as positive out of the 18 positive samples by the golden standard real time PCR and one extra sample detected as negative by real time PCR as positive giving a total of 18 positive samples by LAMP and a diagnostic sensitivity of 94.44% (95% CI = 94.11 to 94.78%) and a positive predictive value of 94.44% (95% CI = 94.11 to 94.78%). Of the 71 samples detected by real time PCR as negative, RT-LAMP identified 70 of them as negative giving a diagnostic specificity of 98.59% (95% CI = 98.50 to 98.68%) and a negative predictive value of 98.59% (95% CI = 98.50 to 98.68%). The kappa value for agreement between screening test, RT-LAMP and gold standard test, real time PCR is 93.00% (95% CI = 83.50 to 100.00%) showing an excellent agreement between the two assays with number of observed agreement of 87 (97.7% of the observations) and number of agreement expected by chance of 60.3 (67.7% of the observations). These results showed that the association between real time PCR and RT-LAMP was extremely statistically significant ($P < 0.0001$) when analyzed by Fisher's exact test.

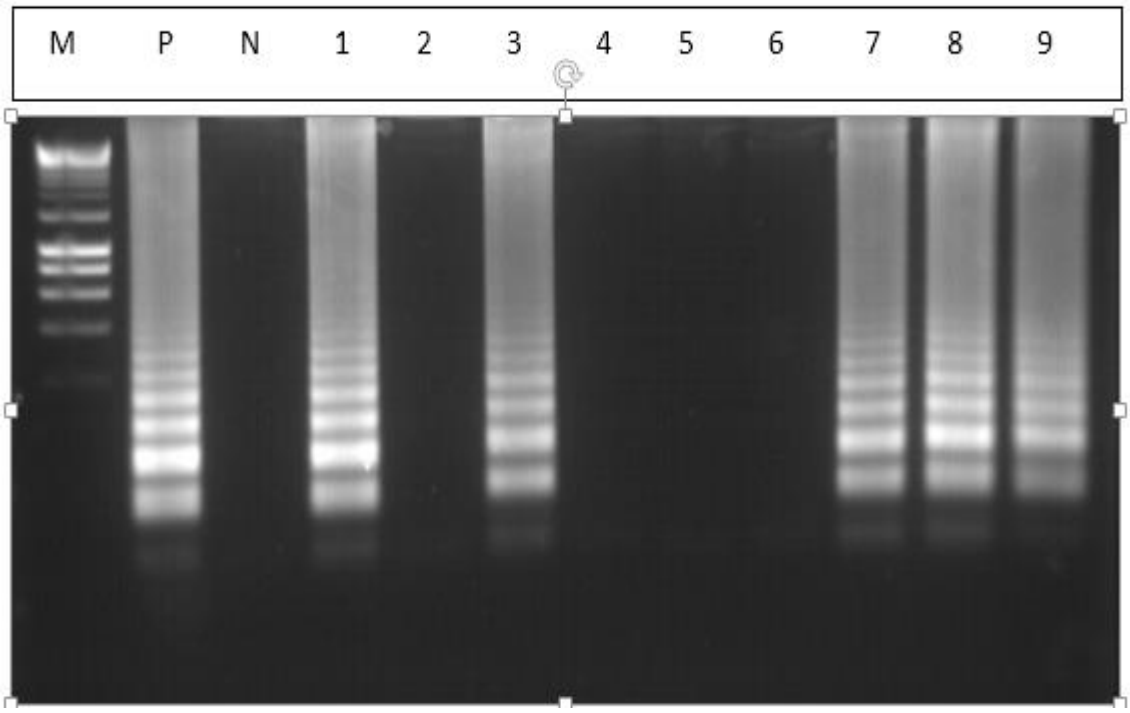

Figure 2. The LAMP representative gel for FMD virus detection. A 2% agarose gel electrophoresis of LAMP products. Lane M is ϕX174 DNA 1000bp marker (Amersham Biosciences, UK), P is positive control (field isolate), N is negative control (nuclease free water), 1, 3, 7, 8, and 9 are positive samples while 2, 4, 5 and 6 are negative samples.

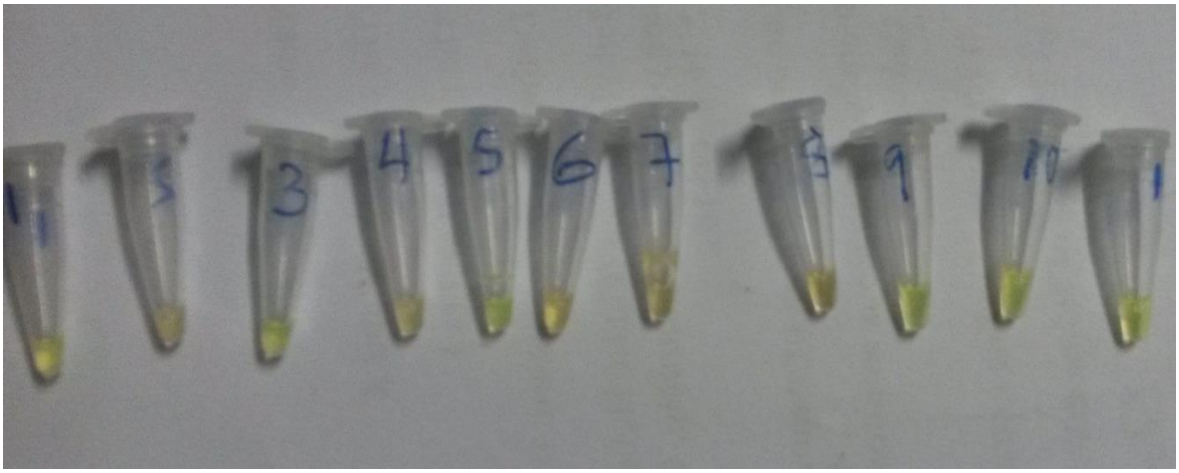

Figure 3. Visualization of FMD virus LAMP products by naked eye using SYBR green. Tube 1 is for positive control (field isolate), 2 is negative control (nuclease free water), 3, 5 9, 10, 11 are positive samples while 4, 6, 7, 8 are negative samples.

Determination of optimal reaction time and temperature for RT- LAMP assay

The optimum temperature and time for efficiency and effectiveness of RT-LAMP assay were determined during optimization prior to screening of all the samples. Results were obtained after 40, 45 and 60 min at a constant temperature of 65°C (Figure 6a) and after 60, 63 and 65°C at a constant time of 1 h (Figure 6b). Amplification was achieved at all the three temperatures (Figure 6b). However clearest signal was attained at 65°C. Therefore the optimal conditions for RT- LAMP assay are 65°C at 1 h/45 min.

DISCUSSION

The aim of this study was to evaluate the previously developed FMD virus LAMP assay (Dukes et al., 2006;

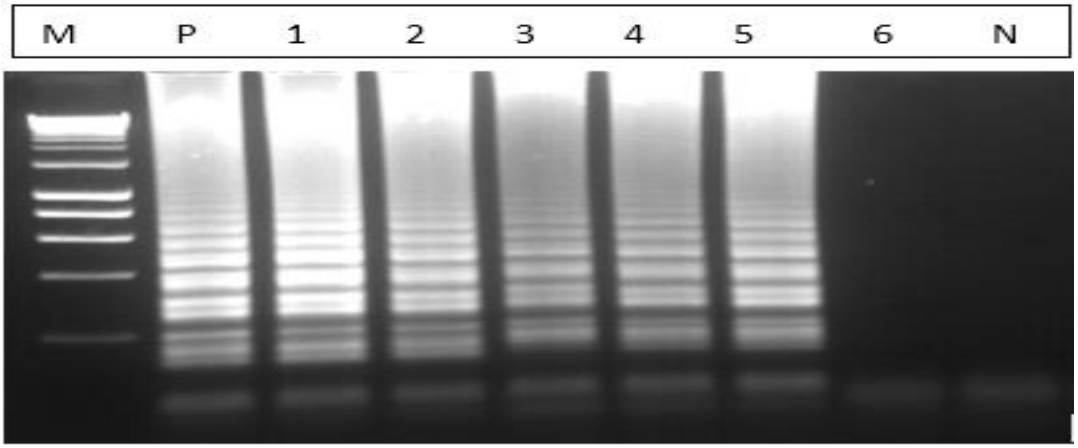

Figure 4. The RT-LAMP representative gel using RNA template. A 2% agarose gel electrophoresis for one tube LAMP reaction products. Lane M is for ϕX174 DNA 1000 bp marker (Amersham Biosciences, UK), P is for positive control (field isolate), lanes 1, 2, 3, 4, 5 are positive samples while 6 and 7 are negative samples.

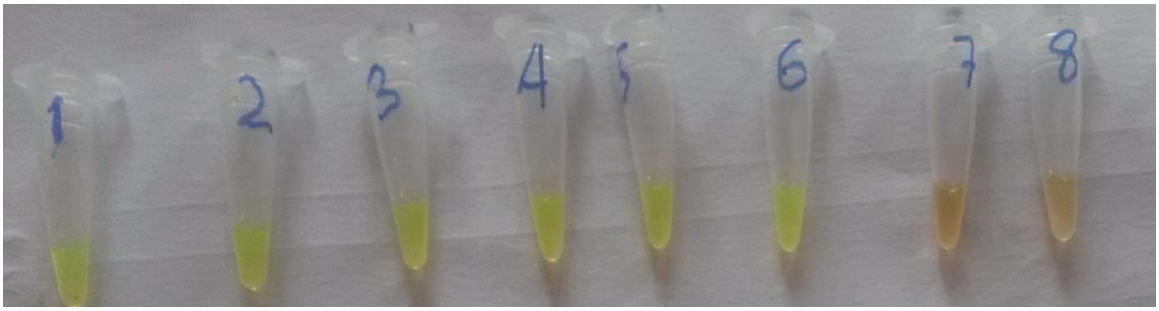

Figure 5. Visualization of FMD virus RT-LAMP products using SYBR Green. Tube 1 is for positive control, tubes 2, 3, 4, 5 and 6 are positive samples while tubes 7 and 8 are negative samples.

Table 4. Comparison of the Real time PCR and LAMP assay in FMD virus detection.

LAMP	Real time PCR (Reference standard)		
	Positive	Negative	Total
Positive	17	1	18
Negative	1	70	71
Total	18	71	89

Hao-tai Chen et al., 2011) in the detection of FMD virus in Uganda as a possible alternative to the OIE recommended conventional PCR using real time PCR as the golden standard. The LAMP assay has been used to diagnose several infections both in humans and animals elsewhere (Zhang, 2012; Ding et al., 2011; Chen et al., 2010; Hao-tai Chen et al., 2011; Curtis et al., 2008). In Uganda LAMP has been used in the detection of African swine fever virus (Atuhaire et al., 2014), Human African Trypanasomiasis (Matovu et al., 2010) and Malaria (Hopkins et al. 2013).

The Loop Mediated Isothermal Amplification (LAMP) assay is based on the principle of strand displacement DNA synthesis (Notomi et al., 2000). The reaction is performed by Bst DNA polymerase with high strand displacement activity and a set of two specially designed inner primers and two outer primers (Chen et al., 2010).

The LAMP is highly specific for the target sequence because of the recognition of the target sequence by six independent sequences in the initial stage and by four independent sequences during the latter stage of the LAMP reaction (Chen et al., 2008). The amplification efficiency of the LAMP method is extremely high because there is no time loss for thermal change because of its

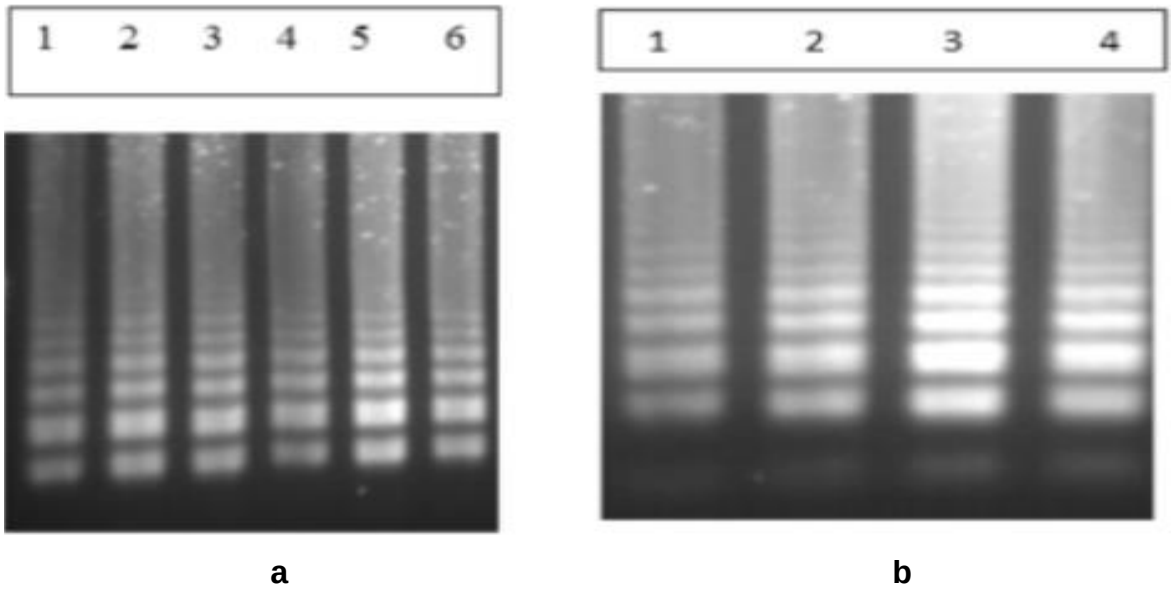

Figure 6. Optimum temperature and time for FMD virus LAMP assay. a: A 2% agarose gel electrophoresis of LAMP products showing representative results for optimum time for the reaction at 65°C. Lanes 1 to 3 are for positive control and lanes 4 to 6 are for a positive sample. Lanes 1 and 4, 2 ad 5, 3 and 6 are for results after 40, 45, 60 min, respectively. b: A 2% agarose gel electrophoresis of LAMP products showing representative results for optimum temperature for the reaction at 60 min. Lanes 1, 2 and 4 are for a positive sample at 60, 63, 65°C respectively while lane 3 is for the positive control (field isolate) at 65°C.

isothermal reaction (Chen et al., 2008). Since the amplification of DNA is directly correlated with the production of magnesium pyrophosphate leading to turbidity, real-time monitoring of LAMP reaction is possible by real-time measurement of turbidity in an inexpensive turbid meter (Mori et al., 2001), or visualization by color changes due to incorporation of SYBR green (Poon et al., 2006) or Calcein and Hydroxynapthol blue (Goto et al., 2009). Further improvements in the time kinetics and sensitivity of the LAMP reaction by the use of two additional loop primers termed accelerated LAMP have been reported (Nagamine et al., 2002). Therefore the LAMP assay has the advantage of high sensitivity, selectivity and rapidity over other nucleic acid amplification (Nagamine et al., 2002). The LAMP assay is also useful for RNA template detection upon the use of reverse transcriptase (RTase) together with DNA polymerase (Chen et al., 2008) termed RT-LAMP and has been used for the detection of RNA viruses (Parida et al., 2004; Hao-tai Chen et al., 2011; Curtis et al., 2008).

In the present study, the proportion of FMD from outbreak was determined by LAMP and then the results were compared with real time PCR. It is noted that 18 (20%) of the 89 samples examined were positive by both techniques. However, each technique missed one sample which was identified as positive by the other. Therefore concordance was with 17 (19.1%) samples for both assays.

The preferred sample for virus detection is epithelial tissue from un ruptured or freshly ruptured vesicle or vesicular fluid (OIE, 2012) because virus multiplication starts from the pharynx epithelium and spreads to the oral mucosa producing vesicles or aphthae (Burrows et al., 1981). So, all samples from epithelial tissues (ET) and the oral swabs were positive by Real-Time RT-PCR (15/15). The virus can also be obtained from the secretions like saliva/oral swabs (Alexandersen et al., 2002). Of the 64 oral swabs from the mouth, only 3 (3/64) were positive using real time RT-PCR. The cattle from which oral swabs were obtained had no clinical symptoms, so the virus could have been missed in the oral swab but still present in the oro-pharyngeal fluid (so the animal may still be positive) giving a potential limitation to this study (Table 3). Alternatively, the viraemia could be too low since the viral multiplication is slow for the first 2½ days after the incubation period yet in the other tissues, there is rapid multiplication of the virus (Alexandersen et al., 2003). Transmission depends on population density (Nishiura and Omori, 2010). However, in villages from which the samples were collected the farmers keep 2 to 3 animals mainly on zero grazing (Table 1). This low population density could explain the slow transmission of the virus. Therefore, very few non symptomatic animals were positive by the gold standard real time RT-PCR (Table 3).

The results show that the FMD virus LAMP assay was highly sensitive for the detection of FMDV when compared with real time PCR as the gold standard. Previous studies have shown a high sensitivity of LAMP assay in FMDV and other diseases 100% (Hao-tai Chen et al., 2011), 95% (Atuhaire et al., 2014), 97% (Hopkins

et al. 2013). In this study, LAMP gave a sensitivity of 94.44%, specificity of 98.59%, and positive predictive value of 94.44% as well as a negative predictive value of 98.59%. The association between real time PCR and LAMP where statistically significant ($P < 0.0001$) when analyzed by Fisher's exact test. The correlation between real time PCR and LAMP gave a kappa value of 93.0% showing a very good agreement.

The LAMP picked up 1 more positive sample (BUD/KAM/065) that was negative by real time PCR indicating a high sensitivity of LAMP which gave a diagnostic sensitivity of 94.44% (Table 3) comparable to diagnostic sensitivities of LAMP in similar studies by Hao-tai Chen et al. (2011) which gave a sensitivity of 100%. However, one sample (KAM/BUD/06) was identified as positive by real time PCR but as negative by LAMP (Table 3). This finding is not surprising because it has been explained in earlier findings by Dukes et al. (2006) due to error prone 3D polymerase RNA dependent RNA polymerase targeted in his study and in the current study preventing probe binding.

The optimum temperature and time for the FMD virus RT- LAMP assay determined in this study were respectively 65°C and 60 min (Figure 6). However, results were obtained even after 45 min, though the clearest signal was obtained after 1 h. These results were consistent with the findings by Dukes et al. (2006) that found an optimal reaction temperature of 65°C. The study by Hao-tai Chen et al. (2011) slightly differed in temperature by giving optimal results at 63°C but was consistent with the optimal time of the current study. Since timely diagnosis is key in preventing spread of any highly infectious disease such as FMD, the fact that RT- LAMP gave results in 45 min makes it a good choice for the timely diagnosis of FMD virus.

Furthermore, in this study, the results of 2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV light were consistent with those obtained when the RT-LAMP product was stained by a double stranded DNA intercalating dye SYBR Green due to appearance of a colour change to green for positive sample and orange colour for negative samples. This suggests that RT-LAMP assay can be used in the field (Dukes et al., 2006).

In conclusion, our study reports the first study in Uganda evaluating the use loop-mediated isothermal amplification assay in the detection of foot-and-mouth disease virus in Uganda. The assay was first optimized in terms of amplification conditions and reagent concentrations then applied on outbreak samples. The RT-LAMP assay has demonstrated a very high sensitivity comparable to that of real time PCR which is recommended as the gold standard in countries whose biosafety levels do not permit them to virus isolate including Uganda. The RT-LAMP assay allows the product to be visualized with naked eyes by using SYBR green which is a double stranded DNA intercalating dye by changing to green in case of appositive sample and to orange in case of a negative sample. Therefore, the FMD virus RT-LAMP assay gives hope as one of the good alternative simple, rapid, sensitive with colorimetric detection of the product without the need of a thermo cycler.

In Uganda where the disease is endemic and with common outbreaks strategies of focal screening and vaccination remain key in the management of foot-and-mouth disease whose success is contingent upon timely and accurate diagnosis. All these strategies have been hampered by lack of highly sensitive, accurate and timely diagnosis at the site of sample collection. The study has demonstrated that LAMP detects viraemia with accuracy similar to that of real time PCR removing the need of using a real time machine in remote settings. The relative simplicity of the LAMP procedure and the low infrastructural costs opens up the opportunities of bringing molecular-level viraemia detection to local village settings.

We suggest further studies to evaluate FMD virus LAMP assay using field samples directly omitting the laborious RNA extraction steps and, or cDNA synthesis but instead using lysis buffer and relevant enzymes as well as a heating block. We also recommend the use of oral pharyngeal fluid (probang) for asymptomatic animals rather than the oral swabs as the swab may fail to capture the virus.

ACKNOWLEDGEMENTS

This work was in part supported by the United States Department of Agriculture (USDA) especially field work during sample collection through a grant to associate professor Frank Norbert Mwiine. The authors are greatly indebted to their contributions.

The authors also thank the Molecular Biology Laboratory staff who helped during sample collection and data analysis.

REFERENCES

Alexandersen S, Brotherhood I, Donaldson AI (2002). Natural Aerosol Transmission of Foot and Mouth Disease Virus to Pigs: Minimal Infection Dose for Strain O1 Lausanne. J. Epidemiol. Infect. 128:301-12.

Alexandersen S, Mowat N (2005). Foot-and-Mouth Disease: Host Range and Pathogenesis. Curr. Top. Microbiol. Immunol. 288:9-42.

Alexandersen S, Quan M, Murphy C, Knight J, Zhang Z (2003). Studies of Quantitative Parameters of Virus Excretion and Transmission in Pigs and Cattle Experimentary Infected with Foot-and-Mouth Virus. J. Comp. Pathol. 129:268-82.

Alexandersen S, Zhang Z, Donaldson AI (2002). Aspects of Persistance of of Foot-and-Mouth Disease Virus in Animals -the Carrier Problem. Microbes Infect 4(10):1099-1110.

Anderson EC, Anderson J, Doughty WJ, Drevmo S (1975). The Pathogenicity of Bonine Strains of Foot-and-Mouth Disease Virus for Impala Nd Wildbeast. J. Wild Dis. 11 (2):248-55.

Anon (2002). Infectious Diseases in Livestock; Apolicy Document 15/20. In *The* Royal Society, London, pp. 685-715.

Atuhaire DK, Afayo M, Ochwo S, Katiti D, Mwiine FN, Nanteza A, Mugasa CM, Olaho-Mukani W, Ojok L (2014). Comparative Detection of African Swine Fever Virus by Loop-Mediated Isothermal Amplification Assay and Polymerase Chain Reaction in Domestic

Pigs in Uganda. Afr. J. Microbiol. 8(23):2322-28.
Ayebazibwe C, Kirsten T, Mwine FN, Muwanika VB, Ademun OH, Siegismund AR, Belsham GJ, Preben N, Alexandersen S (2010). The Role of the African Buffalos (Suncerus Caffer) in the Mantainance of Foot-and-Mouth Disease in Uganda. Vet. Res. 6: 54.
Balinda SN, Belsham GJ, Masembe C, Sangula AK, Siegismund H, Muwanika VB (2009). Molecular Characterization of SAT 2 Foot-and-Mouth Diseases Virus from Post-Outbreak Slaughtered Animals: Implications for Disease Control in Uganda. J. Epidemiol. Infect. 138:1201-10.
Balinda SN, Sangula AK, Heller R, Muwanika VB, Belsham GJ, Masembe C, Seigismond HR (2010). Diversity and Transboundary Mobility of Serotype O Foot-and-Mouth Disease Virus in East Africa: Implications for Vaccination Policies. Infect. Genet. Evol. 10:1058-65.
Barnett PV, Cox SJ (1999). The Role of Small Ruminants in the Epidemiology of Foot-and-Mouth Disease. Vet. J. 158:6-13.
Berger HG, Straub OC, Ahl R, Tesar M, Marquardt O (1990). Identification of Foot-and- Mouth Disease Virus Replication in Vaccinated Cattle by Antibodies to Non-Structural Virus Proteins. Vaccine 8:213-16.
Boothroyd JC, Highfield PE, Cross GA, Rowlands DJ, Lowe PA (1981). Molecular Cloning of Foot and Mouth Disease Virus Genome and Nucleotide Sequences in the Structural Protein Genes. Nature 290:800-802.
Buderer NM (1996). Incorporating the Prevalence of Disease into the Sample Size Calculation for Sensitivity and Specificity. Acad. Emerg. Med. 3:895-900.
Burrows R, Mann JA, Garland AJ, Greig A, Goodridge D (1981). The Pathogenesis of Natural and Simulated Natural Foot-and-Mouth Disease Infection in Cattle. J. Comp. Pathol. 91(4):599-609.
Chen HT, Jie Z, Yong-sheng L, Xiang-Tao L (2011). Detection of Foot-and-Mouth Disease Virus RNA by Reverse Transcription Loop-Mediated Isothermal Amplification. Virol. J. 8(1). BioMed Central Ltd: 510. doi:10.1186/1743-422X-8-510.
Chen HT, Zhang J, Ma LN, Ma YP, Ding YZ, Liu XT, Cai XP, Ma LQ, Zhang YG, Liu YS (2010). Rapid Pre-Clinical Detection of Classical Swine Fever by Reverse Transcription Loop-Mediated Isothermal Amplification. Mol. Cell Probes. 24:104-6.
Chen HT, Zhang J, Ma LN, Ma YP, Ding YZ, Liu XT, Chen L, Ma LQ, Zhang YG, Liu YS (2009) Rapid Pre-Clinical Detection of Classical Swine Fever by Reverse Transcription Loop-Mediated Isothermal Amplification. Mol. Cell Probes 23(2):71-74.
Chen HT, Zhang J, Sun DH, Ma LN, Liu XT, Quan K, Liu YS (2008). Reverse Transcription Loop-Mediated Isothermal Amplification for the Detection of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus. J Virol. Methods 153(2):266-68.
Condy JB, Hedger RS, Hamblin C, Barnett IT 1(985). The Duration of the Foot and Mouth Disease Virus Carrier State in African Buffalo (i) in the Individual Animal and (ii) in a Free-Living Herd. Comp. Immunol. Microbiol. Infect. Dis. 8(3-4):259-65.
Curtis KA, Rudolph DL, Owen SM (2008). Rapid Detection of HIV-1 By Reverse- Transcription, Loop-Mediated Isothermal Amplification. J. Virol. Methods 151:264-70.
Ding YZ, Yong-sheng L, Jian-hua Z, Hao-tai C, Jie Z, Li-na M, Gang W (2011). A Highly Sensitive Detection for Foot-and-Mouth Disease Virus by Gold Nanopariticle Improved Immuno-PCR. Virol. J. 8:148. doi:10.1186/1743-422X-8-148.
Dukes JP, King DP, Alexandersen S (2006). Novel Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of Foot-and-Mouth Disease Virus. Archiv. Virol. 151(6):1093-1106.
Gloster J, Freshwater A, Seller RF, Alexandersen S (2005). Re-Assessing the Likelihood of Airborne Spread of Foot-and-Mouth Disease at the Start of the 1967-1968 UK Foot-and-Mouth Disease Epidemic. Epidemiol. Infect. 133:767-83.
Goto M, Honda E, Ogura A, Nomoto A, Hanaki KI (2009). Colorimetric detection of loopmediated isothermal amplification reaction by using hydroxy naphthol blue. BioTechniques 46:167-172.
Hopkins H, Iveth JG, Spencer DP, Patrick A, John A, Caroline A, Bosco A (2013). Highly Sensitive Detection of Malaria Parasitemia in a Malaria-Endemic Setting: Performance of a New Loop-MediatedIsothermal Amplification Kit in a Remote Clinic in Uganda. The J. Infect. Dis. 208 (4):645-52. doi:10.1093/infdis/jit184.
Kasambula L, Belsham GJ, Siegismund VB, Muwanika HR, Ademun-Okurut AMC (2012). Serotype Identification and VP1 Coding Sequence Analysis of Foot-and-Mouth Disease Viruses from Outbreaks in Eastern and Northern Uganda in 2008/9. Transbound. Emerg. Dis. 29(4):323-30.
Longjam N, Deb R, Sarmah AK, Tayo T, Awachat VB (2011). A Brief Review on Diagnosis of Foot-and-Mouth Disease of Livestock: Conventional to Molecular Tools. Vet. Med. Int. 2011:905768.
Margo E, Chase-Topping HI, Nicholas DB, Bankowski JM, Gibson D, Sarah JC, Miriam AW, Elizabeth R, Doel C (2013). Understanding Foot-and-Mouth Disease Virus Transmission Biology: Identification of the Indicators of Infectiousness. Vet. Res. 44:48.
Matovu E, Kuepfer I, Boobo A, Kibona S, Burri C (2010). Comperative Detection of Trypanasomal DNA by Loop-Mediated Isothermal Amplification and PCR from Flinders Technology Associates Cards Spotted with Patient Blood. J. Clin. Microbiol. 48(6):2087-90.
Matovu E, Irene K, Alex B, Stafford K, Christian B (2010). Comparative Detection of Trypanosomal DNA by Loop-Mediated Isothermal Amplification and PCR from Flinders Technology Associates Cards Spotted with Patient Blood 48 (6):2087-2090. doi:10.1128/JCM.00101-10.
Mori Y, Nagamine K, Tomita N, Notomi T (2001). Detection of Loop-Mediated Isothermal Amplification Reaction by Turbidity Derived from Magnesium Pyrophosphate Formation. *Biochem.* Biophys. Res. Commun; 289:150-154.
Mwiine FN, Ayebazibwe C, Olaho-Mukani S, Alexandersen W, Balinda SN, Masembe C, Ademun Okurut AR, Christensen LS, Sorensen KJ, Tjornehoj K (2010). Serotype Specificity of Antibodies against Foot-and-Mouth Disease Virus in Cattle in Selected Districts in Uganda. Transbound. Emerg. Dis. 57:365-374.
Nagamine K, Hase T, Notomi T (2002). Accelerated Reaction by Loop Mediated Isothermal Amplification Using Loop Primers. Mol.cell Probes 16:223-229.
Nishiura H, Omori R (2010) An Epidemiological Analysis of the Foot-and-Mouth Disease Epidemic in Miyazaki, Japan,. Transbound. Emerg. Dis. 57(6):396-403.
Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000). Loop-Mediated Isothermal Amplification of DNA. Nucleic Acids Res. 28:63.
Office International des Epizooties (OIE) (2008). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.
Office International des Epizooties (OIE) (2009). Foot-and-Mouth Disease. In Mannual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Edn, Paris.
Office International des Epizooties (OIE) (2012). Foot-and-Mouth Disease. In Mannual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees) 7th Edn, Paris France 1.
Parida M, Posadas G, Inone S, Morita K, Hasebe F (2004). Real-Time RT-LAMP for Detection of West Nile Virus. J. Clin. Microbiol. 42:257-263.
Poon LL, BW Wong, EH Ma, KH Chan, LM Chow, W Abeyewickren, N Tangpukdee (2006). Sensitive and Inespensive Molecular Test for Falciparum DNA Directly from Heat Treated Blood by Loop-Mediated Isothermal Amplification,. Clin. Chem. 52:303-6.
Reid SM, Ferris NP, Hutchings GH, Zhang Z, Belsham GJ, Alexandersen S (2002). Detection of All Seven Serotypes of Foot-and-Mouth Disease Virus by Real-Time Fluorogenic Reverse Transcription Polymerase Chain Reaction Assay. J. Virol. Methods 105:67-80.
Rodriguez A, Martinez-Salas E, Dopazo J, Davila M, Saiz JC, Sobrino F (1992). Primer Design for Specific Diagnosis by PCR of Highly Variable RNA Viruses : Typing of Foot- and-Mouth Disease Virus. Virology 189:363-367.
Rweyemamu MM (1982). Developments in the Biochemical and Immunoassays Assessment of Foot and Mouth Disease Antigen, in Proceedings of the International Conference on the Impact of Viral Disease on the Development of Latin America Countries and Caribbean Region. Riode Janeiro Brazil 2:78-79.
Sanson RL, Gloster J, Burgin L (2011). Reanalysis of the Start of the

UK 1967 to 1968 Foot-and-Mouth Disease Epidemic to Calculate Airborne Transmission Probabilities. Vet. Rec. 169:336.

Seller R, Gloster J (2008). Foot-and-Mouth Disease: A Review of Intranasal Infection of Cattle, Sheep and Pigs. Vet. J. 177:159-168.

Sutmoller P (1992). Vesicular Diseases in Foreign Animal Diseases, Foreign Animal Diseases of the United States,.

Syed MJ, Belsham G (2013). Foot-and-Mouth Disease: Past, Present Anf Future. Vet. Res. 44:116.

Vosloo W, Bastos AD, Sangare O, Hargreaves SK, Thomson GR (2002) Review of Status and Control of Foot-and-Mouth Disease in Sub-Saharan Africa. Rev. Sci. Tech. 21:437-449.

Zhang H (2012). Evaluation of Reverse Transcription Loop-Mediated Isothermal Amplification Assays for Rapid Detection of Human Enterovirus 71 and Coxsackievirus A16 in Clinical Samples. Adv. Infect. Dis. 02(04):110-18. doi:10.4236/aid.2012.24019.