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RESEARCH ARTICLE

Development and Application of TaqMan qPCR Assays for Detecting Monkeypox Virus, Lumpy Skin Disease Virus, Goatpox Virus, Fowlpox Virus and Swinepox Virus in the Border Areas of Yunnan Province, China

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A B S T R A C T

The re-emergence and spread of animal poxviruses pose a significant threat to global livestock economies and public health, highlighting the need for high-precision diagnostic and surveillance tools. In this study, we developed a panel of five independent TaqMan qPCR assays utilizing adenovirus-packaged pseudovirus standards for the detection of five major animal poxviruses: monkeypox virus (MPXV), lumpy skin disease virus (LSDV), goatpox virus (GTPV), fowlpox virus (FWPV), and swinepox virus (SWPV). The constructed pseudovirus standards demonstrated good stability, with coefficients of variation (CV) below 3.46%. All five assays showed high specificity and sensitivity. The assay was further evaluated through a large-scale epidemiological survey of clinical samples collected from the transboundary high-risk border areas of Yunnan Province, China. The surveillance revealed a baseline prevalence of 13.05% for LSDV in cattle and 2.84% for SWPV in pigs. This newly established qPCR assay offers a highly sensitive, specific, and biologically safe diagnostic toolset, which may serve as a potentially useful tool for poxvirus active surveillance and transboundary animal disease control.

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INTRODUCTION

The *Poxviridae* family encompasses a variety of significant pathogens, including monkeypox virus (MPXV), lumpy skin disease virus (LSDV), goatpox virus (GTPV), fowlpox virus (FWPV), and swinepox virus (SWPV), which can infect a broad range of livestock and poultry (Safini *et al*., 2021). Animal poxviruses can induce severe clinical manifestations in livestock, such as high fever, decreased milk production, abortion, and mortality, thereby causing substantial economic losses to the animal husbandry industry. In addition, monkeypox virus (MPXV) has attracted considerable public health concern due to its capacity to trigger outbreaks in human populations (Sprygin *et al*., 2018; Donofrio *et al*., 2021; Safini *et al*., 2021). The global epidemiological landscape of poxviruses has evolved significantly in recent years, posing unprecedented threats to both animal agriculture and public health(Tahir et al., 2025; Hossain et al., 2025).

Since May 2022, a global outbreak of clade IIb MPXV has spread to over 118 countries, with more than 97,000 confirmed cases reported worldwide as of June 2024 (Prompetchara *et al*., 2024). The World Health Organization (WHO) declared mpox a Public Health Emergency of International Concern (PHEIC) twice—first in July 2022 and again in August 2024 (Vakaniaki *et al*., 2024; Ahmed and Islam, 2025). The zoonotic nature of MPXV significantly complicates containment efforts; although its primary reservoir remains unidentified (Moss, 2024), the virus has been isolated from African squirrels, and its antigens or antibodies have been detected in various species, including wild rodents, shrews and pigs (Khodakevich *et al*., 1988; Reynolds *et al*., 2010; Salzer *et al*., 2013; Orba *et al*., 2015; Doty *et al*., 2017), highlighting its broad cross-species transmission potential (Prompetchara *et al*., 2024). Concurrently, animal-specific poxviruses have emerged as major transboundary threats. Lumpy skin disease has emerged as a major transboundary animal disease affecting cattle populations globally. Since 2019, LSD has rapidly expanded across Asia, with major outbreaks reported in Bangladesh, India, China, and

subsequently in multiple Southeast Asian countries (Lu *et al*., 2021; Uddin *et al*., 2022; Nugroho *et al*., 2025). By 2023, over 1,385 LSD outbreaks were recorded across South, East, and Southeast Asia, causing estimated economic losses of approximately 1.45 billion dollars (Lu *et al*., 2021; Nugroho *et al*., 2025). In China, LSDV was first detected in Xinjiang in August 2019 and has since spread to multiple provinces (Nugroho *et al*., 2025). According to the World Organisation for Animal Health (WOAH), LSDV remains widespread across Africa, Asia, and Europe. The virus's exceptional environmental resilience—particularly its resistance to heat and desiccation—facilitates its persistent transmission and severely disrupts international livestock trade (Lubinga *et al*., 2013). Goat pox, a deadly goat disease with up to 100% mortality, is prevalent in Africa, the Middle East, and Asia (Roy *et al*., 2018)**Error! Reference source not found.**. Swinepox, first identified in 1842 (Kasza *et al*., 1960), is now a global swine disease. Similarly, FWPV remains a highly contagious threat to poultry (Zeng *et al*., 2016; Hamdi *et al*., 2021). The clinical management of FWPV is further complicated by the integration of the avian reticuloendotheliosis virus into most FWPV genomes, which exacerbates pathogenesis and challenges routine detection (Hamdi *et al*., 2021). Collectively, the expanding host ranges, environmental persistence, and cross-border spread of these diverse poxviruses underscore an urgent need for robust, multiplex diagnostic strategies.

Currently, multiple diagnostic approaches are available for poxviruses, including etiological, serological, and molecular detection methods. Although virus isolation remains the classical gold standard owing to its definitive specificity, its labor-intensive nature and prolonged turnaround times severely restrict routine high-throughput screening(Lin et al., 2024). Electron microscopy can identify poxvirus particles morphologically but requires expensive equipment and highly trained staff. Serological tests such as ELISA can characterize immune responses but suffer from limitations such as high false-positive rates and long processing times. Among modern approaches, PCR and quantitative real-time PCR (qPCR) technologies, known for their speed and accuracy, have become the core methods in laboratory diagnostics. TaqMan real-time qPCR is characterized by high sensitivity and multiplexing capability, enabling parallel screening of multiple poxviruses and effective discrimination among closely related strains (Wang *et al*., 2023), thereby demonstrating broad applicability in veterinary disease diagnostics.

Although single-plex qPCR assays have been reported for each of the five poxviruses, a standardized detection panel that unifies the reaction conditions, reagent formulation, and testing workflow for individual screening of all five viruses—and that has been rigorously validated through large-scale field surveillance in high-risk transboundary regions—has not yet been described. To address this gap, we developed a TaqMan qPCR system that employs adenovirus-packaged pseudoviruses as reference standards, enabling a parallel processing format for separate detection of each of the five animal poxviruses. This novel assay combines high throughput, high specificity, and high sensitivity, ensuring accurate viral identification in a practical and reproducible manner. Moreover, substituting conventional live virus controls with biologically secure pseudovirus standards substantially mitigates biosafety risks while enhancing assay stability and reproducibility. The system is also operationally convenient, making it suitable for routine monitoring at resource-limited border sentinel posts. Collectively, the establishment of this system provides critical technical support for epidemiological surveillance and the implementation of prevention and control measures against poxviruses, while effectively filling the gap in poxvirus monitoring data in the China–Southeast Asia border region.

MATERIALS AND METHODS

Experimental materials: Key reagents included a viral DNA/RNA extraction kit (Tianlong Technology), AceQ® Universal U+ Probe Master Mix V2 (Vazyme), 2×TransTaq High Fidelity PCR SuperMix I/II (TransGen Biotech), T4 DNA ligase and restriction endonucleases (Thermo Scientific), a gel extraction kit and a plasmid miniprep kit (Tiangen), as well as DMEM medium (Gibco) and fetal bovine serum. Primers and TaqMan probes (with 5′-FAM and 3′-MGB modifications) were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The cell line used in this study was human embryonic kidney 293T cells (ATCC CRL-3216). Cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100U/mL penicillin and 100μg/mL streptomycin, at 37°C in a 5% CO_2 atmosphere. Cells were passaged every 2–3 days when they reached 80–90% confluence. The plasmids and vectors employed included the universal expression vector PUC-SP (constructed by the Infectious Disease Laboratory of Yunnan Agricultural University), the adenovirus shuttle vector pac-Ad5 K-N pA and the adenovirus backbone vector pac-Ad5 9.2-100 (kindly provided by Zhongkai University of Agriculture and Engineering).

Construction of Pseudovirus standards: The *GFP* gene (1005 bp) was amplified from the PUC-SP plasmid and, after double digestion with *Kpn* I and *Xho* I, cloned into the pMD18-T vector. Following sequence verification of the resulting plasmid, the GFP fragment was subcloned into the adenovirus shuttle vector pacAd5 K-N pA to gene of fowlpox virus (FWPV) and the *P4a* gene of lumpy skin disease virus (LSDV) (Table 1). Each target gene was amplified from the corresponding poxvirus reference strain (or synthetic plasmid) and cloned into pacAd5 K-N pA-GFP via Xho I and Not I restriction sites, yielding five recombinant shuttle plasmids: pacAd5 K-N pA-GFP-F3L, -C14L, -J3R, -P4b and -P4a. The sequences and reading orientations of all inserted fragments were confirmed by sequencing.

To generate pseudoviruses, each recombinant shuttle plasmid was co-transfected into 293T cells together with the adenovirus backbone plasmid pacAd5 9.2-100. A transfection mixture containing 2μg of shuttle plasmid and 1μg of backbone plasmid was combined with 6μL of Lipofectamine 3000 in 200μL of Opti-MEM, incubated at room temperature for 20 min and then added to 293T cells

at 70% confluence. After 6h, the medium was replaced with DMEM supplemented with 2% FBS. Cytopathic effects (CPE) were observed daily, and the proportion of GFP-positive cells was recorded to evaluate packaging efficiency. On day 7 post-transfection, the samples were harvested, and viral supernatants were obtained after three freeze-thaw cycles followed by centrifugation at 3,000×g for 10 min at 4°C. Subsequently, viral titers were quantified by qPCR.

Establishment of the Poxvirus detection assay: TaqMan probes (5′-FAM, 3′-MGB modified; see Table 2) were designed for each target gene. Primer and probe concentrations were systematically optimized using a matrix design in a 20μL reaction volume, testing six equimolar settings from 1.5 to 4.0pmol with intervals of 0.5pmol. The ratio that yielded the lowest Ct value, the highest fluorescence signal and no primer-dimer formation was selected for subsequent experiments.

The qPCR reactions were performed in a total volume of 20μL, containing AceQ® Universal U+ Probe Master Mix V2, primer/probe mixture, and template (Table 3). The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15s and 55–59°C for 30s (the annealing temperature was optimized via gradient PCR). Standard curves were generated using ten-fold serial dilutions of plasmid standards ranging from 10^4 to 10^{11} copies/μL.

The assay was subsequently validated in terms of sensitivity, specificity, and repeatability. For sensitivity assessment, ten-fold serial dilutions of each pseudovirus standard (ranging from 10^1 to 10^9 copies/μL) were tested in replicate; the limit of detection (LOD) was defined as the lowest concentration that consistently yielded positive results (Ct≤35). Specificity was evaluated by testing nucleic acids from relevant and non-relevant pathogens, including GTPV, LSDV, FWPV, PRV, ENTV-2, varicella-zoster virus (VZV), *Escherichia coli* and Salmonella spp., to assess cross-reactivity. Repeatability was examined by analyzing high, medium and low-concentration standards within a single run (10 replicates) and across three independent runs on different days. Precision was expressed as the coefficient of variation (CV), calculated as CV (%) = (standard deviation / mean) × 100%, with a CV<5% considered acceptable.

Sample Collection: Samples (n=3,996) were collected from 25 border counties across five prefecture-level cities (Lincang, Pu'er, Xishuangbanna, Honghe, Wenshan) in Yunnan, adjacent to Myanmar, Laos, and Vietnam. A combined random and risk-based targeted sampling strategy prioritized areas with high livestock density, prior disease outbreaks, or active cross-border animal movements. Sampled species included cattle, pigs, goats, and chickens.

Samples were placed in PBS. Tissues were frozen in liquid nitrogen, ground, centrifuged (12,000×g, 4°C, 5 min), and supernatants used for nucleic acid extraction. Throat swabs/oral fluids were resuspended in PBS and extracted with a commercial kit. Each batch included positive (pseudovirus) and negative (DEPC-water) controls.

Results: Ct≤35 = positive; 35<Ct<40 = retest; Ct≥40 = negative. Suspicious samples (Ct 35–40, atypical curves, or from outbreak areas) were confirmed by Sanger sequencing using the same qPCR primers; ≥95% identity to reference sequence was considered positive (Fig. 1).

RESULTS

Preparation of Pseudoviruses for five Poxviruses: In this study, a 1005 bp GFP reporter gene fragment was obtained by PCR amplification. Agarose gel electrophoresis analysis confirmed that the band size was consistent with the expected size. Double digestion of the cloning plasmid pMD-18T-GFP with *Kpn* I and *Xho* I yielded two fragments of 2692 bp and 1005 bp. Sequence alignment verified the integrity of the GFP gene sequence.

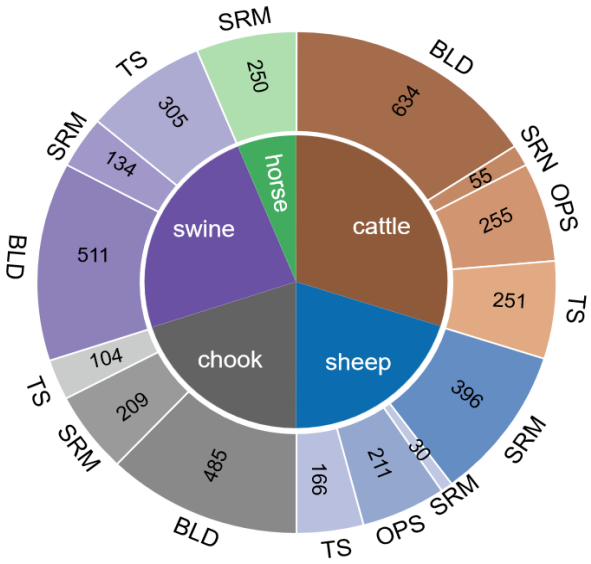

Fig. 1: Statistical chart of samples collected in Yunnan Province. SRM (Serum samples), BLD (Blood samples), SWB (Swab samples), and TIS (Tissue samples).

Table 1: Primer sequences used in the current study

Primer name	Nucleotide sequence (5'-3')	gene	length (bp)	Reference sequence
HD-1F	CCCTCGAGATGGAGAAGCGAGAAGT	F3L	480	PP093698.1
HD-1R	TTGCGGCCGCTCAGAATCTAATGATGA			
NJJ-1F	CCCTCGAGATGATGCCCATTAACGC	P4a	1608	AF409137.1
NJJ-2R	ATCCCATTGCCGAAAAAAGGAGAATGGA			
YD-1F	CCCTCGAGATGGAAGCAGTATCTAT	J3R	1020	OR239060.1
YD-1R	TTGCGGCCGCTTACTTATTATAGCGTA			
JD-1F	CCCTCGAGATGGAATCTGATTCTAATAT	P4b	1992	M25781.1
JD-1R	TTGCGGCCGCTTAAACAAATATTTC			
ZD-1F	CCCTCGAGATGGAGCCTATTCTTC	C14L	843	L22013.1
ZD-1R	TTGCGGCCGCTTATATACATTGAGA			

GFP-F	GGTACCGAGGGCCTATTTCCCAT	GFP	1005	LN515608.1
GFP-R	CTCGAGATGCTCTACTAGTGATGG			

Table 2: TaqMan qPCR Primer Information for Animal Poxvirus

Virus	Primer name	Nucleotide sequence (5'-3')	Reference sequence)
MPXV	F3L-F	CTCATTGATTTTTCGCGGGATA	PP093698.1
	F3L-R	GACGATACTCCTCCTCGTTGGT	
	F3L- Probe	FAM-CATCAGAATCTGTAGGCCGT-MGB	
GTPV	J3R-F	GCTTCAACCATTTGCGCCTA	OR239060.1
	J3R-R	TCATCCTTTGTGATGCATCTAAGC	
	J3R- Probe	FAM-TGAGGTTAATAAGTATTTATAGC-MGB	
FWPV	P4b-F	TCAGCAGTTTGTTACAAGACA	M25781.1
	P4b-R	CCATTTCCGTGAATAGAATAGTAT	
	P4b- Probe	FAM-ATCTCCGCCGTCGCAACTTCCA-MGB	
LSDV	P4a-F	TCAGGAAAGACGGTATCAAAACT	AF409137.1
	P4a-R	TGGACGCTGGTCTTTTTGGA	
	P4a- Probe	FAM-CTGAAGTGTTAGAAAGTGTAAACTCT-MGB	
SWPV	C14L-F	TGGACGCTGGTCTTTTTGGA	L22013.1
	C14L-R	GCTGATTGGGCCAGAAAATG	
	C14L-Probe	FAM-TTCCCTCCACAGCTGCAAATGCTACT-MGB	

Table 3: TaqMan qPCR Detection System

Reagent Name	Volume
AceQ® Universal U+ Probe Master Mix V2	10.0μL
Forward primer	0.8μL
Reverse primer	0.8μL
Probe	0.8μL
Nuclease-Free Water	(20-x)μL
Template	xμL
Total	20μL

After insertion of the GFP gene into pac-Ad5 K-N pA, double enzyme digestion analysis revealed two bands at approximately 7000 bp and 1005 bp, confirming the successful construction of the recombinant plasmid pac-Ad5 K-N pA-GFP. Using species-specific primers, PCR amplification of the MPXV *F3L*, SWPV *C14L*, GTPV *J3R*, FWPV *P4b*, and LSDV *P4a* genes yielded fragments of 480 bp, 843 bp, 1020 bp, 1992 bp and 1608 bp, respectively. Following insertion of the target genes into pac-Ad5 K-N pA-GFP, sequencing analysis confirmed the correct integration of the exogenous gene fragments. Typical grape-like clusters of cytopathic effects (CPE) and GFP fluorescence appeared on day 5 (Fig. 2). The packaging efficiency, calculated based on the percentage of GFP-positive cells, ranged from 80 to 90%. After repeated freeze-thaw cycles and long-term storage, the intra- and inter-batch coefficients of variation for MPX, SWP, GTP, FWP, and LSD pseudoviruses were ≤1.66%, ≤2.28%, ≤3.46%, ≤2.12%, and ≤1.47%, respectively (Fig. 3), indicating excellent stability.

Establishment and evaluation of the TaqMan qPCR detection assay: Through gradient optimization, the optimal primer-to-probe ratio was determined to be 1:1 (Table 4 and Table 5). The final annealing temperatures for each detection system were established as follows: 59°C for MPXV, 59°C for SWPV, 55°C for GTPV, 57°C for FWPV, and 59°C for LSDV (Fig. 4).

Standard curves were constructed using ten-fold serial dilutions of the positive standards. The results showed that the amplification efficiencies ranged from 90 to 110%, with correlation coefficients (R^2) between 0.98 and 1.00, and slopes ranging from 3.58 to 3.10 (Fig. 5; Table 6). To evaluate the diagnostic performance of the TaqMan qPCR assay established in this study, parallel comparisons were conducted against the respective reference methods for each virus. For monkeypox virus (MPXV), the reference method was the MPXV Nucleic Acid Typing Detection Kit (Fluorescent PCR) recommended by the Africa Centres for Disease Control and Prevention and manufactured by Bioperfectus Technologies. For lumpy skin disease virus (LSDV), the reference was the Chinese national standard GB/T 39602-2020 was adopted. The clinical sample concordance rates were 99.67, 98.00, 93.75, 91.43 and 92.86%, respectively, indicating good agreement between the developed method and each reference method. Furthermore, sensitivity evaluation revealed that the limit of detection of the present method was one order of magnitude lower than that of each corresponding reference method (MPXV: 1.84 copies/μL vs. 10 copies/μL; LSDV: 3.07 copies/μL vs. 22.6 copies/μL; SWPV: 22.4 copies/μL vs. 100 copies/μL; GTPV: 1.34 copies/μL vs. 15 copies/μL; FWPV: 3.70 copies/μL vs. 625 copies/μL) (Fig. 6), demonstrating that the newly developed assay possesses superior detection sensitivity.

No cross-reactivity was observed between the established TaqMan qPCR assay and pseudorabies virus (PRV), enzootic nasal tumor virus type 2 (ENTV-2), varicella-zoster virus (VZV), *Escherichia coli*, or *Salmonella* spp. (Fig. 6). In the repeatability assays, the coefficients of variation for the MPXV, SWPV, GTPV, FWPV, and LSDV detection systems were ≤2.04%, ≤2.43%, ≤2.50%, ≤1.28%, and ≤1.33%, respectively (Fig. 6), demonstrating satisfactory stability and repeatability.

Investigation of Poxvirus infection status in Yunnan Province (China–Myanmar, China–Laos, and China–Vietnam Border Regions): All 3,996 samples tested for MPXV were negative (0.00%, 0/3996). A total of 950 swine samples tested for SWPV yielded an overall positivity rate of 2.84% (27/950). The 27 positive samples were distributed across 5 counties: Menglian County (Pu'er City) had the highest number with 8 positive samples (30.0% positivity rate), followed by Jiangcheng County (Pu'er City) with 6 positive samples (15.0% positivity rate), Xishuangbanna County with 5 positive samples (10.0% positivity rate), Honghe County with 4 positive samples (8.0% positivity rate), and Wenshan County with 4 positive samples (6.7% positivity rate). The remaining 10 counties had no SWPV-positive samples. Additionally, 1,195 bovine samples tested for LSDV showed a total positive rate of 13.05% (196/1,195). The 196 positive samples were

distributed across 7 counties: Gengma County (Lincang City) had the highest positivity rate at 58.54% (48/82 samples), followed by Cangyuan County (Lincang City) at 35.29% (36/102), Mengla County (Xishuangbanna) at

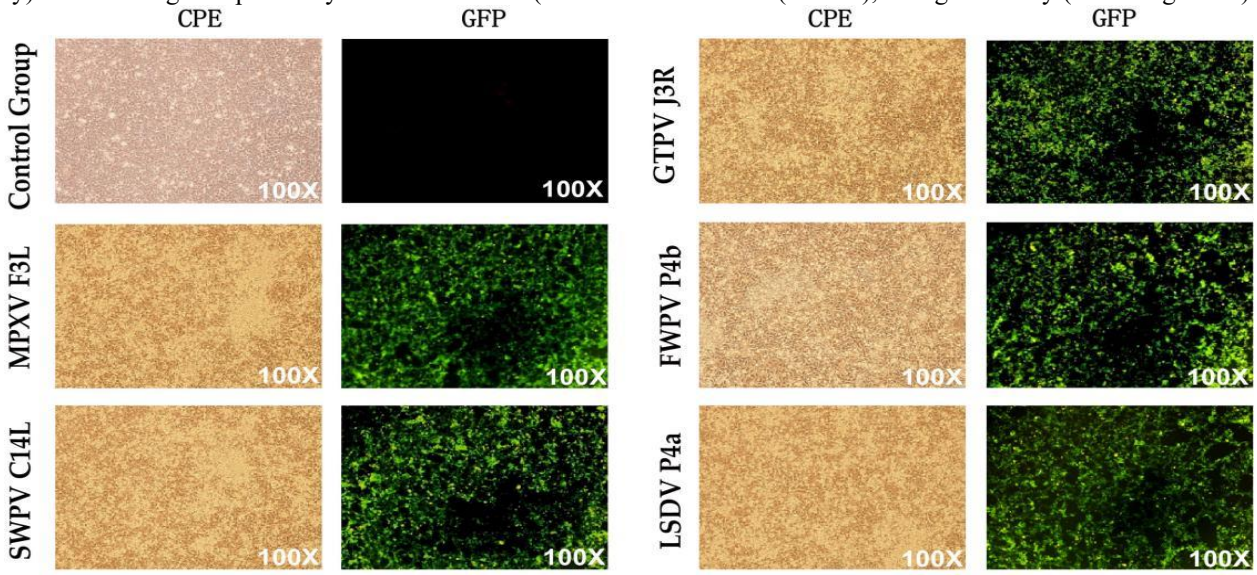

Fig. 2: CPE and GFP expression in pseudovirus packaging.

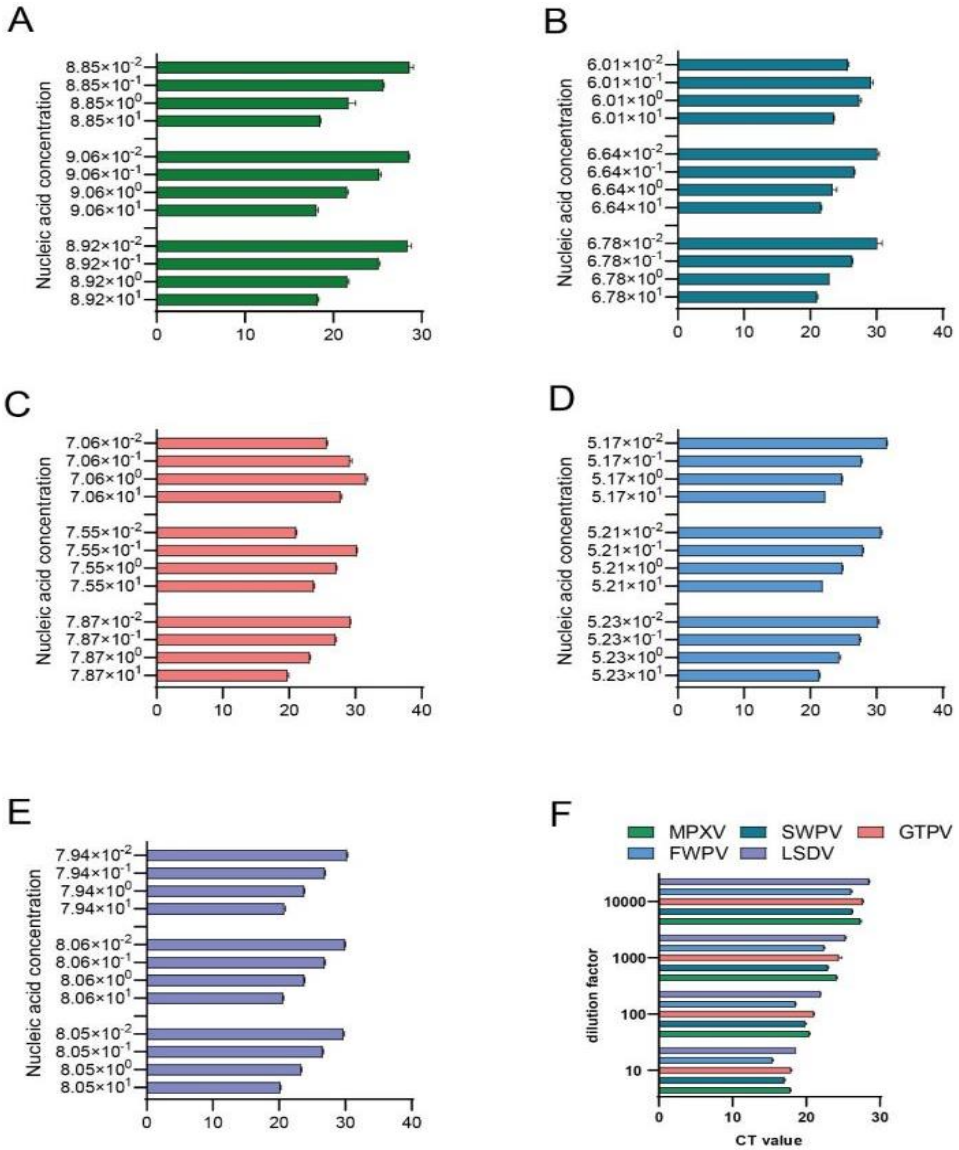

Fig. 3: Stability assay. A: MPX pseudovirus stability assay; B: SWP pseudovirus stability assay; C: GTP pseudovirus stability assay; D: FWP pseudovirus stability assay; E: LSD pseudovirus stability assay; The Y-axis represents the coefficient of variation (CV) percentage. The three groups on the X-axis represent: (1) Intra-batch variation (within the same batch); (2) Inter-batch variation (between different batches); (3) Long-term storage variation (after storage at -80°C for 6 months). F: Coefficient of Variation (CV).

Table 4: TaqMan qPCR assay system

Virus	Reagent name	Volume
MPXV/FWPV/LSDV	AceQ® Universal Probe Master Mix V2	U+10.0μL
	Forward Primer	0.8μL
	Reverse Primer	0.8μL
	Probe	0.8μL
	Nuclease-Free Water	5.6μL
	Template	2μL
	Total	20μL

18.75% (30/160), Jinghong County (Xishuangbanna) at 12.50% (20/160), Menghai County (Xishuangbanna) at 8.75% (14/160), Malipo County (Wenshan) at 6.67% (16/240), and Hekou County (Honghe) at 4.17% (10/240). The remaining 13 counties had no LSDV-positive samples. All 803 ovine samples and 798 poultry samples tested negative for GTPV and FWPV, also resulting in a detection rate of 0%. No infections of GTPV or FWPV have been detected in the border regions of Yunnan Province. This indicates that livestock poxvirus infections along China's borders with Myanmar, Laos, and Vietnam are primarily dominated by LSDV, followed by SWPV. Furthermore, no infections of MPXV have been identified in livestock within Yunnan Province during this study (Fig. 7).

Table 5: TaqMan qPCR assay system

Virus	Reagent name	Volume
SWPV/GTPV	AceQ® Universal U+ Probe Master Mix V2	10.0μL
	Forward Primer	0.6μL
	Reverse Primer	0.6μL
	Probe	0.6μL
	Nuclease-Free Water	6.2μL
	Template	2μL
	Total	20μL

Table 6: TaqMan qPCR assay system

Virus	Linear relationship equation	Amplification efficiency (E)	Correlation coefficient (R^2)	Y-intercept
MPXV	y = -3.32x + 50.969	8.3%	0.999	50.957
SWPV	y = -3.42x + 50.559	5.8%	1.000	50.545
GTPV	y = -3.32x + 47.499	9.9%	1.000	47.492
FWPV	y = -3.35x + 49.679	9.0%	0.999	49.670
LSDV	y = -3.33x + 47.949	9.3%	0.999	47.943

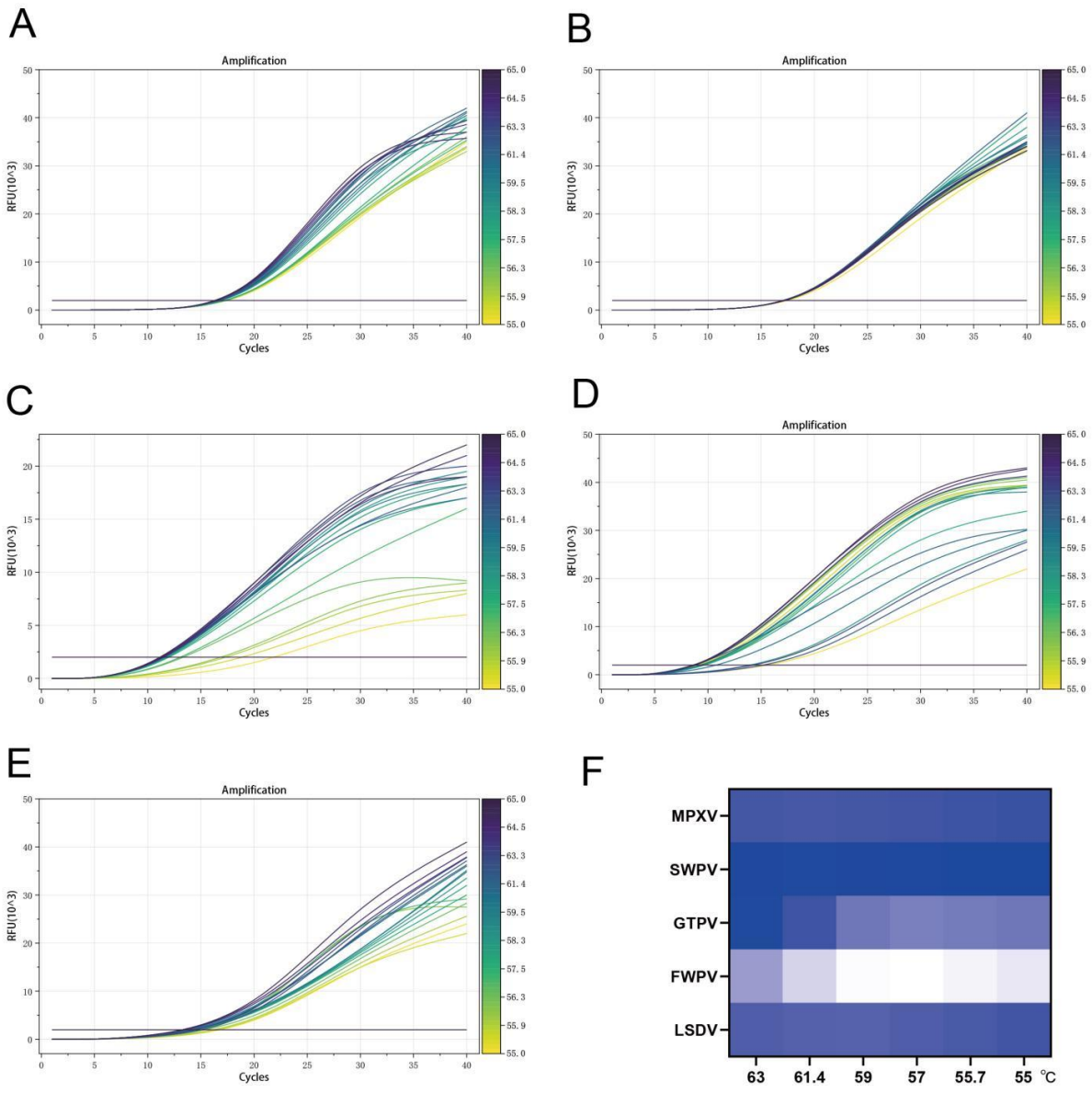

Fig. 4: Optimization of the temperature gradient. A: Sensitivity evaluation of the MPX detection assay; B: Sensitivity evaluation of the SWP detection assay; C: Sensitivity evaluation of the GTP detection assay; D: Sensitivity evaluation of the FWP detection assay; E: Sensitivity evaluation of the LSD detection assay; F: Optimal annealing temperature.

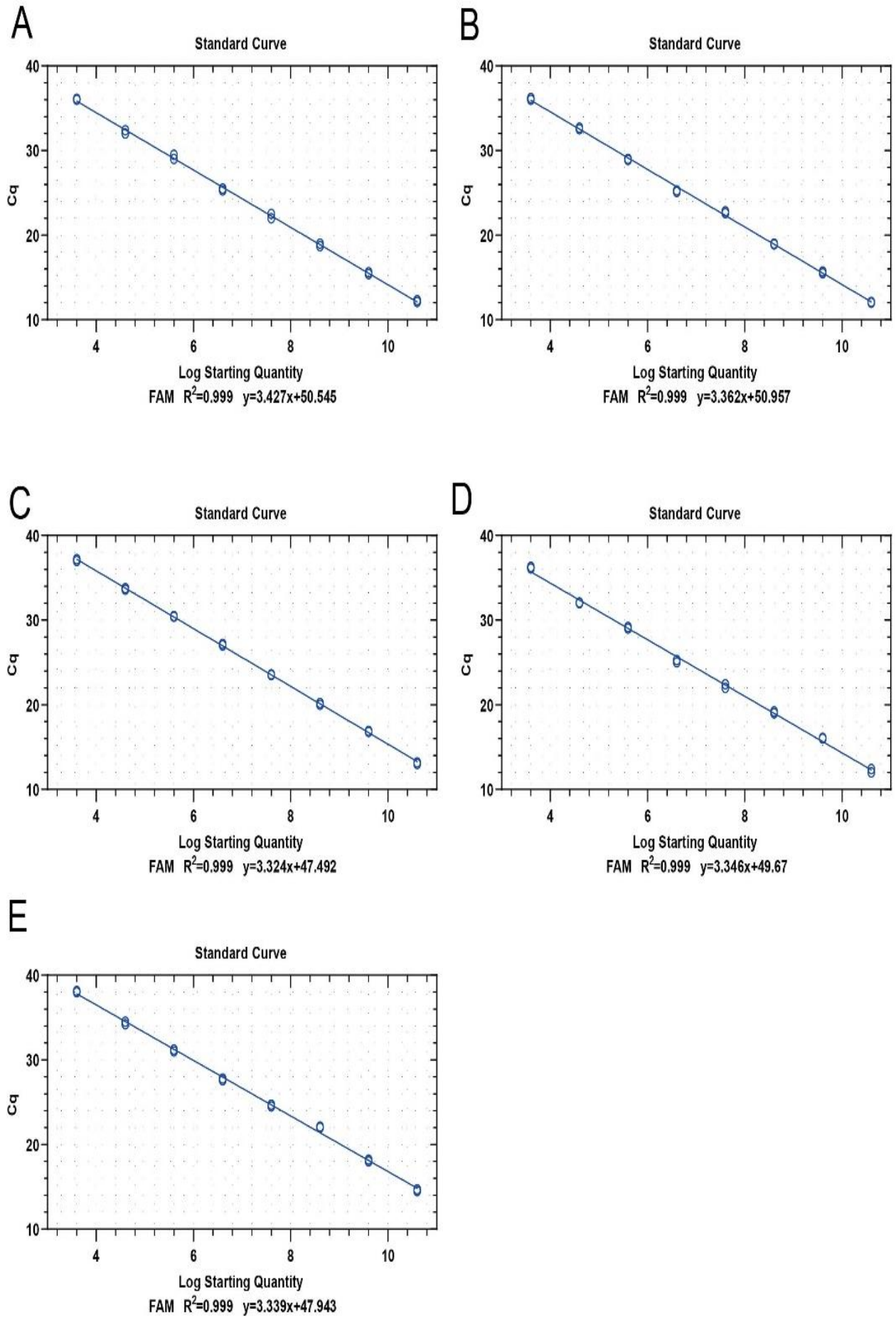

Fig. 5: Establishment of the standard curve for the detection method. A: Standard curve for MPX; B: Standard curve for SWP; C: Standard curve for GTP; D: Standard curve for FWP; E: Standard curve for LSD.

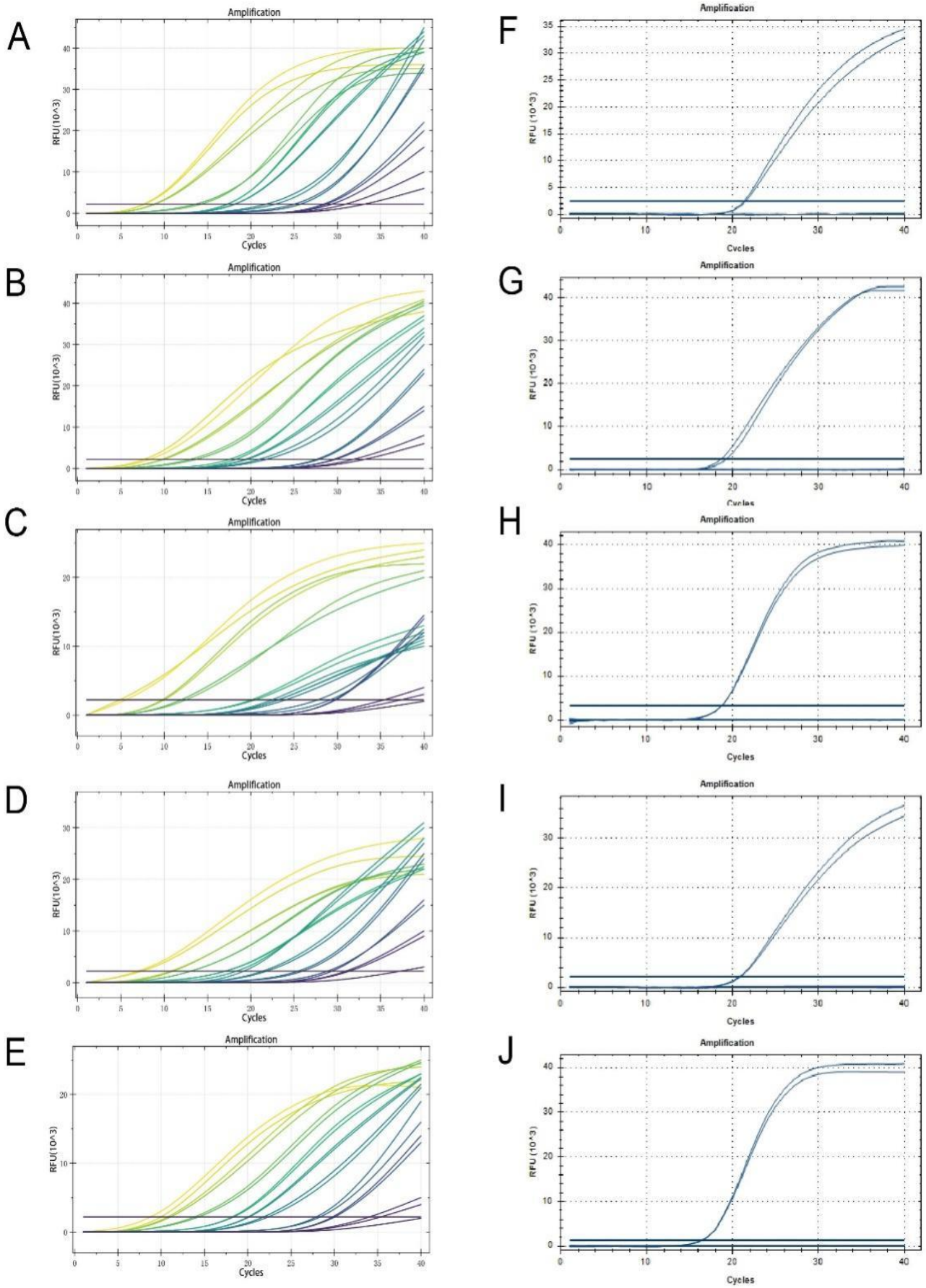

Fig. 6: Sensitivity and specificity testing. A: Sensitivity evaluation of the MPX detection assay; B: Sensitivity evaluation of the SWP detection assay; C: Sensitivity evaluation of the GTP detection assay; D: Sensitivity evaluation of the FWP detection assay; E: Sensitivity evaluation of the LSD detection assay; F: Specificity testing of the MPX detection assay; G: Specificity testing of the SWP detection assay; H: Specificity testing of the GTP detection assay; I: Specificity testing of the FWP detection assay; J: Specificity testing of the LSD detection assay.

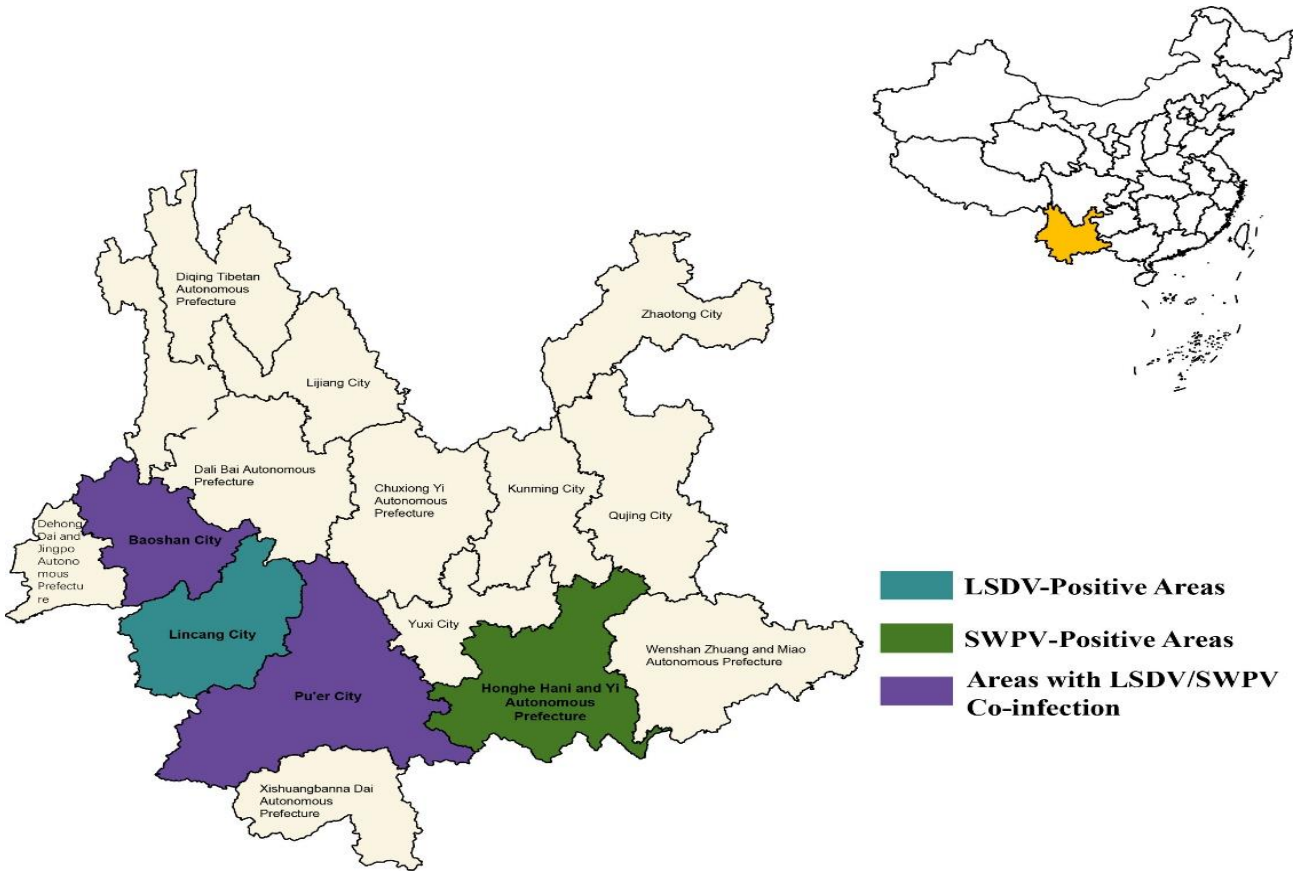

Fig. 7: Map of areas with positive Poxvirus detection results in Yunnan Province**.**

DISCUSSION

Compared with previously reported qPCR assays for poxviruses, our panel offers several distinct advantages. Kulesh *et al*. (2004) developed a tetraplex real-time PCR assay targeting pan-orthopoxvirus and MPXV-specific genes, but their system does not cover LSDV, GTPV, FWPV, or SWPV. Wang *et al.* (Wang *et al*., 2021) established a multiplex real-time PCR method for the simultaneous detection and differentiation of GTPV, SPPV, and LSDV, with a detection limit of 10^2 copies/μL. Nan *et al*. (2023) recently developed a triplex qPCR assay capable of distinguishing LSDV, GTPV, and SPPV in a single reaction. Wolff *et al*. (2021) established several duplex qPCR assays for reliable differentiation of the three capripoxvirus species. In addition, a Chinese patent (Primers, methods and kit for multiplex TaqMan-MGB probe real-time fluorescence quantitative PCR detection of capripoxviruses 2014) described a multiplex TaqMan-MGB probe real-time PCR method for LSDV, GTPV, and SPPV. However, none of these platforms simultaneously address all five poxviruses (MPXV, LSDV, GTPV, FWPV, and SWPV) that threaten cattle, pigs, goats, poultry, and humans in the transboundary context. Our five-assay panel not only expands the diagnostic spectrum but also achieves superior sensitivity (LOD: 1.84–22.4 copies/μL) compared to existing reference methods. Furthermore, the use of adenovirus-packaged pseudoviruses as positive controls represents a significant advancement over live virus or plasmid standards, offering enhanced biosafety, stability (CV≤3.46%), and visual quality control via GFP fluorescence. These features make our platform particularly suitable for routine surveillance in resource-limited border settings.

Compared with existing detection technologies, the TaqMan qPCR system established in this study offers notable advantages. The limit of detection ranges from 1.84 to 22.4 copies/μL, enabling effective identification of early-stage or low-viral-load infections, and rendering it suitable for large-scale screening and early warning in settings such as farms and live animal markets. Moreover, the assay employs highly conserved virus-specific target genes (MPXV *F3L*, SWPV *C14L*, GTPV *J3R*, FWPV *P4b*, and LSDV *P4a*), thereby circumventing the cross-reactivity issues commonly encountered in conventional serological methods. In addition, this study utilized pseudoviruses carrying a GFP reporter gene as positive reference standards. These pseudoviruses not only exhibit excellent biosafety and stability (coefficients of variation ≤ 3.46%) but also allow visual monitoring of packaging efficiency via GFP fluorescence, thus providing reliable quality control materials for standardization and quality assurance of detection reagents. This approach overcomes the biosafety risks and batch-to-batch variability associated with live viruses or clinical specimens.

This epidemiological survey decrypts the unique ecological characteristics of animal poxvirus infections in the border regions of Yunnan Province. The LSDV positivity rate in cattle reached a striking 13.05% across seven surveyed counties. This pronounced prevalence aligns with recent epidemiological trajectories documented across multiple Asian nations and several

Chinese provinces (Chihota *et al*., 2001; Tian *et al*., 2010; Gupta *et al*., 2020), strongly indicating that the Yunnan borderlands have transitioned into a critical epicenter for LSDV incursion into mainland China. Concurrently, SWPV was detected in pig populations (overall positivity rate of 2.84%, positive in five counties), indicating that its geographic distribution and transmission burden may be substantially underestimated. This finding warrants intensified surveillance of hematophagous ectoparasites, such as hog lice (*Haematopinus suis*), which potentially drive localized horizontal transmission (Chihota *et al*., 2001; Tian *et al*., 2010; Gupta *et al*., 2020). However, no MPXV was detected in the sampled specimens in this survey. This result may be attributable to the host specificity of MPXV (with rodents and primates serving as its primary hosts) and the fact that the sampling in this study was mainly targeted at apparently healthy livestock and poultry. Nevertheless, this empirical absence does not eliminate the latent risk of spill-over from wildlife vectors or imported human cases into domestic animal populations. The establishment of this "negative" baseline data provides an important reference for monitoring potential future incursions of MPXV. Meanwhile, the absence of GTPV and FWPV may reflect robust regional vaccination coverage, geographical containment, or inherent sampling limitations (Hosamani *et al*., 2006; Kaiser *et al*., 2021).

In summary, this study established an efficient and specific molecular differential detection system for animal poxviruses, while providing the first comprehensive mapping of LSDV and SWPV prevalence along the Yunnan border.

Conclusions: This study established a robust, biologically safe TaqMan qPCR platform utilizing adenovirus-packaged pseudovirus standards for the parallel detection of five major animal poxviruses, achieving exceptional stability and sensitivity. Large-scale epidemiological screening in the border areas of Yunnan Province revealed, for the first time, a 13.05% positivity rate for LSDV in cattle and 2.84% for SWPV in pigs, while no MPXV, GTPV, or FWPV infections were detected. This high-throughput toolset effectively fills a critical geographical monitoring gap, providing pivotal technical support for the early warning and control of transboundary emerging poxvirus diseases.

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