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I-CReST 2025: 001-041 – Differentiating Infected from Vaccinated Animals (DIVA) Strategy for Lumpy Skin Disease (LSD) Cases in Pahang via Real-Time PCR Assay

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ABSTRACT

Lumpy skin disease (LSD) is an emerging transboundary viral disease listed by World Organisation for Animal Health (WOAH) as notifiable disease due to its significant economic losses and the potential of rapid spread. The etiological agent called lumpy skin disease virus (LSDV); could infect cattle and water buffalo by causing high fever, swollen lymph nodes, cutaneous nodules of 2 to 5 cm in diameter on the head, neck, limbs, udder, genitalia and perineum; lachrymation, reduction in milk production, and sometimes death. In response to a widespread LSD outbreak in Malaysia in 2021 which caused substantial economic losses, the authority has intensified control measures including vaccination program. Since 2022, LSD has become endemic in several states, including Pahang. As a result, distinguishing between naturally infected and vaccinated animals; an approach known as DIVA (Differentiating Infected from Vaccinated Animals) is essential for effective disease management and eradication. Thus, this study aims to evaluate LSD-positive cases in Pahang using a molecular DIVA strategy based on a real-time polymerase chain reaction (qPCR) assay. Following this, clinical samples collected from suspected LSD cases were processed and tested for the presence of LSDV. The confirmed positive samples were then subjected to DIVA qPCR testing to identify the viral strain. Our findings revealed that all positive cases were due to wild-type LSDV, indicating ongoing circulation of the virus in Pahang. We have successfully established a molecular-based method for DIVA LSD via qPCR assay which enables accurate identification of infection sources and supports targeted control interventions. Hence, it is an urge for continuous efforts of LSD awareness, strict regulation on animal movement and import protocol, as well as proper vaccination programs to curb the spread of LSD in Pahang.

Keywords: Lumpy skin disease (LSD); lumpy skin disease virus (LSDV); differentiating infected from vaccinated animals (DIVA); real-time polymerase chain reaction (qPCR); cattle disease

1. INTRODUCTION

Lumpy skin disease (LSD) is an emerging transboundary viral disease which listed by World Organization for Animal Health (WOAH) as notifiable disease due to its significant economic losses and the potential of rapid spread [1]. Lumpy skin disease virus (LSDV); an enveloped double-stranded DNA virus of *Capripoxvirus* genus within *Poxviridae* family is capable of infecting cattle and water buffalo by causing high fever, swollen lymph nodes, cutaneous nodules of 2-5 cm in diameter on the head, neck, limbs, udder, genitalia and perineum;

lachrymation, reduction in milk production and sometimes death [2][3]. Originated from Africa continent, LSD has never been reported in Malaysia before and the massive outbreak started in May 2021 is categorized as an emerging disease. The source of invasion was due to illegal movement of smuggled animals from infected neighbouring countries [4].

Following this outbreak, Department of Veterinary Services (DVS) Malaysia has intensified multiple control measures including import and movement control, surveillance, public awareness, vector control, stamping out, strengthen laboratory capacity, disease reporting and vaccination [5]. Vaccination is vital in veterinary medicine since it is one of the most effective technique for control and prevention of diseases as well as to prevent, eliminate and eradicate diseases at the population and the weakening of clinical signs in infection [6][7]. There are two types of vaccine used for LSD in Malaysia which are attenuated and homologous vaccines that covered cattle and buffalo for both dairy and beef [8]. Only the approved vaccines are allowed to be brought in and all the farmers are encouraged to vaccinate their animals voluntarily [5]. As a result, these action plans have showed significant reduction of LSD outbreak thus leading to endemicity of the disease since August 2022 [4].

However, there are several positive cases were newly reported in Pahang started year 2025; which urges an immediate response to any potential outbreak in Malaysia. One of the factors that need to be looked into is sustainability of the vaccination control. Even though vaccination is a useful tool in fighting LSD since their efficacy in the field was proven successfully, the sustainable application of vaccination is determined by several factors. These factors include safety and efficacy as well as accompanying diagnostics. Due to the antibodies formed in serum after vaccination, it is difficult to distinguish between antibody titers resulting from conventional vaccination and exposure to the actual agent [9]. Thus, marker vaccines or DIVA vaccines have been developed to solve this problem.

In veterinary medicine, DIVA stands for Differentiating Infected from Vaccinated Animals. It refers to a strategy that allows for the differentiation between animals that have been vaccinated against a disease and those that have been naturally infected with the disease. This is typically achieved through the use of a marker vaccine and accompanying diagnostic tests. However, the importance of accompanying diagnostic, on the other hand, is often overlooked [10]. The DIVA concept is based on the fact that antibody response against the causative agent antigens involved in DIVA vaccines is different from the antibody response to actual microorganisms in the field [9].

This DIVA principle was subsequently contributed to the development of DIVA real-time PCR for LSDV as a promising tool in controlling the epidemic in Asia. Due to its sensitivity, specificity, and rapidity, real-time polymerase chain reaction (PCR)-based technologies are at the leading edge of diagnostics [10]. The development of DIVA PCR-based tests for LSDV has been facilitated by the fact that the most commonly used commercial live attenuated homologous vaccines are all based on the Neethling vaccine strain [10]. Therefore, this is crucial step for higher authority in order to execute an appropriate contingency plans earlier thus allowing accurate control interventions. Hence, one of the control measures that need to be equipped is capability and capacity of veterinary laboratory to provide diagnostic test that enable to distinguish the source of LSD infection. Thus, this paper aims to evaluate LSD-positive cases in Pahang using an established molecular DIVA strategy based on a real-time polymerase chain reaction (qPCR) assay.

2. MATERIALS AND METHOD

2.1 Samples

A total of three clinical samples from cattle namely whole blood were obtained in the duration of January until February 2025 with highly suspected of LSD. Nucleid acid was extracted from

200µL of whole blood in EDTA by using Indispin Pathogen Kit (Indical Bioscience, Germany).

2.2 Real-time PCR Assay for LSDV Detection

A quantitative real-time PCR (qPCR) Taqman assay that amplified and detected an 89bp region, which encodes the intracellular mature virion envelope protein P32 within LSDV ORF074 was used to determine the viral DNA in clinical samples. Forward primers CaPV-074F1 5'- AAA ACG GTA TAT GGA ATA GAG TTG GAA-3' and Reverse primers CaPV-074R1 5'- AAA TGA AAC CAA TGG ATG GGA TA-3' were used with the Taqman probe CaPV-074P1 5'-6FAM-TGG CTC ATA GAT TTC CT-IABkFQ-3' [11]. DNA amplification was performed with Quantinova Probe PCR (Qiagen, Germany) and operated using Bio-Rad CFX real time PCR machine. Results were generated by determination of the threshold cycle (Ct). Clinical sample templates with Ct values less than 37 were considered as positive result. The positive and negative controls were also included in all PCR reactions.

2.3 Real-time PCR Assay for DIVA LSD

The DIVA duplex real-time PCR (qPCR) assay that detect Neethling-based vaccine strains (referred to as the vaccine reaction (VR)) and wild-type field LSDV strains (referred to as the wild-type reaction (WTR)) were used to determine the source of LSDV infection in positive samples [10]. DNA amplification was performed with Quantinova Probe PCR (Qiagen, Germany) and Bio-Rad CFX Opus qPCR machine was employed for real-time analysis. Results were generated by determination of the threshold cycle (Ct) with Ct values less than 40 were considered as positive result. The positive and negative controls for both types of reaction were also included in all PCR reactions. The selected probes and primers used for the WTR and VR were summarized in Table 1.

Table 1. Probe and primer sequences for WTR and VR

Wild-Type Reaction	**Sequences**
WTR_Frw	5-GGAATCTGTGCAGAAATAAAGTACGA-3
WTR_Rev	5-CCGAAGGGAACGCACTG-3
WTR_Pr	56-FAM-+CTCATCAAATCCCTCTATTTTATG-BHQ1-3
Vaccine Reaction	**Sequences**
VR_Frw	5-GGATTTATTTATATTGTGGGTGGAATT-3
VR_Rev	5-TTTTTGTATGTCGTAATGGGTTC-3
VR_Pr	5-HEX-CTCTCGGAATAGGCTATGAAGG-BHQ1-3

3. RESULTS AND DISCUSSION

Based on real-time PCR analysis for LSDV detection, all three whole blood samples (illustrated as Sample A, B and C) were positive for *Capripoxvirus*; as demonstrated in one of the examples of amplification plot for Sample C in Fig. 1. All of these positive samples were later proceeded for DIVA testing. The results have revealed that all three whole blood samples were positively infected by wild-type strains of LSDV; as summarized in Table 2. This DIVA real-time PCR was designed by Haegeman et. al [10] as a duplex PCR to detect Neethling-based vaccine strains and wild-type filed LSDV strains, including both classical and recombinant wild-type strains. In addition, the primers and probes used were designed in conserved regions of a DNA-ligase-like (LD133) gene and a kelch-like protein (LD144) gene; after the alignment process of 42 capripox genomes, including 29 LSDV of different Asian countries, 7 GTPV and 6 SPPV sequences [10]. Thus, it is showed that this newly designed DIVA real-time PCR is capable to detect our LSD samples accurately, as shown in one of the samples tested in Fig. 2.

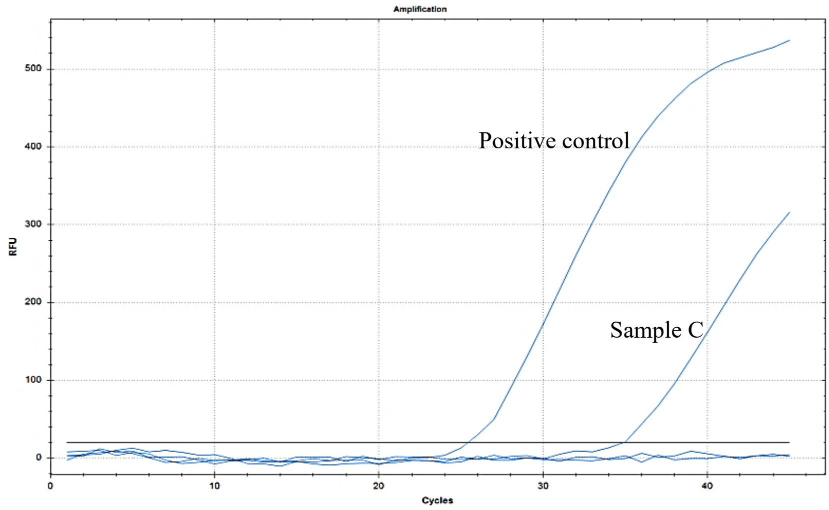

Fig. 1. Relative fluorescence against cycle number of amplification process generated from real-time analysis of LSDV detection in sample C

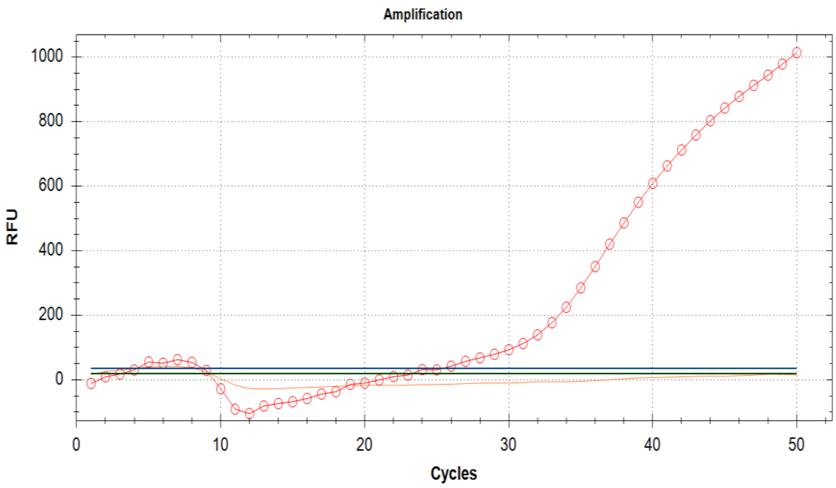

Fig. 2. Relative fluorescence against cycle number of amplification process generated from real-time analysis of DIVA LSD in sample B

Table 2. Detection of LSDV in whole blood samples with their source of infection via DIVA LSD

Sample ID	LSDV (Ct values)	Interpretation	DIVA LSD (Ct values)	Interpretation
A	34.59	Positive LSDV	38.67	Positive WTR
B	31.88	Positive LSDV	25.29	Positive WTR
C	34.85	Positive LSDV	31.16	Positive WTR

Looking through the results in this study, it was confirmed that the infection from wild-type strains have showed that LSDV is still circulating at the investigated area in Pahang. This is correlates with the information provided from the case worksheet which stated that all of these three samples were obtained from unvaccinated cattle in Bentong, Pahang. These farms were operated by various method of livestock production system with and without proper vaccination; which proved that the infection source is not from the vaccine strains. Consequently, improper farm management will lead to disease invasion and biosecurity issues at the cattle farms [12]. However, the level of circulation might not able to cause disease outbreak since there is no suspected case of LSD submitted to our laboratory afterward. This is because, any positive cases that are confirmed from diagnostic report were immediately notified to the Veterinary Health Division of Pahang and Epidemiology and Surveillance Section (DVS). Next, case investigation was conducted immediately by Rapid Action Team (RAT) as well as subsequent control measures such as disinfection, movement control, surveillance and also vaccination. All of these mitigation plans have successfully curbed the spread of the disease which led to zero new case presented to our laboratory.

The availability of reliable diagnostic tools like real-time PCR assay is crucial, particularly during disease outbreaks to enable the early detection of pathogens within affected populations. In addition to their role in early diagnosis, these tools are also essential when vaccination is implemented as a control measure. The ability to distinguish between infected and vaccinated animals, in accordance with the DIVA principle, is important for preventing trade restrictions and ensuring the long-term efficacy and sustainability of disease control programs [10]. Therefore, the developed DIVA real-time PCR for LSD that are used in this study is really suitable and coincide with the laboratory preparedness and capability to support targeted control interventions during outbreak. In addition, DIVA has significant role in eradication scenario in which emergency vaccination using DIVA vaccines could be one control tool for disease outbreaks in densely populated livestock area such as Pahang. In addition, DIVA vaccination might limit the number of culled animals in the process of disease eradication, thereby enhancing public acceptance for disease control measures and limiting economic damage [13]. In essence, DIVA allows for more targeted approach to disease management, improving the efficiency and effectiveness of vaccination and control programs.

However, there is one critical factor that need to be addressed in this study which is type of specimens submitted for diagnostic testing. In our cases, all samples tested are from the whole blood; which demonstrated moderately weak positive results even though the cattle have been reported to show several clinical signs on their skin. It is suggested that the most preferable specimens tested for LSD is scab since the virus replicates in epithelial cells [14] and could be detected on skin biopsies up to 92 days after infection while LSDV in blood was only positive for 4 to 11 days after infection [3]. As a result, the virus may likely undetected if inappropriate sample was tested. Hence, it is clearly important to sample the suitable type of specimens so that our diagnostic results would not be biased and accurately reported to DVS.

4. CONCLUSION

In summary, we have evaluated the positive cases of LSD occurrence in Pahang by using the newly established DIVA real-time PCR that is able to differentiate between wild-type LSDV strains and Neethling-based vaccine strains. It is found that all the positive cases of LSD in Pahang are caused by the infection from wild-type strains; suggesting that LSDV is currently circulated in Bentong, Pahang specifically. By adhering to the multiple control measures, this disease is not capable to cause an outbreak towards cattle populations in Pahang since the disease reporting was done immediately and subsequent preventive measures were taken promptly. Hence, it is clear that this developed test is promising, indicating that it can be a valuable diagnostic tool to help in sustaining the endemicity of LSD in Malaysia.

However, there are few limitations are observed in this study which are type of samples tested, case history and vaccination status of the cattle. Unsuited type of sample will lead to false negative results while inadequate information of the case history and vaccination status will compromise the appropriate control measures that should be taken by DVS. Moreover, it is also suggested that all the positive samples should be proceeded to genomic analysis so that the higher authority will be equipped with valuable understanding of the origin, transmission and genetic diversity of the virus; thus, assist in targeted biosecurity measures and mitigate the economic impact of LSD in the region.

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REFERENCES

[1] F. Namazi and A. Khodakaram Tafti, "Lumpy skin disease, an emerging transboundary viral disease: A review," *Veterinary Medicine and Science*, vol. 7, no. 3, 2021.
[2] WOAH, "Lumpy Skin Disease", 2021. Available: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.04.12_LSD.pdf
[3] E. S. M. Tuppurainen, E. H. Venter, and J. A. W. Coetzer, "The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques," *Onderstepoort J Vet Res*, vol. 72, no. 2, pp. 153-164, 2005.
[4] WAHIS (WOAH), 2022. Follow up report Lumpy Skin Disease virus, Malaysia. 1-24.
[5] S. D. Abdullah, "Preparedness by the Country at the Risk of Lumpy Skin Disease (LSD) Incursion", OIE, 2021. [Online]. Available: https://rr-asia.woah.org/app/uploads/2021/06/03_ malaysia_abdullah.pdf.
[6] A. E. ERDEM and B. SAREYYÜPOĞLU, "DIVA (Differentiating Infected from Vaccinated Animals) Vaccines and Strategies," *Etlik Veteriner Mikrobiyoloji Dergisi*, 2022, doi: https://doi.org/10.35864/evmd.932993.
[7] E. N. T. Meeusen, J. Walker, A. Peters, P.-P. Pastoret, and G. Jungersen, "Current Status of Veterinary Vaccines," *Clinical Microbiology Reviews*, vol. 20, no. 3, pp. 489–510, 2007.
[8] A. Ngah Hamid, "Lumpy Skin Disease (LSD): An Update on LSD Prevention & Control with Focus on LSD Vaccination", OIE, 2021. [Online]. Available: https://rr-asia.woah.org/app/uploads/2021/12/03_malaysia_lsd_control_vaccination_update_anh.pdf
[9] M. J. Day and R. D. Schultz, Veterinary Immunology– Principles and Practice. Second edition. CRC Press, 2014.
[10] A. Haegeman, I. D. Leeuw, W. Philips, and N. D. Regge, "Development and Validation of a New DIVA Real-Time PCR Allowing to Differentiate Wild-Type Lumpy Skin Disease Virus Strains, Including the Asian Recombinant Strains, from Neethling-Based Vaccine Strains," *Viruses*, vol. 15, no. 4, pp. 870–870, 2023.
[11] T. R. Bowden, S. L. Babiuk, G. R. Parkyn, J. S. Copps, and D. B. Boyle, "Capripox virus tissue tropism and shedding: A quantitative study in experimentally infected sheep and goats". *Virology*, vol. 371, pp.380-393, 2008.
[12] V. Renault, M.-F. Humblet, P. N. Pham, and C. Saegerman, "Biosecurity at Cattle Farms: Strengths, Weaknesses, Opportunities and Threats," *Pathogens*, vol. 10, no. 10, p. 1315, 2021.
[13] A. Sanyal, "DIVA Strategy in Modern Vaccinology: Shaping Disease Surveiilance, Transmission, and Control in the Amrit Kaal Era", ICAR-National Institute of High Security Animal Diseases, Bhapal, MP, 2024. Available: https://dahd.gov.in/sites/default/files/2024-11/DrASanyal.pdf
[14] H. Irshad, A. Ahsan, M. Hussain, M. U. Zaheer, U. Farooq, L. A. Asadullah, R. W. Ullah, N. H. Kalharo, S. M. H. Andrabi, and M. Akram, "Epidemiological findings of Lumpy

skin disease outbreak investigations carried out in Cattle Colony Karachi, Pakistan," *Agricultural Sciences Journal*, vol. 4, no. 2, pp. 28–34, 2022.