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The prevalence of capripoxvirus causing lumpy skin disease in beef cattle with no clinical signs on a well-managed cooperative farm

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Abstract Capripoxviruses were confidently detected in cattle, exhibiting a prevalence of 1.17% (9 out of 770 samples). The PCR products from the infected cattle consistently ranged from 1,000 to 1,500 base pairs, even at very low viral loads. Importantly, no significant differences in infection rates were found between the summer and rainy seasons ($P > 0.05$), and the prevalence was not associated with specific geographical regions or the timing of blood sample collection ($P > 0.05$). Notably, infected cattle displayed no clinical signs, such as skin nodules, emphasising that the disease can be present without causing visible harm. These findings strongly reinforce the necessity for stringent disease prevention measures in beef cattle production to effectively mitigate potential losses.

Keywords: LSD, PCR, Cow, Asymptomatic, Molecular technique

Introduction

Lumpy skin disease (LSD) is a viral infection that affects cattle but cannot be transmitted to humans. It causes nodules and lesions on the skin, along with other symptoms (Seerintra *et al.,* 2022; Hamdi *et al.*, 2021). The disease is transmitted by blood-feeding insects and close contact between animals.

Prevention methods include insect control, cleaning of pens, and quarantine of new animals (Pasusart News, 2021). There is no specific treatment for LSD, and the main impact is reduced productivity (Kitjakoson, 2021).

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LSD was first documented in Zambia in 1929 and has spread widely across various African and Asian countries, causing significant economic losses. Recently, there have been outbreaks in Southeast Asian countries such as Vietnam (Tran *et al*., 2020), Laos, Cambodia, Myanmar (Das *et al*., 2021; Azeem *et al*., 2022), and Malaysia (Khoo *et al*., 2022). In March 2021, Thailand reported its first LSD outbreak in Roi Et province, which quickly spread to other provinces (Arjkumpa, 2022; Sariya *et al*., 2022; Seerintra *et al*., 2022).

The outbreak of LSD has hindered the advancement of beef cattle farming in Thailand. The Department of Livestock Development has encouraged farmers to form cooperatives, centralize marketing efforts, and maintain disease-free herds to tackle this. Additionally, the department aims to expand the market for Thai beef by exporting it to neighbouring countries.

The Cowboy Burapha Project aims to encourage 6,000 farmers to breed beef cows, with a goal of reaching 30,000 cows (Khaosod online, 2018). The Ministry of Agriculture and Cooperatives has instructed its affiliated agencies to support farmers in Sa Kaeo province by promoting cattle raising. The project focuses on fostering collaboration among farmers to improve their livelihoods and establish sustainable professions (Thaneeto, 2018). The ministry's ultimate objective is to designate the project area as a disease-free zone to facilitate exports to partner countries.

The strategy for producing high-quality beef cattle may not meet expectations if there are asymptomatic infections within the herd, potentially leading to disease spread. Since Capripoxvirus is transmitted by vector insects, it is important to investigate whether beef cattle herds managed according to standards still experience Capripoxvirus infections. This information will help guide future prevention strategies.

The research aimed to assess the prevalence of Capripoxvirus in healthy beef cattle on a well-managed farm, and improved beef cattle farming's disease monitoring and prevention strategies.

Materials and methods

Ethical approval

This study adhered to the guidelines outlined in "The Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes," as edited by the National Research Council of Thailand. The study was approved by the Institutional Animal Care and Use Committee at Burapha University (Approval number: IACUC 001/2567).

Blood sample collection

As part of the Cowboy Burapha Project in the Watthana Nakhon District of the Sa Kaeo Province, blood samples were collected from female crossbred beef cattle that were at least 18 months old. Participating farmers from 11 sub-districts provided these samples, with 7 farms taken from each sub-district. Each farm provided 5 samples, each comprising 5 ml, resulting in a total of 770 samples. The sample size was calculated using the method developed by Krejcie and Morgan (1970). Blood was collected from the jugular vein using a puncture method and placed in tubes containing EDTA to prevent coagulation. The samples were collected during two periods, from March to April and from June to July, and were stored at -20ºC until DNA extraction.

DNA extraction and amplification

The acid phenol extraction method (Chomczynski and Sacchi, 1987) was applied to extract 200 ml of beef cattle blood samples. Specific primers for the capripoxvirus genes, which cause lumpy skin disease (Elhaig *et al*., 2017), were then used to amplify the required amount of DNA through the Polymerase Chain Reaction (PCR) technique. The PCR reaction involved an initial denaturation at 95ºC for 1 min, followed by denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 70 sec. This process was repeated for 40 cycles, with a final extension step at 72ºC for 5 min.

To create the PCR mixture (100 ml), 10 ml of DNA templates, 10 ml of 10X buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 3 ml of 10mM dNTPs, 5 ml of 50 mM $MgCl_2$, 4 ml each of 10 nmol of forward and reverse primers, and 1.5 ml of DNA polymerase (Invitrogen, USA) were combined. The PCR machine used was the Primus 96 plus thermocycler. The amplified DNA fragments were then separated on a 1.5% agarose gel to determine their size.

The prevalence of capripoxvirus

Through analysis using the Chi-square test, the prevalence of capripoxvirus as the root cause of lumpy skin disease in beef cattle from the Cowboy Burapha Sa Kaeo Project was determined. Positive samples were collected from all sub-districts to calculate the percentage of capripoxvirus in beef cattle of the Cowboy Burapha Sa Kaeo Project, located in the Watthana Nakhon District of the Sa Kaeo Province. The study also examined the relationship between spatial factors and the season or month during which samples were collected to detect capripoxvirus infection.

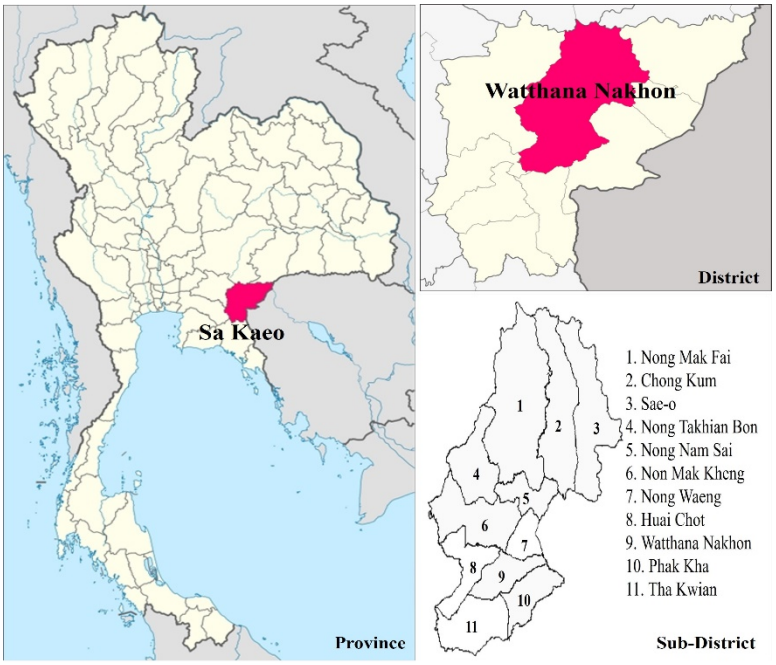

Figure 1. The area of each sub-district in Watthana Nakhon District, Sa Kaeo Province

Results

DNA detection

The DNA extraction process was performed on blood samples taken from beef cattle. The process utilized the acid phenol extraction method, which is a commonly used technique for isolating DNA from various sources. After the extraction, the DNA was amplified using two specific primers: LSD43U and LSD1262L. These primers target specific regions of the DNA with an expected size of 1,238 bp.

To confirm the accuracy of the amplification, PCR products were loaded onto a 1.5% agarose gel. The gel electrophoresis technique allowed the visualization of DNA fragments based on their size, confirming the size of the amplified fragments. As depicted in Figure 2, the length of the PCR products varied from 1,000 to 1,500 bp.

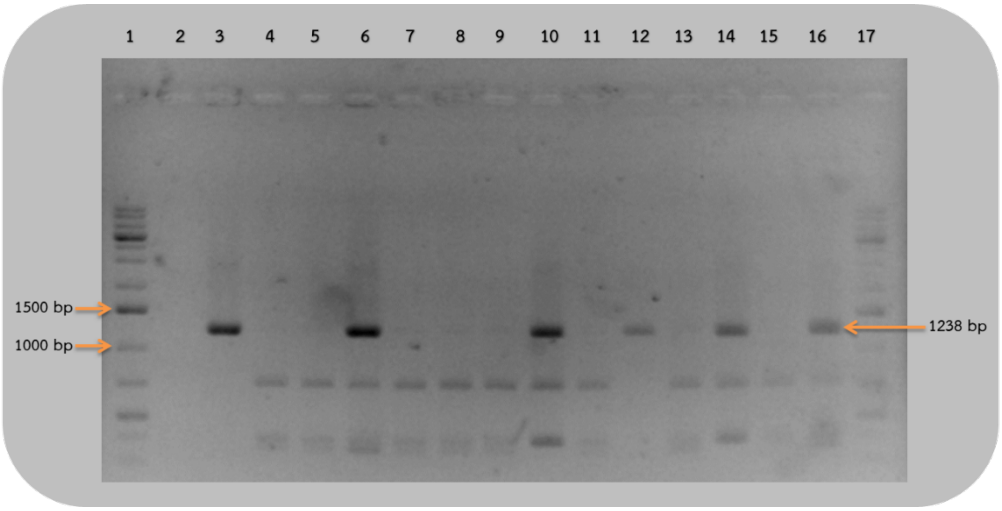

Figure 2. Analysis of PCR products of capripoxvirus that causes lumpy skin disease in beef cattle using 1.5% agarose gel electrophoresis. A 10 µl of PCR mixture was loaded onto each lane of agarose gel. Lanes 1 and 17 = DNA marker (1 kb plus), Lane 2 = Negative control, Lane3 = capripoxvirus positive control, Lane4-16 = PCR product of beef cattle from Cowboy Burapha Sa Kaeo Project, Watthana Nakhon District, Sa Kaeo Province

The prevalence of capripoxvirus causing lumpy skin disease in beef cattle of the Cowboy Burapha Sa Kaeo project

According to the data presented in Figure 2, blood samples taken from beef cattle infected with capripoxviruses, which are known to cause lumpy skin disease, exhibit a DNA band measuring 1,238 base pairs, as reported by Elhaig *et al*. (2017). On the other hand, samples that do not contain capripoxviruses do not show this DNA band. These diagnostic results helped in determining the prevalence of capripoxviruses in several sub-districts, specifically Nong Mak Fai, Chong Kum, Sae-o, Nong Takhian Bon, Nong Nam Sai, Non Mak Kheng, Nong Waeng, Huai Chot, Watthana Nakhon, Phak Kha, and Tha Kwian. The resulting prevalence of capripoxviruses in each sub-district is as follows: 0.00%, 1.43%, 1.43%, 0.00%, 1.43%, 0.00%, 2.86%, 1.43%, 1.43%, and 0.00%, respectively. Furthermore, Table 1 indicates that the prevalence of capripoxviruses in Watthana Nakhon District is 1.17% (Table 1 and Figure 3).

Table 1. Prevalence of capripoxvirus that causes lumpy skin disease in beef cattle blood from each farm in Cowboy Burapha Sa Kaeo Project, Watthana Nakhon District, Sa Kaeo Province

Farm location (Sub-district)	Samples (n)	43U/1262L primer		Prevalence (%)
		Negative	Positive	
Nong Mak Fai	70	70	0	0.00
Chong Kum	70	69	1	1.43
Sae-o	70	69	1	1.43
Nong Takhian Bon	70	70	0	0.00
Nong Nam Sai	70	69	1	1.43
Non Mak Kheng	70	70	0	0.00
Nong Waeng	70	68	2	2.86
Huai Chot	70	69	1	1.43
Watthana Nakhon	70	69	1	1.43
Phak Kha	70	70	0	0.00
Tha Kwian	70	68	2	2.86
Total	**770**	**761**	**9**	**1.17**

The correlation between various factors concerning capripoxvirus infection, such as geographical location and time of sample collection, was investigated. The analysis was conducted at a sub-district level using positive and negative blood samples., The results indicated that the sub-districts where the samples were collected did not significantly affect capripoxvirus infection (P>0.05) as shown in Table 2. Additionally, the researchers examined whether the season of sample collection (March-April or June-July) had any impact on capripoxvirus infection and found that it did not (P>0.05).

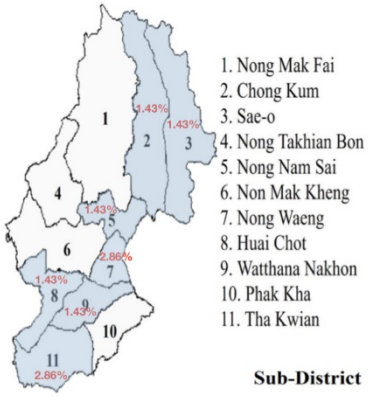

Figure 3. Prevalence of capripoxvirus in beef cattle blood from each sub-district in Watthana Nakhon District, Sa Kaeo Province

Table 2. Analysis of blood collection factors to the prevalence of LSDV

Factor	Sample (n)	Positive (%Prevalence)	Statistic value		
			χ^2	df	*P*-value
Farm location (Sub-district)			**6.9704**	**10**	**0.7282**
Nong Mak Fai	70	0(0.00)			
Chong Kum	70	1(1.43)			
Sae-o	70	1(1.43)			
Nong Takhian Bon	70	0(0.00)			
Nong Nam Sai	70	1(1.43)			
Non Mak Kheng	70	0(0.00)			
Nong Waeng	70	2(2.46)			
Huai Chot	70	1(1.43)			
Watthana Nakhon	70	1(1.43)			
Phak Kha	70	0(0.00)			
Tha Kwian	70	2(2.46)			
Timing of blood collection			**0.1124**	**1**	**0.7374**
March-April	385	4(1.04)			
June-July	385	5(1.30)			
Total	**770**	**9 (1.17)**			

Discussion

There has not been studied on the prevalence of LSD infection in well-managed and healthy beef cattle herds. However, considering that the transmission occurs via blood-feeding insects as vectors, it is interesting to consider whether good farm management practices would still lead to LSD infections, possibly resulting in losses over time. Moreover, detecting the LSD virus in asymptomatic animals is challenging due to the low viral load. As a result, the research team is looking for appropriate diagnostic methods.

The LSDV genomic DNA was targeted in a specific study using primers 43U and 1262L, as it is commonly targeted due to its conserved nature and importance in viral replication. The PCR product was approximately 1,238 bp in length and was confirmed using 1.5% agarose gel electrophoresis. The DNA sequence of the PCR product was validated through sequencing. This method showed high sensitivity in detecting asymptomatic LSD with low viral load in blood samples, which is crucial for timely and effective disease control measures to prevent its spread.

The initial diagnosis of LSD in cattle is typically based on clinical signs like skin nodules, fever, enlarged lymph nodes, and oedema. Viral isolation can be performed by culturing samples from suspected cases in cell lines or embryonated eggs to confirm the diagnosis. However, this method is time-consuming and requires specialized laboratory facilities. Alternatively, serological assays like ELISA can detect antibodies against the Lumpy Skin

disease virus (LSDV) in serum samples. However, these tests may not be reliable for detecting current infections. PCR is a highly sensitive and specific diagnostic method for detecting LSDV. It involves amplifying specific regions of the viral genome to detect even low levels of the virus in clinical samples. Real-time PCR, also known as quantitative PCR (qPCR), is an advanced form of PCR that provides rapid and accurate results and is useful for monitoring the viral load and the effectiveness of control measures (Tuppurainen and Oura, 2012). However, qPCR can be more expensive due to the equipment and reagents required for quantification.

Specific primers targeting the viral genome of LSDV, part of the Capripoxvirus genus, are used to diagnose Lumpy Skin disease using PCR. These primers are designed to target regions of viral genes, such as the P32 envelope protein gene, the G-protein-coupled chemokine receptor (GPCR) gene (Heine *et al*., 1999; Elhaig *et al*., 2017; Selim *et al*., 2021; Koirala *et al*., 2022; Stram *et al*., 2008), the RPO30 gene that encodes the RNA polymerase subunit (Bowden *et al*., 2009), and the F gene encodes a protein that is involved in the viral replication cycle those are conserved among capripoxviruses (Balinsky *et al*., 2008). It is important to select appropriate primers to ensure high sensitivity and specificity in detecting LSDV, especially when working with samples containing a low viral load.

The DNA of the virus causing LSD can be extracted from lesions, lymph nodes, wounds, milk, exudates, semen, and blood of infected cattle (Zeedan *et al*., 2019; Bedekovi *et al*., 2017; Wolff *et al*., 2021; Ghalyanchilangeroudi *et al*., 2021). The viral load of LSDV can vary depending on the sample source due to various factors. LSDV tends to favour specific tissues. For example, skin lesions' viral load might be higher than blood or other bodily fluids. This tissue-specific replication can result in varying concentrations in different sample types (OIE, 2021). The timing of sample collection in relation to the infection stage can significantly impact viral load. Skin lesions and lymph nodes from acute infections may show higher loads compared to blood collected later in the disease course (Babiuk *et al*., 2008). Different sampling methods can lead to variability, such as swabs from lesions versus blood draws. Lesion samples may yield higher viral loads due to direct contact with infected tissue, while systemic samples like blood may show lower concentrations (Haegeman *et al*., 2020). A study found that the highest detection rate was in skin nodule samples collected from animals with visible skin lesions, at 70.13% (54 out of 77), compared to 42.57% (26 out of 55) for nasal swab samples and 20.78% (16 out of 77) for blood samples collected in EDTA (Sariya *et al*., 2022). Furthermore, the copy number of the p32 gene in skin nodule samples was significantly higher than in nasal swabs and EDTA blood samples, suggesting that skin nodule samples may be a more

sensitive sample type for detecting LSDV, indicating a higher viral load (Zeedan *et al*., 2019; Ochwo *et al*., 2020). Tuppurainen *et al*. (2005) reported that PCR can detect the virus DNA in blood during the short viremia period that occurs 4-11 days after infection.

Capripoxviruses have been discovered in healthy beef cattle at the well-managed cooperative farm in the Cowboy Burapha Sa Kaeo Project. The Cowboy Burapha Project is a large-scale agriculture promotion project and a crucial initiative in response to the government's operational policy. The Ministry of Agriculture and Cooperatives has a policy for its affiliated agencies to strengthen farmers by encouraging villagers to raise cattle in the Sa Kaeo province. This project emphasizes cooperation with farmers and encourages them to work together to strengthen their livelihoods, create new sustainable professions, and improve their economic stability. The project operates in Khok Sung, Watthana Nakhon, and Aranyaprathet.

The Ministry of Agriculture and Cooperatives aims to make the project area of the Cowboy Burapha Project free from serious animal epidemics. Lumpy skin disease has caused significant damage in the past year, so bringing cattle or animals into the area must be monitored from the source, with disease prevention and quarantine measures in place 30 days before bringing the animals into the area. If the area in the eastern region can be declared disease-free, it will be possible to export and benefit from the acceptance of trading partner countries (Khaosod Online, 2018). Disease testing is necessary for reconfirmation in cases where animals are carriers without showing symptoms of the disease but can spread the infection through blood-feeding insects.

According to this study, there is no significant correlation between the transmission of LSDV infection and either the seasons or the areas. Reports indicate that LSD can spread within a week in a 30 km radius. It's important to isolate livestock areas and keep them away from external animals. Proper control of blood-sucking insects is crucial in minimizing the risk of LSDV infection. The study found that the disease is more likely to spread if there is an outbreak in the area and breeders have not implemented the necessary measures to manage the occurrence factors.

Despite varying topography and community presence, widespread infections have been revealed throughout the Watthana Nakhon district. The northern section of the district is mountainous, adjacent to the protected Sankamphaeng Range, while the southern part is flat and more densely populated. These findings indicate that blood-feeding insects, particularly mosquitoes, ticks, and fleas, are the primary culprits in spreading diseases in the area. Blood-feeding insects, such as mosquitoes, ticks, and fleas, can spread diseases varying distances, typically travelling 1-3 miles (1.6-4.8 km) from their breeding sites.

However, certain species, like the saltmarsh mosquito, can travel up to 100 miles (160 km) with the wind (Hill and MacDonald, 2022). Ticks and fleas generally do not travel far by themselves but can be transported over long distances by attaching to hosts, such as animals or humans, leading to the spread of ticks and tick-borne diseases over wide areas.

The research also found that there was no significant difference in the rate of LSDV infection in cattle of different ages or genders. Elhaig *et al*. (2017) reported that there was no significant difference in infection between sexes: males (18%) and females (16.3%). Additionally, there was no significant difference between age and LSDV infection: more than 3 years (21.8%, n= 36), 1-3 years (16%, n= 32), and less than 1 year (11.8%, n= 10). This indicates that all ages have the same risk of LSDV (Ochwo *et al*., 2019; Abera *et al*., 2015). However, various other factors have been reported to contribute to the spread of LSDV infection, such as carrier animal movement (BDCVS, 2021; Gari *et al*., 2010; Sevik and Dogan, 2017), poor biological safety practices around farms, feeding areas with dense populations of blood-sucking insects, and improper waste management on farms (Gezahegn *et al*., 2015).

This study discovered that the season indirectly influences LSDV infection by impacting the life cycles of blood-feeding insects. The presence of infections during both study periods indicates that blood-feeding insects have multiple species in the humid tropics of Thailand, each with different life cycles that vary across seasons. For example, mosquito larvae develop in water and are more likely to thrive after rainy seasons when standing water is more abundant (Rocklov and Dubrow, 2020). Ticks go through four life stages: egg, larva, nymph, and adult, each with specific seasonal activity patterns. For instance, many species of ticks lay eggs in the spring, and the larvae emerge in late spring or early summer (Pfaffle *et al*., 2013). Ticks are most active during warmer months, with nymphs active in late spring and summer when temperatures rise. Adult ticks may be more prevalent in the fall, seeking hosts for mating and blood meals.

In summary, it can be identified effective methods for detecting pathogens in asymptomatic animals. Testing with primers 43U and 1262L is found to be highly effective in diagnosing the presence of pathogens in animals. Therefore, it is recommended that beef cattle on farms undergo this diagnostic technique at least once a year. Tests do not need to be limited to the rainy season when mosquitoes are expected to be abundant. Infections can also occur during the summer due to the spread of pathogens by blood-feeding insects such as ticks, fleas, and mosquitoes.

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