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Article

Preliminary results of the development of a portable visual loop-mediated isothermal amplification assay to detect Lumpy skin disease virus

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Abstract

In recent decades, emerging and re-emerging diseases have been spreading worldwide. Lumpy skin disease is one of the most significant economic transboundary animal diseases. It has been reported in several areas of the world.

The study supports the adoption of the portable Loop-Mediated Isothermal Amplification (vLAMP) assay as a primary diagnostic tool for lumpy skin disease virus. Main diagnostic methods such as Polymerase Chain Reactions (PCR) and Enzyme-Linked Immunosorbent Assay (ELISA) for detecting Lumpy Skin Disease (LSD) are sensitive and reliable but are often labor-intensive and time-consuming. In contrast, the portable vLAMP assay offers significant advantages for field application. It eliminates the need for specialized expertise or sophisticated laboratory equipment and can detect Lumpy Skin Disease Virus (LSDV) within 60 minutes under constant temperature conditions. This study aimed to develop and optimize a rapid, portable vLAMP assay for the on-site detection of LSDV. In addition, the sensitivity and specificity of this assay are comparable to conventional PCR and other tests. Thirty-two samples, including tissues, whole blood, serum, and swabs were analyzed using two DNA extraction kits and molecular methods: conventional PCR and vLAMP assay. The sensitivity and specificity of the vLAMP assay were estimated using a two-by-two contingency table and found to both be 100%. The vLAMP had a kappa value of 1.0 against the conventional PCR. Therefore, this vLAMP assay can be adopted as a timely and simple method for the early detection, monitoring, and control of LSDV outbreaks in field settings.

Keywords: Diseases, infectious, diagnosis, assay

Introduction

Lumpy skin disease (LSD) affects cattle and is caused by Capripoxviruses. Cattle strains of Capripoxvirus do not infect and transmit between small ruminants (OIE). The main transmission route is insect vectors and blood-sucking arthropods such as certain mosquitoes, stable flies, biting-midges and ticks [1, 2]. In addition, the genus *Capripoxvirus* within the subfamily Chordopoxvirinae of the family Poxviridae causes sheep pox, goat pox, and lumpy skin disease in cattle [3, 4]. These viruses are antigenically indistinguishable and identical to strains causing sheep pox and goat pox on a biological level [5]. LSD is a serious viral infectious disease in domestic cattle and it can even infect buffalo and wild ruminants [1]. The main clinical signs are fever, raised, circular, firm nodules on the skin, mucosal surfaces and internal organs with a diameter of 1-5 cm, enlargement of superficial lymph nodes, and swelling of the limbs or lower body [2]. As well as a significant economic loss due to temporary reduction in milk production and temporary or permanent sterility in bulls, the disease can also cause damage to hides and sometimes death [3, 7, 8]. Therefore, LSD is categorized as a notifiable disease by the World Organization for Animal

Health [7, 9] and has a substantial negative economic impact on all stakeholders of cattle industries during its outbreaks [6, 8, 10,11]. Since it was first reported in Zambia in 1929it has spread to the Middle East, Europe, and several Asian countries. It has spread to Bangladesh, China, India, Nepal, Pakistan, Vietnam, Myanmar, Malaysia, Hong Kong, Laos, Taiwan, and Mongolia between 2019 and 2022. The virus has an endemic status in Africa and the Middle East [4, 8, 12, 13]. The first Mongolian outbreak was reported in the Eastern parts of Mongolia in 2021 and the causative agent was subsequently identified using molecular methods [14]. Regular surveillance for prevention and control measures of LSD and early detection tools for LSDV diagnosis are still needed. Therefore, this study aimed to establish rapid detection of the Lumpy skin disease virus using a portable loop-mediated isothermal amplification assay. Minor objectives were comparing results between LAMP and conventional PCR and DNA extraction between commercial kits and pen-side methods using magnetic beads.

Materials and Methods

Study design

This study was conducted at the Department of Infectious Diseases and Microbiology, School of Veterinary Medicine, Mongolian University of Life Sciences. This cross-sectional study has been conducted in LSD outbreak areas between 2023-2025. Dornod province in the Eastern part was targeted since the first LSD outbreaks were reported in 2021 in Eastern Mongolia.

Sampling and sample size

The study employed a combination of non-probability and probability sampling techniques. Provinces, subdistricts (soums), households, and individual livestock were randomly selected. Three soums were purposely selected while households were selected proportional to the size of the cattle population in Dornod province. The sample size was calculated to estimate the design effect (DE) within-cluster correlation of the study, and the livestock number within each cluster was defined by a 95% confidence interval, at a 5% significance level. The calculated sample size for each cluster was 10 herds, each with at least 10-12 cattle.

Sample collection

Samples were collected from cattle in Dornod Province for further analysis between 2023 and 2024. Samples (blood, swabs, and serum) were collected from cattle with non-apparent clinical signs in each household. In addition, four tissue samples in the liver, spleen, skin nodular lesions, and 2 serum samples as reference positive controls were used. Four tissue samples from the first LSD outbreaks in Mongolia in 2021 were kept in the pathology laboratory of the School of Veterinary Medicine (SVM). Additionally, two positive serum samples were stored in the virology laboratory of the Institute of Veterinary Medicine.

A couple of blood samples were collected from the jugular vein for each cattle using a vacutainer tube with a disposable needle with ethylenediaminetetraacetic acid (EDTA) for whole-blood collection and without anticoagulant for serum collection. Each serum was separated and pipetted from the top layer after centrifugation at 1500-3000 rpm for 10 minutes, and the serum was transferred into a 2 ml labeled Eppendorf tube. The nasal swab sample from the mucosal surface was gathered by gently rotating a sterile cotton swab for a few seconds after inserting it into the nostril. Collection of skin nodular lesions/ or scabs, and internal organs (liver, lung, spleen) was done by using sterile scalpels or forceps under aseptic conditions by biopsy. All samples were transported in a cooling box with ice packs to provincial veterinary laboratories. They were kept at -20℃ until they were shipped to the laboratory of the School of Veterinary Medicine.

Sample collection in this study was approved by the ethics committee for the use of animals' experiments, Institute of Veterinary Medicine (protocol number VMBMR 21/01/08), and the scientific committee, School of Veterinary Medicine, Mongolian University of Life Sciences (protocol number 01/18) in accordance with their guidelines.

DNA extraction

DNA was extracted from various sample types-including swabs, serum, and tissues from the lung, liver, and skin nodular lesions-using different protocols appropriate to each sample. DNA

extraction from all samples was performed using a commercial kit (G-spin™ Total DNA Extraction Kit, Cat. No. # IBT-QMS-GT1704, iNtRON, South Korea) according to the manufacturer's protocol. In addition, DNA extraction of the above samples was performed using magnetic-beads methods by Zhang et al. All DNA samples were kept at -20^{0}C before being used in the assay.

Solid-state visualization loop-mediated isothermal amplification assay

LSDV Solid-state Visualization Detection Kit (Product No.:HK-LSDV-S48 shelf, Hebei Normal University of Science and Technology) was used according to the manufacturer's instructions. The final volume of 20 µl LAMP-PCR reaction mixture contained 17 µl primer mix (the LSDV solid state reaction tube consists of forward inner primer "FIP includes two binding targets: F1c & F2"; backward inner primer "BIP includes two binding targets: B1c & B2"; forward outer primer "F3"; backward outer primer "B3", dNTP Mix, plus isothermal amplification buffer, and nuclease-free water), 1 µl Bst polymerase, and 2 µl template DNA. The primer sequences are not provided in this paper due to the application of intellectual properties from Chinese entities.

The LAMP amplification took place at 65^{0}C in portable thermo-temperature equipment for 60 minutes and held at 4^0 C until use. The amplification products of LAMP were judged according to color changes determined by the naked eye.

According to the manufacturer's instructions, positive and negative controls appeared yellow and purple after the reaction. Sample results were interpreted by comparing the color change to the control tubes-samples matching the positive control were yellow and samples matching the negative control were purple.

Conventional PCR assay

The commercial primers for the conventional PCR assay used the following gene sequences per the recommendation of the WOAH's manual: Forward primer 5'-TCCGAGCTCTTTCCTGATTTTTCTTACTAT-3', Reverse primer 5'-TATGGTACCTAAATTATATACGTAATAAC-3'. DNA amplifications were carried out in the final volume of 50 µl PCR mixture containing 2 µl of DNA template, 1 µl forward primer, 1 µl reverse primer, 8 µl of premix (Bioneer, South Korea), and 38 µl of ddH_2O. The PCR condition was used as recommended in the WOAH's manual and held at 4^0 C until analysis. The PCR products were verified by loading 1,5% into a TAE buffer and in a parallel lane with a 100 bp DNA-marker ladder for 40 minutes at 100V. The representative DNA products were visualized in a UV transilluminator.

Validation assays and statistical analysis

A two-by-two contingency table was used to estimate the sensitivity (SE), specificity (SP), positive predictive value (PPV), and negative predictive value (PNV) of the LAMP assay (Table 1). A 2x2 contingency table was created as follows: TP (true positive), FP (false positive), FN (false negative), TN (true negative), TTP (total test positive), TTN (total test negative), and N (total).

Table 1

Estimation of the positive predictive value, negative predictive value, sensitivity, and specificity of the LAMP assay

		Diseased	Non-diseased	Total
Test results	Positive	TP	FP	TTP
	Negative	FN	TN	TTN
	Total	Sub-total	Sub-total	N

$$PPV = TP/TTP * 100 \quad (1)$$

$$NPV = TN/TTN * 100 \quad (2)$$

$$SE = \frac{TP}{TP+FN} * 100 \quad (3)$$

$$SP = \frac{TN}{TN+FP} * 100 \quad (4)$$

Cohen's kappa statistic (k) was used to calculate the agreement between the two assays. Kappa statistic is used to assess the level of agreement between observed and predicted calculations, taking into

account the agreement occurring by chance. Kappa values range from less than 0 to 1, where values <0 indicate no agreement, 0.01–0.20 indicate minimal agreement, 0.21–0.40 indicate limited agreement, 0.41–0.60 indicate moderate consistency, 0.61–0.80 indicate strong agreement and 0.81–1.00 indicate near-perfect agreement.

$$k = \frac{Pr\,(a) - PrPr\,(e)}{1 - PrPr\,(e)} \quad (5)$$

$$Pr_{(a)} = \frac{FN+TTP}{N} \quad (6)$$

$$Pr_{(e)} = \frac{((TP+FN)*(TP+FP))+((FP+TN)*(FN+TN))}{N} \quad (7)$$

Results

DNA samples from 32 cattle (12 blood, 10 serum, 6 swabs, and 4 tissues) were extracted using magnetic beads by Zhang et al and commercial extraction kits. In addition, all DNA samples were tested using both LAMP and conventional PCR. A total of four DNA samples extracted from gross lesions in the lung, spleen, skin nodules, and 2 serum samples were used as positive reference controls. In addition, DNA from two of these reference serum samples was used as a positive control during the assays, while the remaining serum, blood, and swab samples tested negative with both LAMP and PCR assays. In contrast, DNA extracted from four tissue samples tested positive with both LAMP and PCR assays**.** A total of four DNA samples were measured using a NanoDrop spectrophotometer (Thermo Fisher), with concentrations ranging from 36.1 to 44.7 ng/µl.

DNA extracted using magnetic beads and commercial extraction kits yielded identical results when tested by both vLAMP and conventional PCR assays (Figure 1).

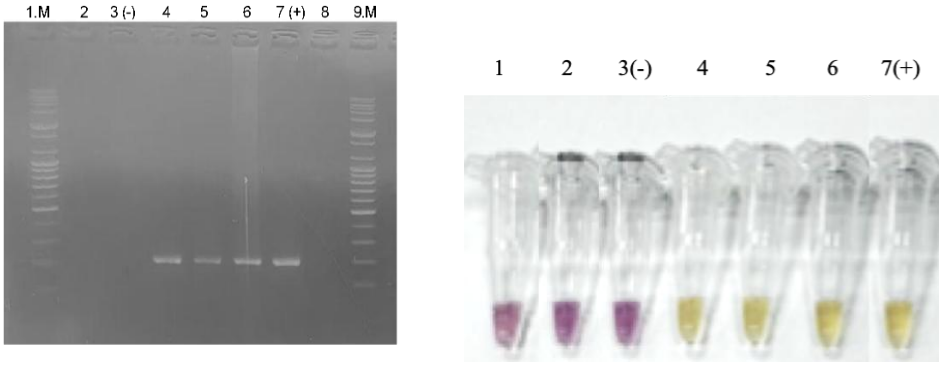

Figure 1. Left: Conventional PCR 1. M-DNA ladder, 3. negative contro (-), 4, 5, 6- positive samples, 7. (+) – positive control, 2&8. no samples, 9. M-DNA ladder. Right: vLAMP assay 1. 1&2. no samples, 3. negative control (-), 4, 5, 6. positive samples, 7. positive control (+).

An analysis of different sample types was conducted using both conventional and solid visualization LAMP-PCR assays (Table 2).

Table 2

Results of conventional and LAMP-PCR assays

Sample Type	Total samples	vLAMP-PCR	Conventional PCR
Blood	12	12 (Neg)	12 (Neg)
Serum	10	10 (Neg)	10 (Neg)
Swabs	6	6 (Neg)	6 (Neg)
Tissues (skin nodules, spleen, lung)	4	4 (Pos)	4 (Pos)
Total	32	32	32

The results of the conventional and solid visualization LAMP-PCR assays were compared. The estimation of sensitivity (SE), specificity (SP), positive predictive value (PPV), and negative predictive value (NPV) for the vLAMP assay using abovementioned formulas was conducted (Table 3). Using conventional PCR as the reference test for analysis.

Table 3

The estimation of sensitivity, specificity, positive and negative predictive value for the vLAMP assay

		Diseased	Non-diseased	Total
Test results (vLAMP)	Positive	4	0	4
	Negative	0	28	28
	Total	4	28	32

$$PPV = \frac{4}{4} * 100 = 100\%$$
$$NPV = \frac{28}{28} * 100 = 100\%$$

Both the positive predictive value (PPV) and negative predictive value (NPV) of the vLAMP assay were both estimated at 100%. A high PPV indicates the assay's ability to correctly identify true positive samples-those that actually have the virus-among all positive test results. Similarly, a high NPV reflects the assay's accuracy in correctly identifying true negative samples-those without the virus-among all negative test results.

$$SE = \frac{4}{4 + 0} * 100 = 100\%$$
$$SP = \frac{28}{28 + 0} * 100 = 100\%$$

The diagnostic sensitivity and specificity of the vLAMP assay were both determined to be 100 %. The specificity indicates that the assay accurately identified all non-infected samples, minimizing false-positive results. Likewise, the sensitivity demonstrates the assay's ability to correctly detect all LSDV-infected samples, thereby minimizing false-negative results.

$$Pr_{(a)} = \frac{4 + 28}{32} = 1$$
$$Pr_{(e)} = \left(\frac{4 * 4}{32}\right) + \frac{\left(\frac{28 * 28}{32}\right)}{32} = 0.78$$
$$Cohen's\ Kappa\ statistic\ value\ (k) = \frac{1 - 0.78}{1 - 0.78} = 1$$

Statistical analysis of the agreement between the vLAMP assay and the reference test (conventional PCR) was performed using Cohen's Kappa statistic (k). The vLAMP assay demonstrated an almost perfect agreement (0.81-1.00) with the conventional PCR, with a kappa value of 1.0.

Discussion

Lumpy skin disease is a transboundary animal disease, and one of the most economically devastating diseases in the cattle industry worldwide [12, 13, 15]. Although the primary host species are bovine and water buffalo, it can also infect some wild animals. Due to outbreaks of LSD, a substantial decline in milk production, temporary or permanent inability to reproduce in bulls, damage to hides, and sometimes death can occur [6].

In addition, the transmission route is predominantly spread rapidly between cattle by insect vectors, particularly biting flies. Direct contact does not significantly facilitate transmission, but it can also be transmitted through direct contact with infected

semen, milk, and placentas [5, 15]. A rapid and simple screening method with high sensitivity and specificity is important for the prevention and early detection of LSD in cattle populations [16].

The detection of LSDV is typically based on laboratory findings that confirm the presence of the virus or its antigens, using PCR assays and virus isolation in cell cultures from various types of cattle samples. In addition, conventional methods such as histopathological examination, immunofluorescence assay, and multiplex enzymatic immunosorbent assays are also used for LSD diagnosis [17]. A study found that the investigation of the diagnosis during the first outbreak of LSD in Mongolia was conducted through clinical manifestation, PCR detection, virus isolation, and histopathological analysis [14].

However, while all the above-mentioned diagnostic methods provide advanced advantages for laboratory findings, they are not available in field settings [17]. In another study, the LAMP assay was used for the rapid detection of LSDV in cattle, and the results were compared with conventional PCR. The LAMP assay showed higher detection accuracy and sensitivity, with results nearly identical to those of the PCR [16].

In this study, a total of 32 DNA samples were extracted using different protocols from a commercial kit, depending on the sample type. Additionally, all 32 DNA samples were re-extracted from various sample types using a single protocol developed by Zhang et al. This DNA extraction protocol utilizes magnetic beads instead of a silica column for DNA separation. Although the DNA yield did not differ significantly between the extraction kit and the magnetic bead-based method, the latter was deemed more suitable due to its efficiency and accessibility under local conditions.

Four samples from gross lesions and two serum samples were used as positive reference controls. A total of four reference tissue samples tested positive in both assays. However, the two serum samples that were serologically positive yielded negative results in both the LAMP and PCR assays, indicating that the LAMP assay may have limited sensitivity for detecting LSD during the later stages of infection.

One study found the LAMP assay with the gold standard qPCR test for the detection of LSDV. The sensitivity and specificity of the LAMP assay were found to be 60% and 86%, respectively. The positive and negative predictive values were 93.5% and 85.7%. Additionally, statistical analysis using Cohen's Kappa test indicated a fair level of agreement between the LAMP assay and the qPCR test ($\kappa = 0.32$) [17].

In this study, when we validated the results of the vLAMP assay, both the diagnostic sensitivity and specificity were 100%. Moreover, the positive and negative predictive values were also 100%. Additionally, the agreement between the two tests, as measured by the Kappa statistic, indicated almost perfect agreement ($\kappa = 1.0$). The assay demonstrates good sensitivity, specificity, positive predictive value, and negative predictive value, indicating its reliability in identifying both infected and non-infected animals. These results suggest a low risk of false-negative and false-positive outcomes, thereby reducing the likelihood of misdiagnosis.

All cattle showed no clinical signs of LSD at the time of sample collection. Consequently, the results of this study indicated that both PCR and LAMP assays could not detect the infection in field samples from cattle herds. However, reference strains collected during the LSD outbreaks in Mongolia in 2021, as well as the positive and negative controls for both assays, performed well.

In contrast, the advantages of the vLAMP method include characteristics such as a lack of cross-reactivity with other common bovine diseases (high specificity), a limit of detection of 10-50 copies of plasmid per reaction (high sensitivity), and ease of operation, as it provides ready-to-use reaction tubes with solidified buffers. The results can also be visualized by the naked eye.

Conclusion

In conclusion, the portable visualization LAMP assay implementation method has been shown to detect LSDV in naturally infected cattle herds.

An accurate and timely diagnostic tool is crucial for the early detection and management of LSDV infection in the laboratory or on the field. The combination of a DNA extraction kit using magnetic beads and a vLAMP assay, along with simplified techniques, could further facilitate the rapid implementation of these methods in low-resource field settings, providing portability to conduct tests.

Thus, portable vLAMP assay and magnetic beads extraction kits do not require expensive equipment,

specialized tools such as electrophoresis and thermocycler, or many reaction components, all while still maintaining accuracy.
In this study, identical results were obtained from both the vLAMP and conventional PCR assays. In addition, both the vLAMP and conventional PCR assays demonstrated almost perfect agreement, as indicated by the Kappa statistic. Therefore, this portable and reliable vLAMP assay enables rapid and early detection of LSDV infection and is a suitable, cost-saving, convenient, and efficient tool for potential pen-side use.

Conflict of Interests:
The authors declare no conflict of interests.

Author's contribution
B.B. writing original draft, conducted laboratory analysis. J.Z. project administration, writing-review and editing. Y.L. methodology. S.Y. review and editing. Z.F. review and editing. A.T. conducted field work. U.T. review and editing. N.G. review and editing. E.O. review and editing. D.N conducted field work and laboratory analysis. O.G. review and editing. O.E. conducted field work. O.M. review and editing. C.B. supervision, conceptualization, methodology, validation, writing-review and editing.

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