Development of a real-time PCR assay for differentiation between recombinant and vaccine strains of lumpy skin disease virus in Thailand

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Abstract

Background: The first emergence of a recombinant strain of lumpy skin disease virus (LSDV) in Thailand in 2021 prompted the use of live-attenuated vaccines for disease control. However, current diagnostic tools are insufficient for rapid differentiation between recombinant and vaccine strains. This study aimed to develop a rapid, highly sensitive and specific real-time PCR assay to distinguish the recombinant LSDV circulating in Thailand from the live-attenuated vaccine strains.

Methods: A real-time PCR assay targeting the *ORF146* gene was designed and optimized. The assay was evaluated using nine samples of Thai recombinant LSDV strains, five Neethling vaccine strain samples, and ten samples of other bovine pathogens to determine analytical sensitivity and specificity.

Results: The developed assay detected the recombinant LSDV with high sensitivity (limit of detection: 15 DNA copies/reaction), excellent linearity (R^2 = 0.9988), and an amplification efficiency of 96.95%. It specifically detected recombinant LSDV in clinical and cultured samples from five regions of Thailand, without cross-reactivity with vaccine strains or other bovine pathogens.

Conclusion: This real-time PCR assay provides a rapid, sensitive, and specific tool for the detection of recombinant LSDV in Thailand and enables reliable differentiation from vaccine strains. Initial screening for LSDV should be conducted using the WOAH-recommended assay, followed by strain differentiation with the real-time PCR method developed in this study.

Keywords: real-time PCR, lumpy skin disease virus, differentiation, recombinant strain, vaccine strain

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การพัฒนาวิธี Real-time PCR สำหรับการจำแนกเชื้อไวรัสโรคลัมปี สกิน ระหว่างสายพันธุ์ลูกผสมกับสายพันธุ์วัคซีนในประเทศไทย

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บทคัดย่อ

ที่มาของการศึกษา: การระบาดของเชื้อไวรัสโรคลัมปี สกิน (Lumpy skin disease virus, LSDV) สายพันธุ์ลูกผสมเกิดขึ้นในประเทศไทยครั้งแรกเมื่อปี พ.ศ. 2564 จึงมีการนำเข้าวัคซีนเชื้อเป็นแบบอ่อนฤทธิ์มาใช้ในการควบคุมโรค อย่างไรก็ตาม ปัจจุบันแทบไม่มีวิธีการตรวจวินิจฉัยที่สามารถจำแนกสัตว์ที่ติดเชื้อ LSDV สายพันธุ์ลูกผสมออกจากสัตว์ที่ได้รับวัคซีนได้อย่างรวดเร็ว การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาวิธี real-time PCR ที่มีความรวดเร็วความไวและความจำเพาะสูง สำหรับใช้จำแนกเชื้อ LSDV สายพันธุ์ลูกผสมที่ระบาดในประเทศไทยออกจากสายพันธุ์วัคซีน

วิธีการ: ได้ออกแบบวิธี real-time PCR ที่จำเพาะต่อยีน *ORF146* และปรับสภาวะการทดสอบให้เหมาะสม วิธีที่พัฒนาขึ้นถูกนำไปทดสอบกับตัวอย่างเชื้อ LSDV สายพันธุ์ลูกผสมจำนวน 9 ตัวอย่าง สายพันธุ์วัคซีนจำนวน 5 ตัวอย่าง และเชื้อก่อโรคอื่นในโคและกระบือจำนวน 10 ตัวอย่าง เพื่อประเมินความไวและความจำเพาะเชิงวิเคราะห์ของวิธี

ผล: วิธี real-time PCR ที่พัฒนาขึ้นสามารถตรวจหาเชื้อ LSDV สายพันธุ์ลูกผสมได้อย่างมีประสิทธิภาพ โดยมีค่าความไวเชิงวิเคราะห์ เท่ากับ 15 DNA copies/reaction ค่าสัมประสิทธิ์การตัดสินใจ (R^2) ของกราฟมาตรฐานเท่ากับ 0.9988 และค่า PCR amplification efficiency เท่ากับ 96.95% วิธีนี้สามารถตรวจหาเชื้อ LSDV สายพันธุ์ลูกผสมได้จากทั้งในตัวอย่างสัตว์ติดเชื้อและเซลล์เพาะเลี้ยง ที่เก็บจาก 5 ภูมิภาคของประเทศไทย โดยไม่พบปฏิกิริยาข้ามกับสายพันธุ์วัคซีนหรือเชื้อก่อโรคอื่นในโคและกระบือ

สรุป: วิธี real-time PCR ที่พัฒนาขึ้นมีความรวดเร็ว มีความไวและความจำเพาะสูง เหมาะสำหรับการตรวจหาเชื้อ LSDV สายพันธุ์ลูกผสมที่ระบาดในประเทศไทย และสามารถจำแนกเชื้อดังกล่าวออกจากสายพันธุ์วัคซีนได้อย่างมีประสิทธิภาพ ทั้งนี้การตรวจวินิจฉัยเชื้อ LSDV ควรเริ่มต้นด้วยการตรวจคัดกรองเบื้องต้นด้วยวิธีที่ WOAH แนะนำ จากนั้นจึงทำการจำแนกเชื้อ LSDV สายพันธุ์ลูกผสมด้วยวิธีที่พัฒนาขึ้นในงานวิจัยนี้

คำสำคัญ: วิธี real-time PCR, เชื้อไวรัสโรคลัมปี สกิน, การจำแนกเชื้อ, สายพันธุ์ลูกผสม, สายพันธุ์วัคซีน

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Introduction

Lumpy skin disease (LSD) is an infectious viral disease of cattle and water buffaloes, caused by the lumpy skin disease virus (LSDV). Clinical signs typically include fever, skin nodules, reduced milk production, and enlarged lymph nodes. The disease is arthropod-borne and primarily transmitted by mosquitoes, biting flies, and ticks. LSD is classified as a notifiable disease by the World Organisation for Animal Health (WOAH) due to its potential for rapid spread and significant economic impact on animal health, productivity, and international trade (WOAH, 2024).

The causative agent, LSDV, belongs to the genus *Capripoxvirus* within the family *Poxviridae*. Its genome consists of double-stranded DNA, approximately 151,000 base pairs (bp) in length, and encodes 156 open reading frames (*ORFs*) (Tulman *et al*., 2001). The *Capripoxvirus* genus also includes sheeppox virus (SPPV) and goatpox virus (GTPV), both of which share high nucleotide identity with LSDV. LSDV exhibits over 97% sequence homology with GTPV (Upton, 2004) and shares similar gene content and genomic organization with both SPPV and GTPV. Although SPPV and GTPV serologically cross-react with LSDV and can occasionally infect each other's hosts, they rarely cause disease outside their natural hosts (Roche *et al*., 2021). In contrast, LSDV remains highly host-specific, with no natural infections reported in sheep or goats to date (Namazi and Khodakaram Tafti, 2021).

Vaccination remains the most effective strategy for controlling LSD, with both heterologous and homologous vaccines available (Calistri *et al*., 2020). Heterologous vaccines derived from SPPV or GTPV are considered suitable for LSD-free regions or eradication programs due to their lower risk of adverse effects, although their immunogenicity in cattle may be limited (EFSA, 2017). Conversely, homologous vaccines based on live-attenuated LSDV strains induce stronger and more protective immune responses in cattle (Kumar *et al*., 2025). However, their use carries a higher risk of vaccine-induced disease and potential reversion to virulence, which may lead to new outbreaks (Sprygin *et al*., 2018; Matsiela *et al*., 2022; Shumilova *et al*., 2024).

In Thailand, the first recorded outbreak of LSD occurred in March 2021 (Arjkumpa *et al*., 2022). The disease subsequently spread rapidly nationwide, affecting more than 600,000 cattle (Suwankitwat *et al*., 2022). That same year, the reported morbidity and mortality rates were 37.1% and 7.3%, respectively (Wilhelm and Ward, 2023). Genetic analyses revealed that Thai LSDV isolates were closely related to recombinant vaccine-like strains previously identified in China, Hong Kong, and Vietnam (Sariya *et al*., 2022; Paungpin *et al*., 2022; Suwankitwat *et al*., 2022). Whole-genome sequencing further demonstrated that recombinant LSDV isolates circulating in China and Southeast Asian countries, including Thailand, belong to cluster 2.5 (Breman *et al*., 2023; Krotova *et al*., 2023; Suwankitwat *et al*., 2024).

Homologous live-attenuated vaccines have been widely used to control LSD outbreaks. In Thailand, Neethling strain-derived vaccines, such as *Lumpyvax*, *Kemin*, and *Lumpyvac*, have been applied in mass immunization programs to reduce cattle losses. However, these vaccines have certain drawbacks, including clinical side effects, and LSDV isolates from vaccinated cattle have clustered with Neethling vaccine strains (Singhla *et al*., 2022). The real-time PCR assays currently used in Thailand cannot differentiate the Thai recombinant LSDV from vaccine strains, necessitating DNA sequencing, which is limited by high cost, complex analysis, and long turnaround times.

Although differentiating between animals infected with LSDV and those vaccinated with the Neethling vaccine strain remains challenging, recent studies have begun identifying and applying potential DIVA (Differentiating Infected from Vaccinated Animals) marker genes, such as *ORF154* (Nokhwal *et al*., 2025), *LD133,* and *LD144* (Haegeman *et al*., 2023). Whole-genome analysis of the Thai recombinant strain, LSDV/Thailand/Yasothon/2021, identified *ORF146* (phospholipase D-like) as a potential alternative DIVA target gene based on multiple single nucleotide polymorphisms (SNPs) and amino acid differences compared with the LSDV vaccine strain (Suwankitwat *et al*., 2022). This finding facilitates the development of molecular methods to distinguish the Thai LSDV strain from Neethling-based vaccines. Accordingly, the aim of this study was to establish a sensitive and rapid *ORF146*-based real-time PCR assay capable of differentiating the recombinant Thai LSDV strain from the live-attenuated vaccines used in Thailand.

Materials and methods

Viruses and vaccines: Nine samples of the Thai recombinant LSDV strain were analyzed, comprising six samples from the National Institute of Animal Health (NIAH; GenBank accession no. OQ253252, OQ267777, OQ253250, OQ253253, OQ511520, and OM033705), and three samples from the Veterinary Research and Development Center (VRDC), Upper Northern Thailand. All samples were verified by genome sequencing. The sample matrices included skin lesions, whole blood, internal organs, and Madin-Darby bovine kidney (MDBK) cell culture fluid. Samples of the Neethling vaccine strain were obtained from three commercial vaccines (Lumpyvax, MSD, South Africa; Kemin, MEVAC, Egypt; and Lumpyvac, Vetal, Turkey), together with two LSD-like clinical samples. These clinical samples were confirmed as vaccine strains by DNA sequencing and phylogenetic analysis and were provided by the Faculty of Veterinary Medicine, Chiang Mai University. In addition, ten nucleic acid samples of other bovine viral pathogens were sourced from the International Atomic Energy Agency (IAEA, Austria), NIAH, and VRDC for inclusion as controls in the analytical specificity assessment.

Plasmid: A recombinant plasmid DNA containing the *ORF146* gene of the Thai recombinant LSDV strain was synthesized by Gene Universal (Newark, DE, USA). The 350-bp target amplicon was cloned into the pUC57-BsaI-Free vector, generating a recombinant plasmid with a total length of 3,070 bp (Figure 1). The purified recombinant plasmid was supplied as a lyophilized powder, with approximately 5 µg per vial. It was prepared by dissolving in 50 µL of

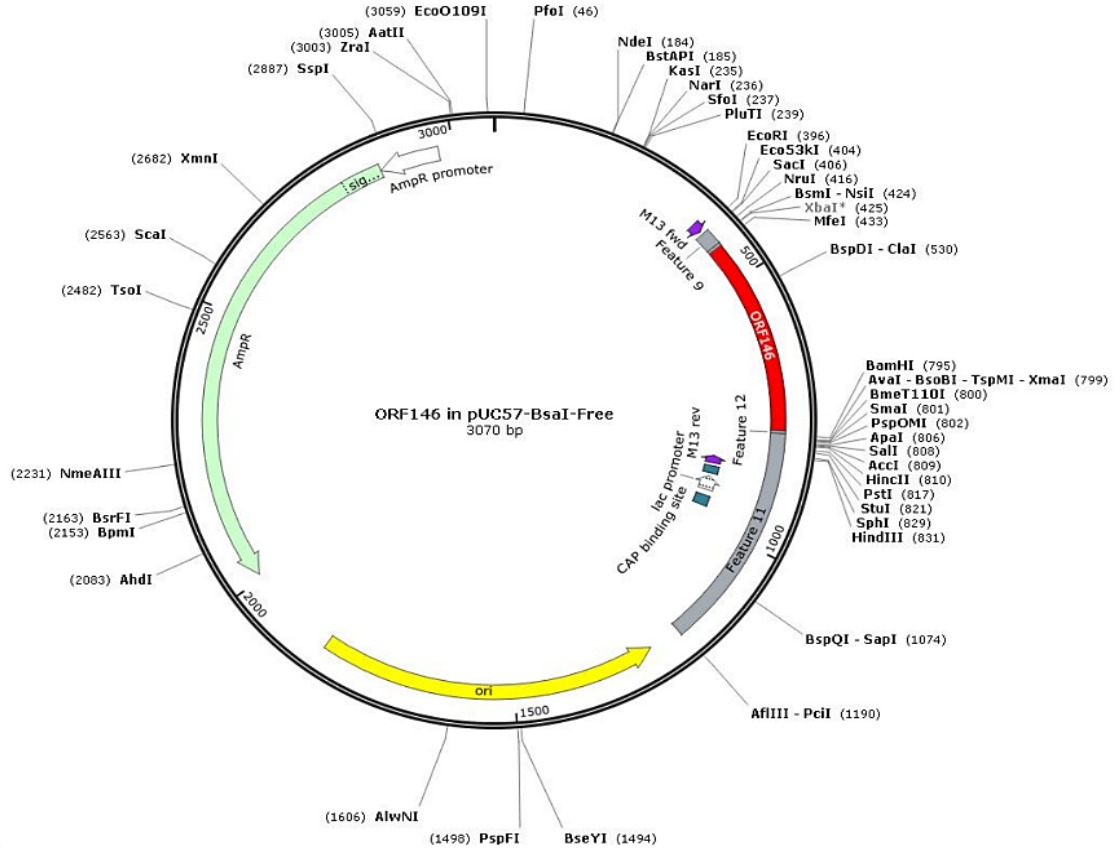

Figure 1 Structure map of plasmid *ORF146* pUC57-BsaI-Free containing the *ORF146* gene of the Thai recombinant LSDV strain.

1X TE buffer to yield a working concentration of approximately 100 ng/µL. This plasmid was used to evaluate the analytical sensitivity of the assay.

Genomic DNA extraction: Viral genomic DNA from LSDV samples (skin lesions, whole blood, internal organs, and MDBK cell culture fluid) was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. Purified nucleic acids were stored at -20 °C until use.

Primers and probe design and *in silico* analysis: Primers and a probe specific to the Thai recombinant LSDV strain were designed from multiple sequence alignment of 17 complete LSDV genomes in GenBank, including four recombinant (accession no. OM033705, MZ577076, MW355944, and MW732649), seven vaccines (accession no. AF409138, KX764643, KX764644, MG972412, KX764645, MW656252 and MH646674), and six classical wild-type strains (accession no. MT134042, AF409137, KX683219, MH893760, AF325528, and MN072619). Sequence alignments were performed using Geneious Prime 2021.2.2 (https://www.geneious.com), and a consensus sequence was used to design primers and probe targeting the *ORF146* gene. The specificity and binding efficiency of the primers and probe were verified using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Optimization of real-time PCR: A novel real-time PCR assay was developed for the specific detection of the Thai recombinant LSDV strain, while excluding Neethling vaccine strains.

Reaction conditions were optimized with respect to primer and probe concentrations, annealing temperature, and cycle number. Primer (0.7–1.6 µL) and probe (0.2–0.4 µL) concentrations and annealing temperatures (55–60 °C) were optimized by triplicate testing. The number of amplification cycles was compared between 40 and 45. Optimal conditions were determined based on average Ct values, with the lowest Ct considered optimal. All PCR reactions were subjected to statistical analysis.

Preparation of Standard DNA: A recombinant plasmid containing the *ORF146* gene of Thai recombinant LSDV strain was used as the control template to evaluate the analytical sensitivity of the assay. Ten-fold serial dilutions of the plasmid, ranging from 2.97×10^{9} to 2.97×10^{-2} double-stranded DNA copies/µL, were prepared. The initial plasmid copy number was calculated from the DNA concentration (ng/µL), plasmid length, and Avogadro's constant using the standard formula described by Whelan *et al.* (2003). These dilutions served as standards for quantifying the Thai recombinant LSDV strain. The quantified DNA was serially diluted and tested to determine the limit of detection (LOD) of the developed assay.

Analytical sensitivity and specificity: The analytical sensitivity of the real-time PCR assay was evaluated using ten-fold serial dilutions of the *ORF146* gene–recombinant plasmid, tested in triplicate to generate a standard curve and determine the LOD and PCR efficiency (%E). The analytical specificity was assessed by performing the real-time PCR using reference samples of the Thai recombinant LSDV strain across four matrices skin lesions, whole blood, internal organs, and cell culture fluid as well as the LSDV Neethling vaccine strains and ten other bovine viral pathogens.

Statistical analysis: All statistical analyses were performed using GraphPad Prism software version 9.3.1. Statistical differences were assessed using one-way ANOVA followed by Tukey's HSD test. Data are presented as mean ± standard error of the mean. $P < 0.05$ was considered statistically significant (*).

Results and discussion

Primers and probe of real-time PCR: A novel real-time PCR assay was developed to differentiate the Thai recombinant LSDV strain from vaccine strains, targeting the alternative DIVA marker *ORF146* gene. *In silico* analysis indicated that the designed primers and probe were suitable for specific detection of the Thai LSDV strain. The nucleotide sequence of the Thai reference strain LSDV/Thailand/Yasothon/2021 (GenBank accession no. OM033705) was aligned with sequences from other LSDV vaccine and wild-type strains. Sequence variation was identified in the *ORF146* region between 139,851 and 140,102 bp. The forward primer was selected based on nucleotide differences from wild-type strains, while the probe was selected based on nucleotide differences from vaccine strains. The positions of primers and probe are shown in Figure 2, with detailed sequences provided in Table 1.

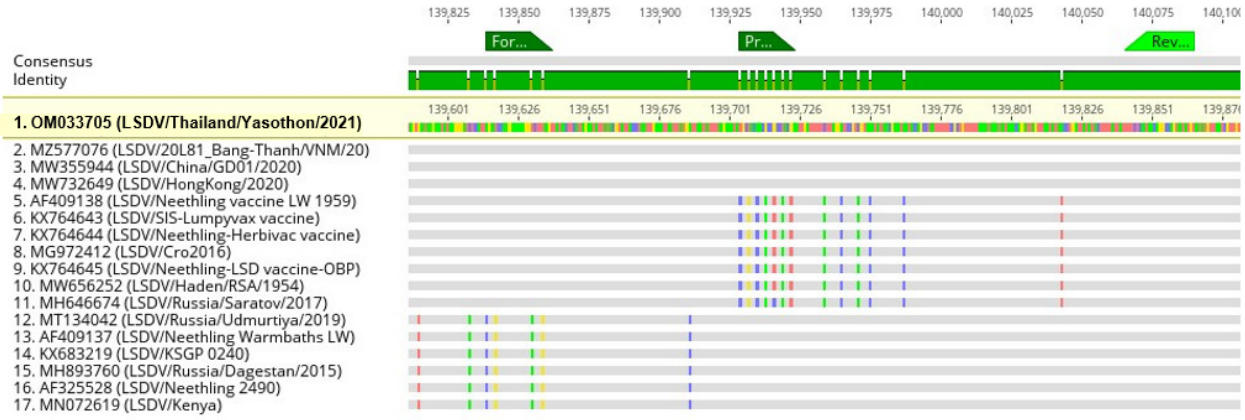

Figure 2 Schematic diagram illustrating the positions of real-time PCR primers and probe targeting *ORF146* gene of the Thai recombinant LSDV strain. Primer and probe orientations are denoted by box colors: dark green for the forward primer and probe, and light green for the reverse primer. LSDV strain groups are represented in different colors: yellow for recombinant, blue for vaccine, and pink for wild type. The forward primer forms four mismatched base pairs with the wild-type strain group, while the probe forms seven mismatch base pairs with the vaccine strain group.

Table 1 Nucleotide sequences and positions of real-time PCR primers and probe targeting the *ORF146* gene of the Thai recombinant LSDV strain.

Name	Type	Sequence (5' -> 3')	Length (bp)	Positions (nt)	Product size (bp)
LSD_For	Forward primer	TGTACCTTGTTTTTGGACAAATGA	24	139851 - 139874	252
LSD_Rev	Reverse primer	TCTATATCAGGCCAGAATAGTGTTT	25	140078 - 140102	
LSD_Pr	Probe	Hex- AGCATCTTCGCCGCCATCGT- BHQ1	20	139968 - 139987	

nt = nucleotide

Optimal novel real-time PCR reaction: The real-time PCR assay was conducted using a QuantStudio™ 5 Real-time PCR System (Applied Biosystems, USA). Each 20 µL reaction mixture contained 10 µL of FastStart Essential DNA Probes Master (Roche, Germany), 5 µL of DNA template, 800 nM of each primer (1.6 µL of 10 µM stock), 125 nM of probe (0.25 µL of 10 µM stock), and 1.55 µL of nuclease-free distilled water (dH_2O). The thermal cycling conditions comprised an initial denaturation at 95 °C for 10 minutes, followed by 45 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 45 seconds. The total amplification time was approximately 90 minutes, indicating a rapid amplification process. Figure 3 illustrates the optimization results for primer and probe concentrations and annealing temperature. The optimal reaction conditions were determined based on the lowest mean Ct value with a statistically significant difference among triplicate reactions.

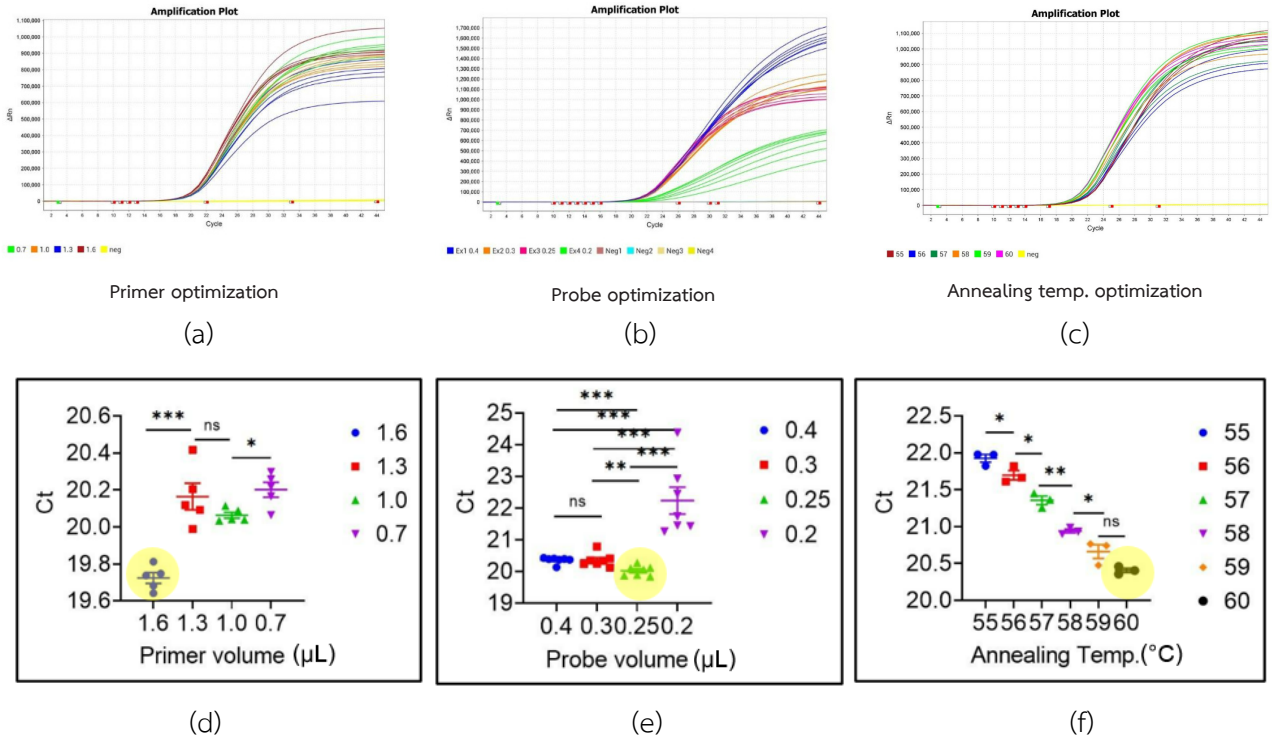

Figure 3 Optimization of primer and probe concentrations and annealing temperature in the real-time PCR assay. Panels (a), (b), and (c) show the amplification curves corresponding to primer concentration, probe concentration, and annealing temperature, respectively. Panels (d), (e), and (f) present the mean Ct values from three to seven replicate reactions, with statistically significant differences indicated by (*). The optimal conditions are highlighted with yellow circles.

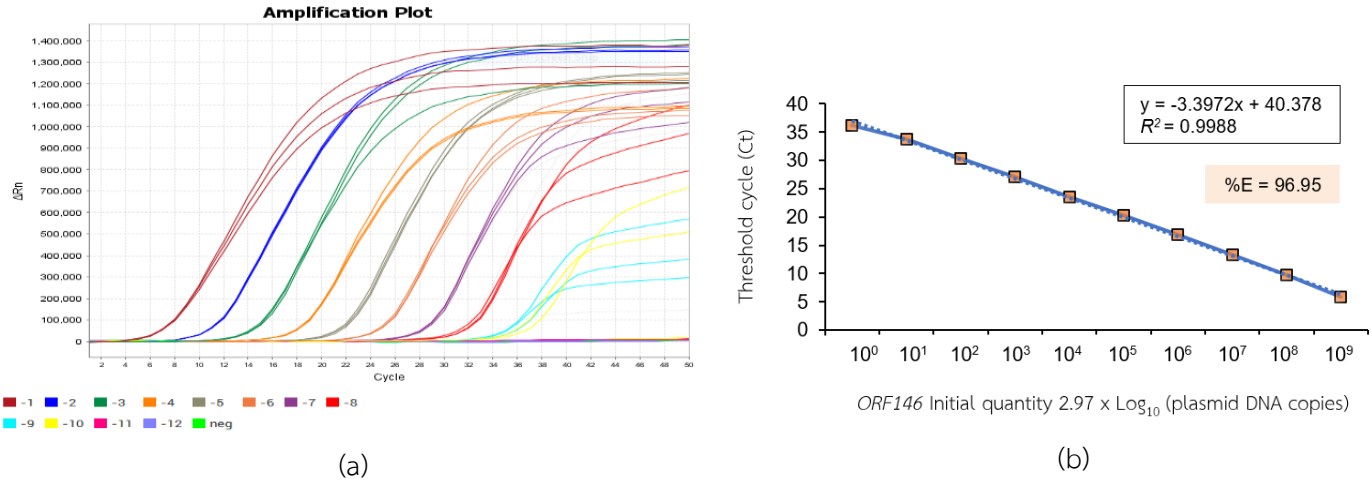

Figure 4 Standard curve of the real-time PCR for detection of the LSDV standard plasmid. (a) Amplification plot of ten-fold serial dilutions of plasmid DNA (-1 to -12) corresponding to concentrations ranging from 2.97 x 10^9 to 10^{-2} copies/µL. (b) Standard curve generated from the mean Ct values of triplicate reactions plotted against the logarithms (base 10) of plasmid DNA concentrations. The standard curve indicates the coefficient of determination (R^2) and PCR efficiency (%E).

Analytical sensitivity of real-time PCR: The amplification curves and corresponding Ct values generated from ten-fold serial dilutions of the *ORF146* standard plasmid were consistent across all triplicate reactions (Figure 4a). The standard curve exhibited excellent linearity, with a coefficient of determination (R^2) of 0.9988 and a PCR amplification efficiency (%E) of 96.95% (Figure 4b). The coefficients of variation (%CV) for all triplicate reactions were below 2%, demonstrating excellent intra-assay precision. The assay demonstrated the LOD of 2.97 copies/µL of template (15 copies per reaction), based on the lowest dilution consistently yielding 100% positive amplification. These findings indicate that the newly developed real-time PCR assay is a highly sensitive and reliable for detecting the Thai recombinant LSDV strain.

The analytical sensitivity observed in this study is comparable to previously developed assay for LSDV detection. Bowden *et al.* (2008) reported a highly sensitive real-time PCR assay targeting *ORF074*, with an LOD of approximately 10 copies per reaction. Although their assay is widely used for *Capripoxvirus* screening, its broad detection capability covering all strains of LSDV, SPPV, and GTPV precludes differentiation among virus species or strains. In contrast, the assay developed in this study was specifically designed to distinguish the Thai recombinant LSDV strain, addressing a crucial diagnostic need in regions where recombinant viruses are now predominant.

DIVA real-time PCR assays require tailoring to the viral strains circulating within each outbreak area. For example, Agianniotaki *et al.* (2017) developed a GPCR-based assay capable of detecting as few as 8 copies per reaction, which differentiates between wild-type and vaccine strains. However, that method was developed for viral populations circulating in Europe and the Middle East. In Thailand, where recombinant LSDV strains have become dominant in recent outbreaks, previously published DIVA assays may not provide optimal discriminatory power.

Analytical specificity of real-time PCR: To evaluate the analytical specificity, nine samples of the Thai recombinant LSDV strain from different matrices including skin lesions, whole blood, internal organs, and MDBK cell culture fluid collected from five regions of Thailand, five samples of the LSDV Neethling vaccine strains, and ten nucleic acid samples of other bovine viral pathogens were tested using the developed real-time PCR assay. The results showed that only the Thai recombinant LSDV strains were detected by the assay, with no cross-reactivity observed with the LSDV Neethling vaccine strain or other bovine pathogens (Table 2). These findings demonstrate that the developed real-time PCR assay exhibits high specificity for differentiating the recombinant LSDV strains circulating in Thailand from the vaccine strain.

Table 2 Viruses and vaccines employed for the evaluation of the real-time PCR assay targeting the Lumpy Skin Disease Virus Thai recombinant strain.

Virus isolate	Source	DIVA real-time PCR
Thai recombinant LSDV strain		Detected
LSDV_OQ253252_Chiang Mai (North)_Skin lesion	NIAH	
LSDV_OQ267777_Khon Kaen (Northeast)_Skin lesion	NIAH	
LSDV_OQ253250_Nakhon Pathom (Central)_Skin lesion	NIAH	
LSDV_OQ253253_Prachuap Khiri Khan (West)_Skin lesion	NIAH	
LSDV_OQ511520_Trang (South)_Skin lesion	NIAH	
LSDV_OM033705_Yasothon (Northeast)_MDBK cell fluid	NIAH	
LSDV_511077/64_Phrae (North)_Skin lesion	VRDC	
LSDV_511116/64_Lamphun (North)_Blood	VRDC	
LSDV_509999/64_Phrae (North)_Internal organs	VRDC	
LSDV Neethling vaccine strains		Not detected
Lumpyvax (commercial vaccine)	MSD Animal Health, South Africa	
Kemin (commercial vaccine)	MEVAC, Egypt	
Lumpyvac (commercial vaccine)	Vetal, Turkey	
LSD-like clinical sample_CMU02 (North)_DNA	Chiang Mai University	
LSD-like clinical sample_CMU03 (North)_DNA	Chiang Mai University	
Other cattle disease viruses		Not detected
Bovine respiratory syncytial virus_RNA	VRDC	
Bovine alphaherpesvirus 1_DNA	VRDC	
Bovine viral diarrhea virus_RNA	VRDC	
Foot and mouth disease virus_RNA	VRDC	
Bovine leukemia virus_RNA	NIAH	
Bovine papillomavirus_DNA	NIAH	
Bovine parainfluenza virus 3_RNA	NIAH	
Cowpox virus_DNA	IAEA	
Bovine popular stomatitis virus_DNA	IAEA	
Pseudocowpox virus_DNA	IAEA	

In this study, we successfully developed and validated a real-time PCR assay capable of differentiating recombinant LSDV circulating in Thailand from Neethling vaccine strains. The assay provides valuable support for molecular epidemiology and outbreak control in the region. Nevertheless, some observations warrant further discussion.

The detection of recombinant LSDV strains in Thailand aligns with reports of recombinant vaccine-like strains emerging across Asia. Vandenbussche *et al*. (2022) observed vaccine-derived recombinants of LSDV in Kazakhstan and neighboring countries, raising concern about vaccine spillover or reversion. Similarly, Krotova *et al*. (2022) reported that five recombinant LSDV lineages circulating in Russia and Southeast Asia belonged to a single expanding lineage from 2020 onwards. These regional findings highlight the relevance of our assay for Thailand, where recombinant strains may complicate diagnostics and control.

The appearance of vaccine-associated genetic changes under field vaccination pressure deserves attention. A Thai monitoring study demonstrated that live-attenuated LSDV

vaccination may lead to emergence of mutated or recombinant strains in non-vaccinated cattle living in vaccinated areas (Suwankitwat *et al.,* 2022). Thus, the ability of our assay to distinguish vaccine from recombinant strains may support not only diagnostics accuracy but also surveillance of vaccination-driven viral evolution. This underscores the utility of real-time PCR tools in monitoring ongoing viral drift and recombination in field settings.

The epidemiological implications of recombinant strain transmissibility are significant. Recent evidence indicates that recombinant LSDV may cause subclinical infection with efficient transmission. Shumilova *et al.* (2024) demonstrated that a recombinant strain led to subclinical infection and indirect contact transmission in experimentally infected cattle. Such findings reinforce the importance of accurate molecular differentiation, as asymptomatic carries may contribute to silent spread.

Currently, LSDV is classified into three genomic groups: classical wild-type strains, Neethling vaccine–derived strains, and the recombinant strains now widespread across Asia. Previous DIVA real-time PCR assays such as those developed by Agianniotaki *et al.* (2017), Pestova *et al.* (2018), and Vidanović *et al.* (2016), which target polymorphisms in *ORF011(GPCR gene)* and *ORF126* (*EEV gene*) have been designed to differentiate wild-type from Neethling vaccine strains. However, these assays cannot reliably distinguish vaccine strains from the recombinant strains circulating in Asia (Flannery *et al.,* 2021). A recently developed DIVA assay targeting the *LD133*, *LD144*, and WTR genes can differentiate wild-type, vaccine, and recombinant LSDV strains and demonstrates improved discriminatory performance; nevertheless, it has not yet been validated using Thai LSDV strains (Haegeman *et al.,* 2023).

In this context, our study is the first design a real-time PCR assay based on *ORF146* specifically for recombinant strains circulating in Thailand, providing an alternative diagnostic tool suited to local epidemiological conditions. However, the limitation of this method is absence of validation using reference wild-type LSDV strains, which were assessed only by *in silico* analysis; therefore, further experimental evaluation will be required in future studies. Furthermore, assay performance could be enhanced by incorporating internal controls such as a bovine housekeeping gene and by further developing the assay into a multiplex real-time PCR capable of differentiating all LSDV strain groups, including wild-type strains, which are not currently present in Thailand but remain important for surveillance of potential introductions.

Finally, although our assay exhibits high sensitivity and specificity under controlled laboratory conditions, challenges remain for long-term field application. The emergence of new SNPs or further recombination events could potentially reduce assay performance if primer and probe sequences are not systematically updated. Ongoing validation using diverse clinical samples, together with regular updates to genomic reference databases are thus crucial to maintaining assay performance amid the rapidly evolving LSDV landscape in the region.

Conclusion and suggestion

This study developed a novel real-time PCR assay targeting the *ORF146* gene for differentiating between recombinant and vaccine strains of LSDV in Thailand. The assay demonstrated high sensitivity (LOD = 15 DNA copies/reaction), specificity, and rapid turnaround, with no cross-reactivity to other bovine pathogens. It provides an effective diagnostic tool for DIVA applications and outbreak surveillance. For initial LSDV detection, the WOAH-recommended assay should be employed prior to differentiation using this method. This assay may also be applied in other regions where recombinant strains genetically similar to the Thai strain are present, such as China, Hong Kong, and Southeast Asian countries.

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