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Development and Validation of a Real-Time RPA Assay for Differentiating Lumpy Skin Disease Virus from Goat Pox Vaccine Strains

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ABSTRACT

In regions where lumpy skin disease virus (LSDV) is endemic, infections lead to major financial setbacks due to poor hide condition, decreased fertility, progressive weight loss, and lower milk production. To curb LSDV outbreaks in China, a heterologous live attenuated goat pox virus (GTPV) vaccine (AV41 strain) has been employed. However, diagnostic approaches that can reliably tell LSDV apart from GTPV vaccine strains remain scarce. This study designed a real-time recombinase polymerase amplification (RPA) assay using primers and probes based on conserved fragments of the ORF132 gene to enable quick and specific detection of LSDV. The reaction required only 20 minutes at a steady 39 °C. The limit of detection was 15 copies/µL, with no detectable cross-reactivity to nucleic acids from goat pox virus, infectious bovine rhinotracheitis virus, Pasteurella multocida, or healthy bovine tissue. When 43 clinical specimens were tested alongside the World Organisation for Animal Health (WOAH) real-time PCR method, a kappa coefficient of 0.94 was achieved. These outcomes verify that this real-time RPA assay is both sensitive and highly specific, offering a practical tool for LSDV diagnosis and surveillance in China.

Keywords: Lumpy skin disease, LSDV, Recombinase polymerase amplification, Targeted detection, Cattle

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Introduction

Lumpy skin disease (LSD) is a transboundary viral illness in cattle caused by the lumpy skin disease virus (LSDV), which possesses a double-stranded DNA genome roughly 151 kb in length. LSDV belongs to the Capripoxvirus genus within the Poxviridae family, sharing close kinship with goat pox virus (GTPV) and sheep pox virus (SPPV) [1]. Although these three capripoxviruses are genetically similar, small variations in their sequences account for differences in pathogenic behavior and host specificity—particularly involving the ORF132 gene, which is unique to LSDV and GTPV [2, 3].

In natural infections, LSDV affects cattle and buffalo, resulting in fever, nodular skin eruptions, swollen lymph nodes, and lesions across internal organs. Additional outcomes include reduced milk yields and fertility issues in breeding bulls [4, 5]. The disease was first identified in Africa [6] and has since expanded due to global trade, spreading into the Middle East, Europe, and Central Asia [7, 8]. In recent years, outbreaks have reached East and South Asia, including China [9], India [10], Bangladesh [11], Thailand [12], and Vietnam [13], causing serious economic disruption to livestock production [14, 15].

The main control measure for LSDV is vaccination, using either heterologous formulations based on goat or sheep pox viruses or homologous LSDV strains such as Neethling and KSGP O240 [16]. Owing to biosafety

considerations, China's Ministry of Agriculture and Rural Affairs authorizes only the heterologous attenuated goat pox AV41 vaccine for susceptible herds. While the live vaccine effectively limits disease spread [17], replication of the vaccine strain can occasionally induce side effects in inoculated animals [18, 19]. Therefore, it is essential to develop a detection technique that can clearly separate LSDV infections from GTPV vaccine responses.

Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification process that functions through three main enzymes: recombinase (facilitating primer–template alignment), DNA polymerase (extending target DNA), and single-stranded DNA-binding protein (stabilizing intermediates). Unlike PCR, RPA operates under constant temperature conditions (37–42 °C) and can be completed within 30 minutes using simple heating devices. When coupled with a fluorescent probe, the assay becomes real-time RPA, allowing continuous monitoring of the amplification [20].

This study introduces a real-time RPA protocol for LSDV detection and compares its performance with the WOAH-recommended real-time PCR. The proposed assay offers a faster and more field-adaptable alternative without compromising accuracy. Importantly, it allows precise differentiation between LSDV field isolates and GTPV vaccine strains, supporting improved disease surveillance and control strategies.

Materials and Methods

Viral and bacterial isolates, and field samples

The laboratory maintained stocks of goat pox virus (GTPV, AV41 strain), infectious bovine rhinotracheitis virus (IBRV, LY strain), and Pasteurella multocida (HY strain). Thirty-two confirmed lumpy skin disease (LSD)–positive field samples—including EDTA blood, nasal and oral swabs, and nodular skin tissues—were provided by the Guangdong Provincial Center for Animal Disease Prevention and Control. Additionally, eleven LSD-negative samples and normal bovine tissue specimens were collected from multiple farms and kept at −20 °C until testing.

Extraction of viral DNA

DNA was purified from the collected specimens using the QIAamp DNA Mini Kit (Qiagen, Dusseldorf, Germany) according to the supplier's recommendations. The extracted DNA was immediately frozen and held at −80 °C until further use.

Design of primers and probe

The ORF132 region of the LSDV genome (GenBank accession no. MH646674; positions 119,870–120,400) was downloaded and aligned with the GTPV AV41 vaccine strain using DNAMAN software (version 7.0.2.176). Based on the conserved and variable regions, a primer–probe set **(Table 1)** capable of distinguishing LSDV from the AV41 strain was created following the TwistAmp exo kit manual (TwistDX, Cambridge, UK). Primer-BLAST on NCBI was used to confirm target specificity. All oligonucleotides were produced by Sangon Biotech Co., Ltd. (Shanghai, China).

Table 1. Primer and probe sequences employed in the RPA assay.

Primer/Probe	Sequence (5′–3′)	Amplicon Size (bp)
LSD-F	TATAMTGGTCTATTTTTAACTTTTTTATGCAAT	176
LSD-R	GAYAACAACACTTTTTCCTTATCTAAAGAGYC	
LSD-Pro	TAATTCACTTTTAACTTTTTTATTAT/i6FAMdT/A/idSp/A/iBHQ1dT/C CATCGATACATGTA-C3 Spacer	

Preparation of standard DNA template

The ORF132 gene fragment from LSDV was cloned into the plasmid pUC57-ORF132, synthesized by Sangon Biotech Co., Ltd. The plasmid DNA concentration was quantified with a NanoDrop Lite spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Copy numbers were estimated using the following equation:

$$Number\ of\ copies \text{ per } \mu L = \frac{c \times 6.022 \times 10^{23}}{n \times 660} \quad (1)$$

where c = plasmid concentration (g/µL) and n = total base pairs of the pUC57-ORF132 molecule.

Real-time RPA procedure

Each recombinase polymerase amplification (RPA) reaction was performed in a 50 µL total volume using the TwistAmp™ exo lyophilized kit (TwistDX, Cambridge, UK). The mixture contained 29.5 µL of rehydration buffer, 1.5 µL of each primer (10 µM), 1 µL of probe (10 µM), 5 µL of DNA sample, 2.5 µL of magnesium acetate (280 mM), and 9 µL of nuclease-free water. Ultrapure water substituted for DNA in the negative control. Reactions were run in an MA-1600 isothermal fluorescence PCR analyzer (Molarray, Suzhou, China) at 39 °C for 20 min using the 6-carboxy-fluorescein (FAM) channel. Threshold time (TT) was determined automatically when fluorescence intensity exceeded the baseline level.

Analytical sensitivity and specificity

Sensitivity assessment employed serial 10-fold dilutions of pUC57-ORF132 plasmid (10^3, 10^2, 10, and 1 copies/µL), tested in triplicate. The preliminary detection limit corresponded to the lowest dilution where all replicates produced detectable TT values. Additional plasmid concentrations near this range were tested 20 times to confirm the limit of detection (LOD), defined as the level yielding ≥95% positive results.

Specificity was verified by testing nucleic acids extracted from GTPV, IBRV, P. multocida, and normal bovine tissues. Both positive and negative controls were included in every run.

Validation with clinical specimens

The diagnostic capability of the RPA method was validated using 43 clinical specimens. For reference, all samples were simultaneously analyzed with the WOAH-approved real-time PCR assay [21]. The RPA results were compared to PCR outcomes to calculate diagnostic sensitivity and specificity. Agreement between both assays was evaluated through kappa statistics and p-values using SPSS version 22 (SPSS Inc., Chicago, IL, USA), following established methods [22, 23]. A p-value < 0.05 was interpreted as statistically significant.

Results and Discussion

Primer and probe optimization

Alignment analysis revealed that the LSDV forward primer (LSD-F) differed from the GTPV AV41 strain by five nucleotides, including a four-base deletion (TTTT). The reverse primer (LSD-R) and probe (LSD-Pro) showed three and seven base mismatches, respectively, when compared to the vaccine strain **(Figure 1)**.

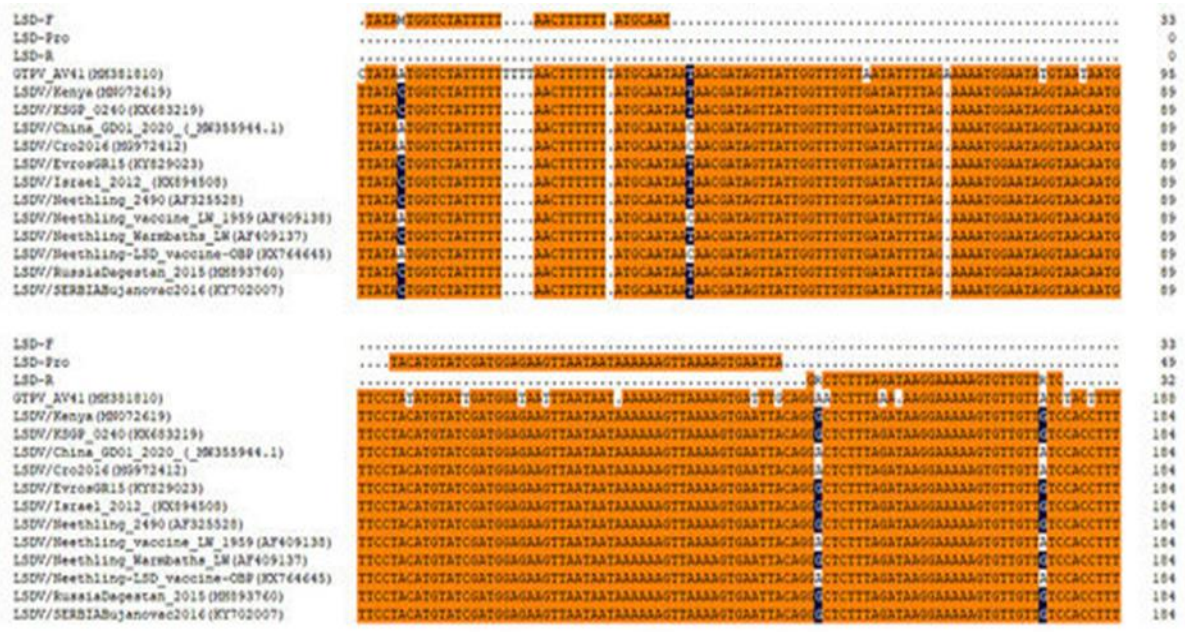
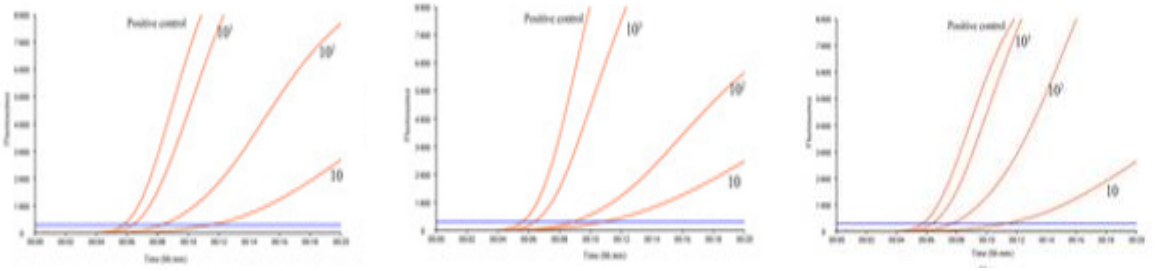


Figure 1. Alignment of the real-time RPA assay target sequences compared with the GTPV AV41 vaccine strain and multiple LSDV field isolates.

Evaluation of analytical sensitivity and specificity

To determine the detection threshold, a serial dilution of the recombinant plasmid pUC57-ORF132 was used. Dilution levels of 10^3 copies/µL, 10^2 copies/µL, and 10 copies/µL all yielded consistent positive signals across triplicate tests, while 1 copy/µL produced no amplification **(Figure 2)**. Consequently, the preliminary detection limit was defined as 10 copies/µL.



a) b) c)

Figure 2. Sensitivity assessment of the real-time RPA assay. Tenfold serial dilutions of the pUC57-ORF132 plasmid, ranging from 10^3 to 10^0 copies/µL, served as templates to determine the detection limit. Panels (a–c) illustrate the three independent replicates.

To confirm this value, additional gradient concentrations of 15, 10, 5, and 1 copies/µL were tested twenty times each. At concentrations of 15 copies/µL and 10 copies/µL, positive amplification occurred in 100% and 90% of replicates, respectively, while 5 copies/µL yielded 50% positivity and 1 copy/µL produced no detectable signal **(Table 2)**. Thus, the final limit of detection (LOD) for the assay was determined to be 15 copies/µL.

Table 2. Evaluation of the minimum detection limit.

Replicate	15 Copies/µL		10 Copies/µL		5 Copies/µL		1 Copy/µL	
	TT (min)	Result	TT (min)	Result	TT (min)	Result	TT (min)	Result
1	00:11:04	+	00:14:42	+	00:17:44	+	Unde	-
2	00:10:16	+	00:14:46	+	00:17:58	+	Unde	-
3	00:10:31	+	00:17:19	+	00:18:13	+	Unde	-
4	00:13:16	+	00:14:26	+	00:16:38	+	Unde	-
5	00:09:51	+	00:15:21	+	00:15:38	+	Unde	-
6	00:09:47	+	00:14:52	+	Unde	-	Unde	-
7	00:10:37	+	00:15:35	+	00:15:56	+	Unde	-
8	00:08:43	+	00:13:37	+	Unde	-	Unde	-
9	00:09:27	+	00:11:25	+	Unde	-	Unde	-
10	00:08:47	+	00:14:24	+	Unde	-	Unde	-
11	00:08:25	+	00:12:44	+	Unde	-	Unde	-
12	00:11:27	+	Unde	-	Unde	-	Unde	-
13	00:08:51	+	00:14:40	+	00:18:01	+	Unde	-
14	00:10:05	+	00:12:28	+	00:17: 12	+	Unde	-
15	00:08:57	+	00:14:00	+	00:17:34	+	Unde	-
16	00:09:17	+	Unde	-	00:16:33	+	Unde	-
17	00:08:18	+	00:09:04	+	Unde	-	Unde	-
18	00:08:17	+	00:14:28	+	Unde	-	Unde	-
19	00:09:03	+	00:12:02	+	Unde	-	Unde	-

20	00:10:44	+	00:13:48	+	Unde	-	Unde	-

Note: "Unde" indicates no fluorescence signal, "+" represents positive amplification, and "–" indicates a negative result.

For analytical specificity, only LSDV nucleic acids produced fluorescence signals, whereas all other viral and bacterial controls—including GTPV, IBRV, Pasteurella multocida, and healthy bovine tissue—tested negative. These findings confirm that the RPA system specifically detects LSDV without cross-reaction **(Figure 3)**.

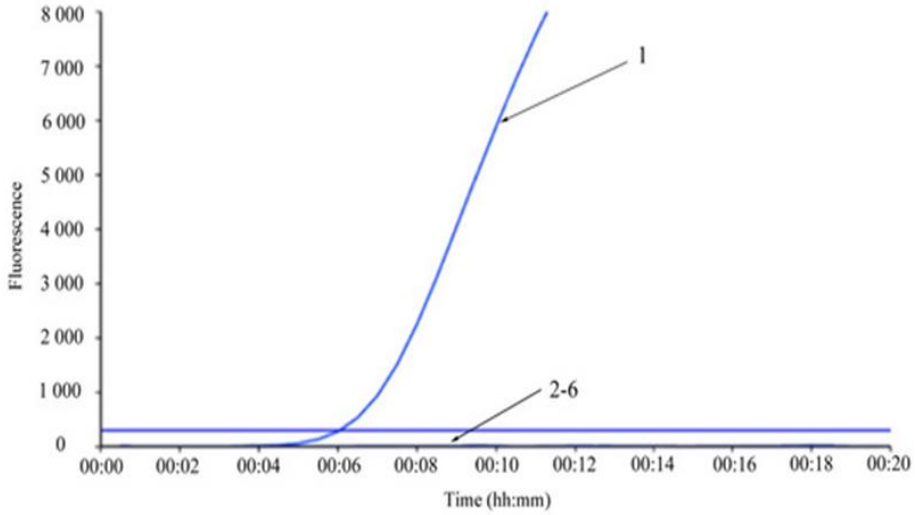


Figure 3. Specificity verification of the real-time RPA assay. Lane 1: LSDV-positive template; lanes 2–6: GTPV, IBRV, P. multocida, healthy bovine tissue, and nuclease-free water (negative control).

Comparison between the real-time RPA assay and WOAH real-time PCR

A total of 43 clinical specimens were analyzed simultaneously using the established RPA assay and the WOAH-endorsed real-time PCR. The RPA test identified 31 positives and 12 negatives, whereas PCR detected 32 positives and 11 negatives. When the WOAH PCR served as the reference, the RPA method achieved diagnostic sensitivity and specificity rates of 96.88% and 100%, respectively **(Table 3)**. The calculated kappa coefficient was 0.94, indicating a strong correlation between both assays, and the p-value (6.4349×10^{-10}) confirmed a statistically significant agreement. These results support the use of the RPA assay for field diagnosis of LSDV.

Table 3. Comparative analysis of 43 clinical samples tested by the real-time RPA assay and the WOAH PCR method.

WOAH Reference Method	Real-Time RPA Positive	Real-Time RPA Negative	Total Samples	Diagnostic Sensitivity (%)	Diagnostic Specificity (%)	Kappa Coefficient
Positive	31	1	32	96.88	100.00	0.94
Negative	0	11	11			
Overall Total	31	12	43			

Note: Diagnostic sensitivity = true positives ÷ (true positives + false negatives); diagnostic specificity = true negatives ÷ (false positives + true negatives).

Lumpy skin disease virus (LSDV) remains a significant poxvirus affecting cattle, particularly across Asian regions where outbreaks are increasingly common [24]. Developing diagnostic assays that can clearly distinguish between LSDV, SPPV, and GTPV—especially field LSDV isolates and vaccine strains—is a high priority.

Over the past decade, multiple molecular approaches have been introduced for LSDV detection. In 2016, an RPA-based real-time assay targeting the GPCR gene was reported [25]. Later, a combined RPA–CRISPR-Cas12a fluorescent detection system focused on the ORF068 gene demonstrated excellent sensitivity (5 copies/μL) with no cross-reactivity to common bovine pathogens [26]. Nonetheless, such assays simultaneously detect SPPV and GTPV, limiting their application in China, where the GTPV AV41 vaccine is widely used in cattle herds.

To overcome this limitation, a real-time high-resolution melting (HRM) PCR method was developed in 2018 to

differentiate field isolates from vaccine strains of LSDV [27]. Additionally, various quantitative real-time RT-PCR assays have been optimized for the same purpose [28, 29]. While these approaches can successfully discriminate between wild-type and vaccine-derived LSDV, they require sophisticated qPCR instruments, costly reagents, and complex protocols. Although effective for regions utilizing homologous LSDV vaccines, these techniques are impractical for China's current vaccination framework, which relies on the heterologous GTPV AV41 strain.

Recently, TaqMan-based systems enabling differentiation between LSDV and GTPV have been reported [30, 31]. Compared with probe-dependent real-time PCR assays, the real-time RPA technique requires simpler, lower-cost instrumentation and significantly reduces reaction duration, thereby accelerating the diagnostic workflow. Consequently, for laboratories or cattle farms that lack advanced thermal cyclers or face budget limitations, the real-time RPA platform represents a feasible and economical alternative.

In this research, we established a novel real-time RPA assay specifically targeting LSDV DNA. The primers and probes were designed based on the ORF132 gene of LSDV, which contains characteristic deletions and nucleotide variations absent in the GTPV AV41 vaccine strain, allowing for selective identification of LSDV field isolates, including recombinant variants currently circulating in China. This fluorescence-probe RPA system enables rapid DNA amplification at a constant temperature of 39 °C, with detection completed in under 20 minutes, making the process both efficient and straightforward.

The assay achieved high analytical sensitivity, with a limit of detection (LOD) of 15 copies/μL for LSDV. No cross-reactivity was observed with GTPV, IBRV, Pasteurella multocida, or healthy bovine tissue samples, confirming its strong specificity. A total of 43 clinical specimens were evaluated using this RPA assay and the WOAH-endorsed real-time PCR, showing excellent concordance between both methods. Since there are no officially approved commercial antibody-based kits for LSDV in China, comparative serological testing could not be performed.

Overall, the developed real-time RPA detection approach demonstrated outstanding performance for the specific and sensitive identification of LSDV, offering advantages such as simple operation, minimal equipment requirements, and rapid turnaround. It holds great promise for routine surveillance and diagnostic applications of LSDV in China.

Conclusion

In conclusion, this study successfully developed a real-time RPA amplification assay designed for the precise detection of lumpy skin disease virus (LSDV). The method exhibits broad applicability in field and laboratory diagnostics, providing a valuable tool for enhanced disease monitoring and control efforts within China.

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Conflict of Interest: None

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Ethics Statement: None

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