ORIGINAL RESEARCH ARTICLE

Development and comparison of a loop mediated isothermal amplification assay for the rapid diagnosis of lumpy skin disease

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Abstract

Lumpy skin disease virus is a poxvirus in the genus *Capripoxvirus* and is closely related to *sheeppox virus* and *goatpox virus*. It's economically important in cattle and a notifiable disease by World Organization for Animal Health. Lumpy skin disease (LSD) is endemic in most parts of Africa with small-scale farmers experiencing the highest loss during outbreaks due to restricted animal trade and costly control and eradication measures. Serological methods of LSD detection are sensitive, inexpensive but can be laborious and time-consuming while, molecular methods such as Polymerase chain reaction (PCR), and real-time PCR/quantitative PCR (qPCR) are sensitive but require expertise and sophisticated laboratories. Loop-mediated isothermal amplification (LAMP) molecular method is advantageous, as it does not require expertise or sophisticated equipment. This study aimed to develop a rapid, simple, specific, and sensitive detection method for LSD. Sixty-two samples that included skin biopsies, whole blood, serum, and cell cultures were used. New LAMP primer (10_LSD) that could detect lumpy skin disease virus, was designed using Genome based LAMP primer designer (GLAPD) software. Samples were analyzed by LAMP assay and a gold standard (real-time PCR). A LAMP field-based extraction method using polyethylene glycol (PEG) was developed and used for the detection of *lumpy skin disease virus*. The 10_LSD had a kappa value of 0.32 against the qPCR gold standard. In terms of limit of detection, qPCR had a detection limit of 10-3 ng/µl while 10_LSD had a limit of detection of 1 ng/µl and. The 10_LSD assay showed sensitivity of 60% and a specificity of 86 %. The LAMP assay did not cross-react with closely related viruses like camelpox, Orf virus, and Pestes des Petit Ruminants but could amplify sheeppox virus and goatpox virus. The average time to positivity was 14-28 minutes. The study supports the adoption of the LAMP assay for rapid *Capripoxvirus* diagnosis as a simpler, effective, and rapid method of detection, monitoring, and controlling outbreaks and the spread of disease in a field set up.

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1.0 Introduction

Lumpy skin disease (LSD) is caused by the *lumpy skin disease virus* (LSDV) a poxvirus in the genus *Capripoxvirus* that is closely related to *sheeppox virus* and *goatpox virus* (Diallo and Viljoen, 2007). The virions are brick-shaped, about 300 × 270 × 200 nm, a dsDNA genome of approximately 150–154 kbp, encoding for 147–156 genes with a 26% G + C content (Biswas *et al.,* 2020). LSD is an emerging disease of cattle that causes substantial economic loss to affected regions and is classified as a notifiable disease by World Organization for Animal Health (OIE) (Abutarbush *et al.,* 2015). The United States government has categorized *Capripoxvirus* as a potential agroterrorism agent (Tuppurainen and Oura, 2012) with losses of up to 65% experienced in intensive and semi-intensive farming units due to production losses, costly control and eradication measures, and restricted trade of animals due to infection. Small-scale farmers in developing countries experience the highest loss during an outbreak (Sherrylin *et al*., 2013).

Initial clinical signs of LSD vary in severity depending on the management practice of the livestock and not related to animal sex or age. Clinical symptoms have two febrile phases (biphasic fever), which appear 4-12 days after variant incubation period (usually 7 days). The infected animals' temperature rises to 40-41.5°C, which may persist for 6 -72hrs but not more than 10 days. The acute phase which is characterized by large skin nodules which suddenly erupt within 1-2 days and may be widespread or restricted to just a few lesions also enlarged lymph nodes, increased nasal discharge, anaroxia, dysgalactia, lachrymation and a disinclination to move.

LSD was first reported in Zambia in 1929 and then spread to other parts of Africa. LSD is endemic and mostly confined in Africa, parts of the Middle East, and Turkey. Since 2012, the disease has spread to some Balkan countries, the Caucasus, and the Russian Federation, (EFSA AHAW Panel, 2022). where the disease continues to spread, making the risk of new outbreaks in previously free regions high. Since 2019, several outbreaks of LSD have been reported in some Asian countries like Bangladesh, India, China, Nepal, Bhutan, Vietnam, Myanmar, Sri Lanka, Thailand, Malaysia, Laos (Khan *et al.,* 2021).

The two main routes of transmission are mechanical transmission by arthropod vectors such as mosquitoes, ticks, and biting flies and the movement of infected animals. It is highly transmissible during the wet season (EFSA AHAW Panel, 2022). The disease can also be spread by fomites through contaminated equipment and in some cases directly from animal to animal. The disease is transboundary which sometimes becomes hard to control due to the nature of transmission. It is usually transmitted to infection-free areas by the transportation of infected animals and or infested with vectors.

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Prevention and control of lumpy skin disease currently rely heavily on vaccination. Several live attenuated vaccines are currently being used that are both affordable and provide over 80% protection in sufficient herd immunity (Tuppurainen *et al.,* 2017). Lack of compulsory and consistent vaccination strategies together with uncontrolled animal movement leads to the rapid spread of *LSDV*.

The availability of proper diagnostic tools enables the early detection and control of infectious diseases. Several methods have been adopted for the diagnosis of LSDV: virus isolation; serological methods; molecular methods; Polymerase chain reaction (PCR), real-time PCR/qPCR, and Loop-mediated isothermal amplification (LAMP) (Haegeman *et al.*, 2019). The type of test employed in diagnosis depends heavily on the infrastructure and resources available. Current serological detection techniques such as ELISA are affordable and do not require expertise but are time-consuming, affected by low antibody expression levels and low specificity as they react with Orf virus and Parapoxvirus. Nucleic acid techniques (PCR and real-time PCR assays) are expensive and require well-equipped labs. Rapid, cheap, and safe tests will increase the immediacy of results from diagnostic testing allowing effective disease control measures against infections to be implemented in time.

Loop-mediated isothermal amplification (LAMP) was developed in 2000 by Notomi. The LAMP assay is a powerful tool that enables rapid, on-field diagnosis thus early detection of diseases during outbreaks. LAMP operates most efficiently at temperatures between 60°C and 65°C, utilizing four main primers to target six unique sequences and a DNA polymerase with strong strand displacement activity such as *Bst* polymerase. Evidence has shown that LAMP on-site testing results are comparable to PCR and qPCR laboratory tests. While LAMP has been developed for Lab analysis of Capripoxvirus (Murray et al., 2013, Venkatesan et al., 2016, Zhao et al., 2014) none exists for on-site detection. The objective of this study was at develop an on-site LAMP assay that can be used for the detection and field-based diagnosis of LSDV.

2.0 Material and Methods

2.1 Study site and design

The study was carried out at the Kenya Agricultural and Livestock Research organization-Kabete (KALRO) - Biotechnology Research Institute in the animal biotechnology lab which is a biosafety level 2 lab. This was a cross-sectional laboratory study design covering two years 2018 and 2020. Due to lack of current information the sample size was determined by the number of samples submitted for testing at the Central Veterinary Laboratories (CVL) – Kabete.

2.2 Sampling method and sample size

The sampling method used was a non-probability sampling technique where convenience sampling was employed as described by Etikan, 2016. Samples that were suspected to be Capripoxvirus positive were selected and analyzed for this study. A sample size of 62 was targeted.

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They included five pure cultures of Capripoxviruses; three lumpy skin disease viruses sourced from cattle, one sheeppox virus sourced from sheep and one goatpox virus sourced from goat. 30 blood samples and 19 skin nodules/skin scrapings from cattle. The samples were sourced from Central Veterinary Laboratories (CVL) – Kabete and Kenya Agricultural and Livestock Research organization-Kabete (KALRO) - Biotechnology Research Institute.

2. 3 DNA extraction

A phenol-chloroform method with extraction buffer modification was used to extract DNA from the samples. The concentration and purity of the DNA were determined using the NanoDrop® ND-2000 (Thermo Fischer Scientific) and the integrity was determined using 2% agarose gel. The extracted DNA was standardized to 30 ng/ul for amplification assays. A quick DNA extraction method was also used with the developed LAMP assay. The extraction method is as described by Chomczynski & Rymaszewski, 2006. Briefly, 60g of PEG200 (Sigma- Aldrich) and 0.93 mL 2M KOH were mixed in 39 mL water and the pH was adjusted to 13.5 to ensure that the isolated DNA remains in solution. A small size, (0.2-0.5g/pea size), of skin nodule was cut and placed in a 4"x6"x6" 100 microns plastic extraction bag as the crushing bag, and 1ml of PEG200 was added. The tissue was then crushed to homogenize into the PEG extraction buffer. One µL of the lysate was then diluted in a ration of 1:10 and used for the LAMP assay. The extracted DNA was not suitable for PCR and qPCR assays.

2.4 Gold Standard

The qPCR assay was used as the gold standard. The assay had previously been evaluated on its simplicity, sensitivity and specificity (Stubbs *et al.,* 2012). Results from this study showed that the assay produced less background fluorescence and lower threshold cycle (Ct) values and was more sensitive than the conventional gel-based PCR assay that is described in the OIE Terrestrial Manual (OIE, 2010).

2.5 Quantitative PCR assays

A qPCR TaqMan assay that amplifies an 89 bp region targeting the viral attachment protein as reported by Bowden *et al.*, (2008), under GenBank accession no. AF325528 was used in this study. The sequences of primers and probes were as follows; Forward primer 5'-AAAACGGTATATGGAATAGAGTTGGAA-3', Reverse primer 5'-AAATGAAACCAATGGATGGGATA-3', TaqMan probe 5'-6FAM-TGGCTCATAGATTTCCT-MGB/NFQ- 3'.

A 25 µL reaction volume was used and contained 2.5 µL of template DNA, 900 nM final concentration of each primer, and 10µM of TaqMan probe A PCR Master Mix Reagents kit (Applied Biosystems) was used. The real-time PCR thermal cycling conditions were an incubation step of 50 °C for 2 min; followed by 95 °C for 10 min; and 45 stepwise cycles of 95 °C for 15 s and 60 °C for 1 min. The samples were run in duplicates on each plate and their threshold cycle (Ct) values were

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averaged during data analysis. A non-template water control (NTC), as well as positive DNA from the cultured isolates, were included. Real-time PCR reactions were performed on Quant-5 studio (Applied Biosystems).
A negative control containing water instead of template DNA and positive control containing pure lumpy skin disease culture was incorporated.

2.6 LAMP primer design and assay validation

Genomic sequences of different strains of *Capripoxviruses* were downloaded from Genbank and grouped into two files one containing the target group which contained sequences of *lumpy skin disease virus* and a background group that contained sequences of *sheeppox virus* and *goatpox virus* genomic sequences. The primers were designed using the whole Genome-based LAMP primer designer software (GLAPD) as described by (Jia *et al.*, 2019). 10 LAMP primers that were meant to be specific to *lumpy skin disease virus* were designed. During primer design, special considerations were made due to the high A-T rich genome of Capripoxviruses, and non-coding regions of the genome of *lumpy skin disease virus* were used for primer design due to the high genetic similarity with *sheeppox virus* and *goatpox virus*.

A set of four primers comprising two outer and two inner primers that recognize eight distinct regions on the target sequence were designed. Primer mapping using the online tool Primer map (https://www.bioinformatics.org/sms2/primer_map.html) was done thereafter to determine the best sets of primers from the 10 generated. Three (3) primer sets were selected for synthesis and sent to a commercial service provider and the sequences are shown in Table 1. These sets of LAMP primers were generated from NC_003027.1_|Lumpy_skin_disease_virus_NI-2490_complete_genome as reference genome from NCBI database.

Table 1: Newly designed and synthesized LAMP primers.

Primer Name	Primer	Sequence (5'-3')	Target Position (Ref:NC_003027.1-LSDV)
4_LSD	F3	ACTGAAACTTTTTTTCTTTTGCC	2293-2315
	B3	TCACATTTTTTAGCCGATGC	2518 to 2537
	FIP	TGGGCAATGATAGGGTTAATGAATC,CGAAAACAGATTTTTTGTCGA	2376-2400
	BIP	TTACCACCAACATGAAAACAAACAC,GCACAACTAAATACATACAAACT	2435-2459
7_LSD	F3	AAAAACACTATCTATGTCTAATGCG	12084-12108
	B3	GTTTTTAACATGGTCGAAAGCT	12284 -12305
	FIP	TGATCGTTGGAGGTTTTCATTATGA,GTTCCATCCTTTTGTTTTATCGT	12169 – 12193
	BIP	TTGATGCATTAAATCTTGGCTGGT,TAATCCTATGTCTTGTAAATGGAGA	12218 -12241
10_LSD	F3	AAATGTTCCTTCTCTTTAGTACTTG	14447 – 14271
	B3	ACTCAGCAATGAAGAGCAATA	14651 – 14671
	FIP	ACAATGGTCAGAAGCTAGATGTTT,AAGTAAGCTATACATTTCAGAGTG	14543 – 14566

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BIP	ACCTCTGAGTAAAATCTTTCGGC,CATTTTTTGCAGCTAGTGATG	14567 – 14589

In initial experiments after ordering and purchase, all three designed LAMP primers were subjected to a gradient LAMP to determine the optimal reaction temperature. All LAMP assays were run on a rechargeable, portable Genie® III (OptiGene Ltd., UK), at 63°C for 30 min. A positive reaction was signified by an exponential increase in fluorescence (δR).

The LAMP reaction assay contained 2.5 µl of the 10× primer mix, 7.5 µl of LAMP master mix ISO-DR002 (Optigene Ltd., UK), and 2.5 µl of target DNA to make a reaction volume of 12.5 µl. The 10× primer mix contained 2.0 µM each of forward and reverse inner primers (FIP and BIP respectively) and 0.5 µM each of forward and reverse outer primers (F3 and B3, respectively).
The 10_LSD LAMP assay was used for subsequent experiments.

2.7 Analytical Sensitivity

The limit of detection of the developed LAMP assay was determined using a pure culture sample *of lumpy skin disease virus* isolate. The concentration of the template DNA was determined and diluted to 100ng/ul. A 10-fold serial dilution was then done and was used as a template for both qPCR and LAMP amplification to measure the sensitivities of these two amplification methods. The reactions for each serially diluted sample were carried out in triplicates and repeated five times to determine the repeatability and reproducibility of the LAMP assay.

2.8 Analytical specificity

To determine the specificity of the assay, closely related poxviruses that included *sheeppox virus, goatpoax virus, camelpox virus, PPR virus*, and *ORF virus* from KALRO biotechnology lab stock were used. DNA was extracted from these isolates and LAMP reaction was carried out in duplicates at the optimal temperature and incubation time. A negative control containing water instead of template DNA was incorporated.

2.9 On-site testing and validation of LAMP assays

Blood samples and skin biopsies were subjected to the PEG extraction method. Blood samples could not be successfully extracted using this method. Skin biopsies were successfully extracted using the PEG method as described in DNA extraction.

Ten samples of skin biopsies were selected to validate the field-based LAMP assay. The samples were extracted using the PEG quick extraction method and subjected to a LAMP assay using the 10_LSD primer.

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Four randomly selected individuals with varying experience (Intermediate=periodic exposure and novice=no prior exposure) in molecular diagnosis were selected. The individuals were trained and given the protocol and blinded samples to perform the LAMP assay.

2.10 Statistical analysis

The diagnostic sensitivity and specificity of the LAMP and PCR was determined using a 2×2 contingency table. The qPCR assay was used as the gold standard for the analysis. Comparison between the extraction methods and performance of the selected individuals was done using a student's t-test at a confidence level of 95 %. Agreement between the tests, that is, between the gold standard and 10_LSD LAMP assay was determined using Cohen's Kappa statistic. The Kappa statistic considers the element of chance and varies from 0 to 1, where <0.00 is a poor agreement, 0 - 0.2 is a slight agreement, 0.21 - 0.40 is a fair agreement, 0.41 - 0.60 is a moderate agreement, 0.61 - 0.80 is substantial agreement, and 0.81 - 1.00 is almost perfect agreement.

3.0 Result

3.1 Gradient LAMP

In initial experiments after purchase, all three designed LAMP primers were subjected to a gradient LAMP. The assays showed an increase in fluorescence in a real-time PCR machine and could successfully identify positive samples. However, these experiments indicated that the 10_LSD LAMP assay was the most rapid and reliable, and therefore this assay was selected for further optimization and evaluation.

3.2 Analytical sensitivity

The sensitivity of optimized qPCR and LAMP assay for LSDV DNA was determined using 10-fold dilutions of target DNA.

Table 2: The limit of detection with qPCR assays

qPCR Detection method	
Concentration (ng/µl)	Threshold Cycle (Ct)
100	34.633
10	31.766
1	33.110
0.1	37.993
0.01	38.812
0.001	41.297

In qPCR, the minimum template concentration limit at which the target DNA could be amplified and detected was 10^{-3}ng/µl with a Cycle threshold (C_t) value of 41, which was considered the cut-off value for the assay (Table 2).

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Table 3: The limit of detection with LAMP assays

LAMP Detection method (10_LSD primer; Designed in this study)	
Concentration (ng/µl)	Time to Positivity
100	15 min : 05 seconds
10	17 min : 05 seconds
1	23 min : 15 seconds
0.1	No Amplification
0.01	No Amplification
0.001	No Amplification

With the LAMP amplification, the minimum template concentration limit at which the target DNA could be amplified and detected was 1ng/µl with a time to positivity of 23 minutes. Therefore, qPCR showed a higher sensitivity than the LAMP assay (Table 3).

3.3 Analytical specificity

The specificity of the LAMP assay was evaluated to determine whether there was potential cross-reactivity with other closely related viruses.

No positive amplification was observed in DNA samples extracted from Orf virus, Pestes-des-petit-ruminants virus (PPRV), and Camel pox. It however amplified sheeppox virus and goatpox virus indicating that the LAMP assay facilitated rapid and specific detection of Capripoxvirus as it could not only detect LSDV (Figure 1).

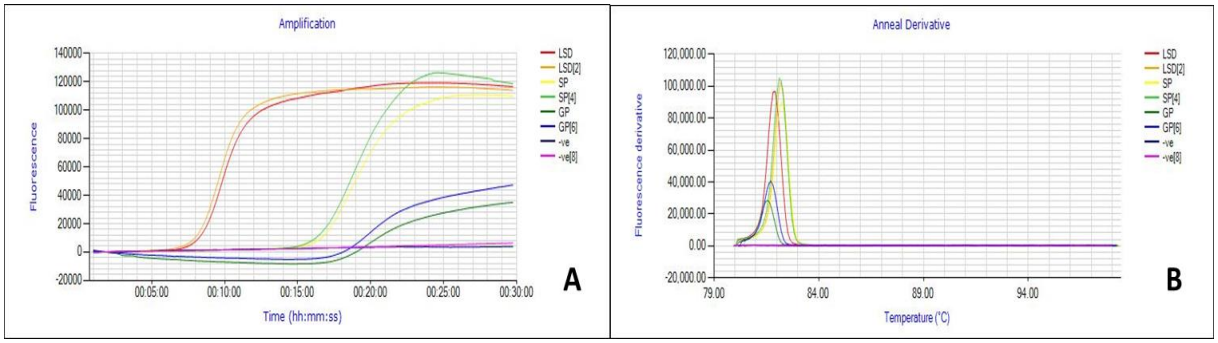

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Figure 1:Real-time detection of LSDV, SPV, and GPV for pure cultures.

A- The amplification curve shows time positivity (TTP) 9.15 – 19.30 min. B – Anneal derivative curves confirming positive reactions of different samples.

3.4 Analysis of different samples types with qPCR and LAMP assay

Table 3: qPCR and 10_LSD LAMP assay results on different sample types

Sample Type	Total Samples	qPCR	LAMP (10_LSD primer)
Blood	34	21	5
Skin Biopsies	19	19	19
Cell Culture	5	5	5
Serum	4	3	2

The 62 samples were subjected to qPCR and LAMP, 48 were positive by qPCR and 31 samples were positive by LAMP (Table 3). The cut-off for the qPCR assay was set at a cycle threshold (C_t) of 41, with skin biopsies and cell culture showing an early C_t of between 15- 30 while blood samples and serum samples showed a late C_t of between 37- 41 (Annex 2)

3.5 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and kappa value

The analytical specificity and sensitivity of the LAMP assay was determined using viral DNA extracted from cell culture-grown virus as the template. To assess the diagnostic accuracy of the LAMP assay, five statistics were determined, i.e., sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and kappa value. Using qPCR as the gold standard.

Table 4: Sensitivity and specificity of 10_LSD primer against qPCR

Test results	Disease	Non-Diseased	Total
Positive	True positive (29)	False positive (2)	Total test positive (31)

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	Negative	False Negative (19)	True Negative (12)	Total Test Negative (31)
		Total Disease (48)	Total Non-Disease (14)	Total (62)

Positive predictive value: True Positive/Total Test Positive x100

29/31 x 100 = 93.5 %

Negative predictive value: True Negative/Total Test Negative x100

12/14 x 100 = 85.7 %

The positive and negative predictive values were 93.5% and 85.7% respectively.

Sensitivity= (true positive)/(true positive + false negative) x 100

= 29/(29 + 19) x 100

Specificity= (true negative) / (true negative + false positive) x 100

= 12/(12+2) x 100

Table 5: Sensitivity, Specificity, Positive and Negative Predictive Values of LAMP assays

Test	Specificity	Sensitivity	PPV	NPV	Kappa Value
10_LSD	86	60	93.5	85.7	0.32

The LAMP assay had a specificity of 86 % in Capripox detection and a sensitivity of 60 %. The 10_LSD showed a fair agreement with the qPCR gold standard with a kappa value of 0.32.

3.6 Evaluation and verification of in-house protocol for On-site application

To validate the 10_LSD LAMP Assay for field application and detection of LSD, blood samples and skin biopsies were subjected to the PEG extraction method (i.e. crude DNA). Blood samples could

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not be successfully detected through this extraction method. Skin biopsies were successfully extracted using the PEG method.

10 skin biopsy samples extracted by the PEG method were run alongside DNA extracted using the phenol method. The crude DNA showed a later time to positivity (14-27 min) compared to the DNA from the phenol method (8-19 min).

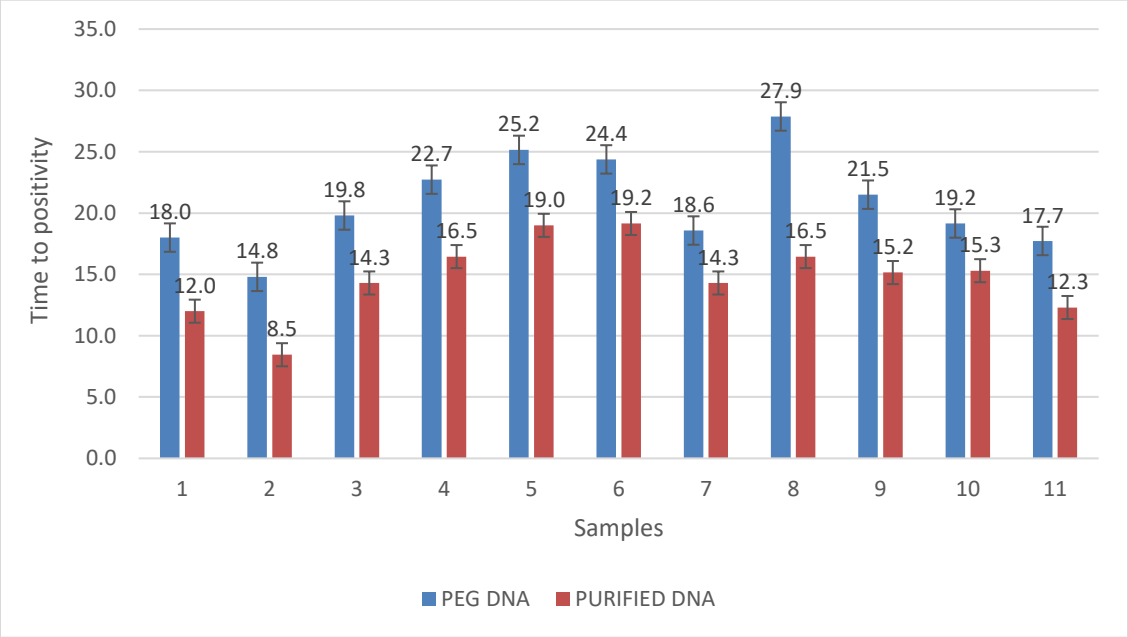

Figure 2: Comparison of time to positivity on phenol extracted samples and PEG extracted samples

Different operators with varying lab expertise were engaged to evaluate the in-house LAMP assay protocol in the detection of Capripoxvirus intended for field deployment. Two intermediate operators with experience in molecular biology took a shorter time for sample preparation as compared to the novice operators who had no experience in molecular biology.

The sample preparation involved extraction using the PEG method and the dilution of the lysate for the run (Figure 3). They took between 13 – 32 minutes for sample preparation depending on the level of experience of the operator compared to 6 -13 minutes for sample loading and running.

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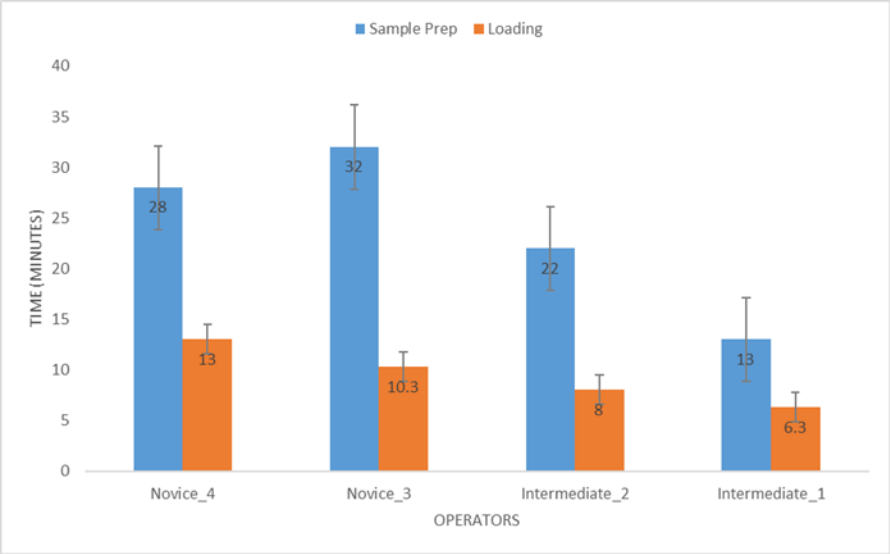

Figure 3: comparison between operators in sample preparation and sample loading

4.0 Discussion

Accurate and timely diagnosis is the key to the effective control of trans-boundary animal pathogens. Clinical diagnosis is not completely accurate; therefore, diagnostic tests with high sensitivity and specificity are required. Various molecular tests are used to diagnose LSDV. These tests are difficult to perform and require expansive laboratory equipment, expertise and chemicals.

An on-site field LAMP assay was developed in this study for the detection of *lumpy skin disease virus* (LSDV), and its operability was further evaluated using field-collected samples. The assay, although meant to be specific to LSDV, was also able to detect *sheeppox virus* and *goatpox virus*. Previous research demonstrated that members of the Capripoxvirus genus are genetically 96% similar (Haegeman *et al.*, 2019) and cannot be differentiated serologically. The developed 10_LSD assay was however very specific to Capripoxvirus as it did not detect viruses that usually at times occur as mixed infections such as *orf virus, camelpox virus*, and *pestes-des-petits-ruminants virus* (results not shown).

This study established that skin nodules are the ideal sample type to be used in field testing as they gave more conclusive results than blood. (Mwanandota *et al.*, 2018) also reported a higher detection rate (DR) with skin biopsies than with blood samples when validating previously designed LAMP assays for Capripoxvirus. A previous study showed that viral DNA could be detected through convectional PCR in blood up to 15 days post-infection and up to 3 months in skin biopsies (Tuppurainen *et al.*, 2005). This is a clinical problem with blood since the virus is only present in the blood during the onset of infection and then moves to the nasal system, lymph

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nodes, and skin where it forms the nodules (Tuppurainen *et al*., 2005). Blood is therefore not a suitable sample type for field detection as it can only give results at the onset of an infection as compared to skin biopsies that can be used to test for both active infections and disease surveillance studies.

The specificity of the specific 10_LSD assay was determined to be 86%. This indicates that the assay correctly identified 86% of samples with LSDV, minimizing false-positive results. It also had a negative predictive value (85.7%) which shows that it correctly identified samples without the virus. This is crucial for ruling out the presence of the virus in samples that test negative, thereby minimizing the risk of false-negative results. A positive predictive value (PPV) of 93.5%, demonstrates the assays ability to correctly identify samples that have the virus among those that test positive. A high PPV is essential for ensuring that samples that test positive on the assay are indeed infected, it is however highly affected by a prevalence of the disease. The sensitivity of the LAMP assay was determined to be 60% indicating that the assay correctly identified 60% of samples that were infected with the virus, minimizing false-negative results.

While the assay demonstrates good specificity and positive predictive value, indicating its reliability in identifying infected animals, its sensitivity appears to be lower. This suggests that there is a risk of false-negative results, potentially leading to missed diagnoses.

The statistical analysis of the LAMP assays against the gold standard using the Cohen's Kappa statistic at a 95 % confidence level indicated there was fair agreement (k 0.32) between the gold standard and 10_LSD specific assays. Despite the 10_LSD LAMP assay having a fair agreement with the gold standard method that is qPCR, its simplicity compared to the gold standard allows its ability to be rolled out to the resource-poor Regional Veterinary Investigation laboratories (RVILs) (Das *et al*., 2012). Determining the level of agreement between various diagnostics tests helps increase the level of confidence in the results presented (Mchugh, 2012). The higher the interrater reliability the higher the confidence in the study results and the higher the accuracy of the results. The Kappa test is however often affected in conditions where the prevalence rates are low (Hoehler, 2000).

The use of crude and pure DNA in LAMP was also assessed. The results of crude and pure DNA on LAMP showed similar results in terms of differentiating negative and positive samples although an earlier time to positivity was recorded using samples that were extracted through the phenol-chloroform (pure DNA) method as compared to the PEG method (crude DNA). This implies that the pure DNA extraction step can be omitted further shortening the time for the test. The crude form of DNA may contain a lot of impurities and that would account for the late time to positivity as the phenol-chloroform method gives a purer form of DNA. PEG extraction method is fast and the resulting lysates can be used directly for LAMP without additional manipulation making it

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suitable for on-site field testing. Complexity of current nucleic acid isolation methods limits the use of many DNA amplification technologies outside of the modern laboratory environment. The process of DNA extraction is time-consuming and complicated, limiting application on pen-side. The speed and simplicity of the PEG method make it ideally suited for nucleic acid amplification-based applications in a field set up with limited resources. PEG has in previous studies been used to isolate DNA for analyses of viral genomic diversity (Colombet and Sime-ngando, 2012), it has also been used to isolate DNA from various bacteria, eukaryotic tissue samples, and whole blood (Chomczynski and M. Rymaszewski, 2006) and also in a field based LAMP assay for detection of sweet potato viruses (Wanjala *et al.,* 2021).

To validate and verify the developed Lamp assay for field adoption, four operators with varying laboratory experiences in molecular work (intermediate operators one and two, novice operators three and four) were selected. The two intermediate operators with experience in molecular biology took a shorter time to perform the assay as compared to the novice operators who had no experience in molecular biology (section 3.6). This shows that the difference noted in the results when running the assay by PEG extraction is dependent on the operator and how they do the extraction and running of the reaction. The most time-consuming part of the field assay was the sample preparation, which can be attributed to the sample homogenization with PEG.

The LAMP molecular assay is a potentially useful alternative to the current molecular tools that require sophisticated equipment and techniques (Parida *et al.,* 2008). Studies have demonstrated that LAMP can be used for Capripoxvirus diagnosis. In this study a field diagnosis assay that uses an instrument-free DNA extraction method was evaluated and verified ready for adoption for the on-site diagnosis of the three Capripoxvirus that is *lumpy skin disease virus, sheeppox virus*, and *goat pox virus*. In conclusion, the developed on-site LAMP can facilitate the rapid use in non-laboratory or low-resource equipment and settings. It is suitable and reliable for the rapid assessment of Capripoxviruses by regional veterinary services and the kit can be deployed in situations where testing is currently impossible or extremely limited. This is of great significance in case of an outbreak when rapid results are required for the detection of Capripoxvirus. In addition, loop primers can be incorporated to accelerate the reaction while using the PEG extraction method and a color change or turbidity method developed to use with the assay. Considering that the interrater reliability in terms of measure of consistency and agreement between 10_LSD and gold standard revealed that the level of agreement between the tests was to a large extent due to fair agreement, it is important that more studies with a greater sample size are conducted (Watson and Petrie, 2010)

5.0 Conclusion

In conclusion, the present study the LAMP assays developed have successfully shown to identify LSDV in naturally infected livestock herds. These LAMP assays together with simplified PEG nucleic

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acid extraction methods could further facilitate the rapid use of these methods in non-laboratory or low-resource settings providing portability to conduct tests in the laboratory or in the field. Maximizing the simplicity of nucleic acid-based testing comprising PEG nucleic acid extraction, amplification and detection would allow these methods to be deployed in situations where testing is currently impossible or extremely limited.

Once DNA extraction was done, the LAMP assay was an effective and time-saving method with an output time of 30 min compared to 3 hrs for PCR and 2-3 weeks for cell culture diagnosis. It is a simple, rapid, sensitive and reliable diagnostic tool and comparable to real time PCR assays for detection of LSDV infection. This is of great significance in case of an outbreak when rapid results are required for the detection of Capripoxvirus.

The sample size was determined by LSDV reported outbreaks and the number of samples submitted for testing at the veterinary laboratories; thus, restricting the number of clinical samples that could be used in the study. The assay offers a test that can be adopted by regional staff in endemic areas with minimal lab experience.

6.0 Recommendation

To reduce the time to positivity using PEG extraction, two more primers; loop forward primer (LF) and loop backward primer (LB) may be included. This increases the sensitivity and acceleration of the reaction, reducing the time by half (Nagamine *et al.,* 2002).

A larger sample size with confirmed positive and negative samples could be used to assess the interrater reliability (Cohen Kappa test) of the LAMP assays in future studies to obtain a better estimate of the agreement due to chance.

Further studies with the use of dry reagents can be tested with the assay to evaluate its use in low resource settings which will be a pre-prepared mix that only requires addition of sample DNA.

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9.0 Annex 1

9.1 List of lamp primers generated (Software GLAPD)

This sets of LAMP primers could be used in 1 genomes for lumpy skin disease virus: they were generated from NC_003027.1_|Lumpy_skin_disease_virus_NI-2490_complete_genome as reference genome from NCBI database.

9.1.1 LSD_1

F3: pos:175,length:25 bp, primer(5'-3'):CCAATTAAGTAGAAGCCAATTAAAC
F2: pos:220,length:22 bp, primer(5'-3'):AAGTAGAAGCCAATTAAACCTG
F1c: pos:280,length:25 bp, primer(5'-3'):TCGCTAGTGAAATCAGCACTAATAA
B1c: pos:323,length:22 bp, primer(5'-3'):GAGGTCTCGAAGCAATACCAAC
B2: pos:368,length:18 bp, primer(5'-3'):TGGGGTGGTGAATCATCT
B3: pos:391,length:20 bp, primer(5'-3'):CATTTTAGCAAGAGCAGCAG

9.1.2 LSD_2

F3: pos:506,length:23 bp, primer(5'-3'):AAACATGTTTTTGACAAAAGCTG
F2: pos:530,length:25 bp, primer(5'-3'):TAGATCATTTCCAAATACAAGTGAG
F1c: pos:574,length:25 bp, primer(5'-3'):GCTGGAAAGGTGTTCTTAGTTTTTG
B1c: pos:602,length:20 bp, primer(5'-3'):CTCCCTGGAGGAAAATGCCA
B2: pos:645,length:18 bp, primer(5'-3'):TATCACAACCGCCATCTC
B3: pos:663,length:22 bp, primer(5'-3'):ACAACTTTTCAGATGATGATGA

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9.1.3 LSD_3

F3: pos:1698,length:25 bp, primer(5'-3'):ATCTTTAAAACTTACACTCTCAACG
F2: pos:1729,length:25 bp, primer(5'-3'):TTCATTTCTGTAGTTACTTGATCAC
F1c: pos:1771,length:22 bp, primer(5'-3'):TGCCACAGTGTGCTAAGAGAAT
B1c: pos:1844,length:24 bp, primer(5'-3'):TACCCAATGCATAAACTGCTTCGT
B2: pos:1886,length:23 bp, primer(5'-3'):CAAAGAAGAAACACAAAACAACT
B3: pos:1909,length:19 bp, primer(5'-3'):TCGCACATGATGATTGTTC

9.1.4 LSD_4

F3: pos:2292,length:23 bp, primer(5'-3'):ACTGAAACTTTTTTTCTTTTGCC
F2: pos:2334,length:21 bp, primer(5'-3'):CGAAAACAGATTTTTTGTCGA
F1c: pos:2375,length:25 bp, primer(5'-3'):TGGGCAATGATAGGGTTAATGAATC
B1c: pos:2434,length:25 bp, primer(5'-3'):TTACCACCAACATGAAAACAAACAC
B2: pos:2490,length:23 bp, primer(5'-3'):GCACAACTAAATACATACAAACT
B3: pos:2517,length:20 bp, primer(5'-3'):TCACATTTTTTAGCCGATGC

9.1.5 LSD_5

F3: pos:6996,length:18 bp, primer(5'-3'):ACTACTGGTGCTACGCAA
F2: pos:7029,length:24 bp, primer(5'-3'):AGTAAATTCTCTACTACAAAACGC
F1c: pos:7084,length:25 bp, primer(5'-3'):TTCATGTAGCTGAAATTGTGTCTCT
B1c: pos:7109,length:24 bp, primer(5'-3'):CTGCAAGGTTGACAAATCTTAACG
B2: pos:7163,length:22 bp, primer(5'-3'):ACTGTATTTGTTTCATCGTTGT
B3: pos:7185,length:23 bp, primer(5'-3'):TGTTTTTACTCCCATTTAGTGTA

9.1.6 LSD_6

F3: pos:7541,length:23 bp, primer(5'-3'):GAACTACAGCTAGGTATCTATCA
F2: pos:7582,length:23 bp, primer(5'-3'):AATGACATGCTATTGTAAAAACC
F1c: pos:7632,length:24 bp, primer(5'-3'):TGGAGTTTAGGAGATTGTTTGTGT
B1c: pos:7683,length:24 bp, primer(5'-3'):AAAAGGAAACACCAACACGAAAAT
B2: pos:7728,length:23 bp, primer(5'-3'):CAATACAGGATATGTTTTTGCTT
B3: pos:7764,length:22 bp, primer(5'-3'):TGTGTTAACTGTTCTTCGTAAA

9.1.7 LSD_7

F3: pos:12083,length:25 bp, primer(5'-3'):AAAAACACTATCTATGTCTAATGCG
F2: pos:12116,length:23 bp, primer(5'-3'):GTTCCATCCTTTTGTTTTATCGT
F1c: pos:12168,length:25 bp, primer(5'-3'):TGATCGTTGGAGGTTTTCATTATGA
B1c: pos:12217,length:24 bp, primer(5'-3'):TTGATGCATTAAATCTTGGCTGGT
B2: pos:12257,length:25 bp, primer(5'-3'):TAATCCTATGTCTTGTAAATGGAGA
B3: pos:12283,length:22 bp, primer(5'-3'):GTTTTTAACATGGTCGAAAGCT

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9.1.8 LSD_8

F3: pos:13128,length:21 bp, primer(5'-3'):GCTCGTAACCTTTTTTTTCCT
F2: pos:13159,length:23 bp, primer(5'-3'):AAAAGGTAAATCTTACAGAGTCA
F1c: pos:13208,length:25 bp, primer(5'-3'):GTGGGGAACTGTCAAAAAAACTAAT
B1c: pos:13247,length:25 bp, primer(5'-3'):AAGAACAAAATCTTCGGTATCGACA
B2: pos:13292,length:23 bp, primer(5'-3'):GAGTTAATGAATTAAGGCCCATA
B3: pos:13329,length:23 bp, primer(5'-3'):AGGTTTATGTATATAAGCAACGA

9.1.9 LSD_9

F3: pos:14115,length:25 bp, primer(5'-3'):AAATTCTTTTTCTATAGACACAGCC
F2: pos:14153,length:25 bp, primer(5'-3'):TCGTAATAATTTCATTAGAAGGAGG
F1c: pos:14199,length:25 bp, primer(5'-3'):CCTTCATTGTGATTTTGCGTGTTTA
B1c: pos:14244,length:25 bp, primer(5'-3'):TTCGTTTGAAAATGTTAATCCTGGC
B2: pos:14289,length:21 bp, primer(5'-3'):TCATTCGCTGCAATATTTTGG
B3: pos:14311,length:20 bp, primer(5'-3'):GAGGGAATATTCTTTTCCGG

9.1.10 LSD_10

F3: pos:14446,length:25 bp, primer(5'-3'):AAATGTTCCTTCTCTTTAGTACTTG
F2: pos:14487,length:24 bp, primer(5'-3'):AAGTAAGCTATACATTTCAGAGTG
F1c: pos:14542,length:24 bp, primer(5'-3'):ACAATGGTCAGAAGCTAGATGTTT
B1c: pos:14566,length:23 bp, primer(5'-3'):ACCTCTGAGTAAAATCTTTCGGC
B2: pos:14609,length:21 bp, primer(5'-3'):CATTTTTTGCAGCTAGTGATG
B3: pos:14650,length:21 bp, primer(5'-3'):ACTCAGCAATGAAGAGCAATA

10.0 Annex 2

Sample No.	Sample Type	Ct Mean	Res.
1	Blood	0.0	Neg
2	Blood	41.1	Pos
3	Blood	0.0	Neg
4	Blood	0.0	Neg
5	Blood	40.5	Pos
6	Blood	38.2	Pos
7	Blood	39.7	Pos
8	Blood	39.3	Pos
9	Blood	40.8	Pos
10	Blood	41.8	Pos
11	Blood	38.3	Pos

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12	Blood	36.8	Pos
13	Blood	39.0	Pos
14	Blood	39.2	Pos
15	Blood	38.2	Pos
16	Blood	39.9	Pos
17	Blood	41.1	Pos
18	Blood	40.0	Pos
19	Blood	40.4	Pos
20	Blood	29.0	Pos
21	Blood	8.8	Pos
22	Blood	0.0	Neg
23	Blood	0.0	Neg
24	Blood	0.0	Neg
25	Blood	37.7	Pos
26	Blood	0.0	Neg
27	Blood	0.0	Neg
28	Blood	39.8	Pos
29	Blood	15.6	Pos
30	Blood	0.0	Neg
31	Skin Biopsy	16.5	Pos
32	Skin Biopsy	37.1	Pos
33	Skin Biopsy	15.3	Pos
34	Skin Biopsy	17.4	Pos
35	Skin Biopsy	33.1	Pos
36	Skin Biopsy	21.7	Pos
37	Skin Biopsy	17.3	Pos
38	Skin Biopsy	32.3	Pos
39	Skin Biopsy	17.9	Pos
40	Skin Biopsy	18.3	Pos
41	Skin Biopsy	20.3	Pos
42	Skin Biopsy	21.8	Pos
43	Skin Biopsy	15.4	Pos
44	Skin Biopsy	35.0	Pos
45	Skin Biopsy	32.3	Pos
46	Skin Biopsy	36.5	Pos
47	Skin Biopsy	17.5	Pos
48	Skin Biopsy	17.9	Pos

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49	Skin Biopsy	33.9	Pos
50	Cell Culture	25.0	Pos
51	Cell Culture	29.4	Pos
52	Cell Culture	39.0	Pos
53	Cell Culture	36.0	Pos
54	Cell Culture	27.0	Pos
55	Blood	0.0	Neg
56	Blood	0.0	Neg
57	Blood	0.0	Neg
58	Blood	0.0	Neg
59	Serum	0.0	Neg
60	Serum	32.3	Pos
61	Serum	35.9	Pos
62	Serum	37.4	Pos

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