ORIGINAL ARTICLE

MOLECULAR CHARACTERIZATION OF LUMPY SKIN DISEASE VIRUS EMERGED IN NORTHERN VIETNAM IN 2020 BASED ON RPO30 AND GPCR GENES

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ABSTRACT

Lumpy skin disease (LSD) is a devastating viral disease of cattle causing economic losses in the cattle industry. LSD was first reported in Vietnam in 2020, therefore, there have not been many studies focused on the detection of LSD virus (LSDV) or the molecular characterization of the virus. In this study, two suspected LSD cattle displaying clinical signs were investigated and the presence of the LSDV genes was confirmed. In addition, the molecular characterization of obtained Vietnamese isolates based on analyzing the RPO30 and GPCR genes was performed. Phylogenetic analysis of the full-length RPO30 and GPCR sequences revealed that the current Vietnamese LSDVs clustered closely with five previously reported Vietnamese and Chinese strains. Furthermore, LSDVs differ from the reported LSDV field isolates in Africa, the Middle East, and Europe. According to deduced amino acid (aa) sequence comparison, some aa substitutions were observed in the RPO30 and GPCR protein of obtained LSDVs. Interestingly, two aa substitutions S98F and L103F were firstly found in the RPO30 of one Vietnamese strain in this study.

Keywords: *Lumpy skin disease virus, Capripoxvirus, RPO30, GPCR, Vietnam*

Philipp. J. Vet Med., 59(2): 155-163

INTRODUCTION

Lumpy skin disease (LSD) is a contagious malady disease of cattle caused by the lumpy skin disease virus (LSDV) within the *Capripoxvirus* genus (CaPVs), the subfamily *Chordopoxvirniae*, and the family *Poxviridae*. LSDV causes some clinical signs such as fever, lymph node swelling, circumscribed nodules on the skin causing severe emaciation, reduction in milk production, and infertility in cattle. LSD is a transboundary and vector-borne disease. The biting flies, mosquitoes, and ticks are indicated as mechanical transmission of the disease thus, is the prime route of its spread. In addition, LSDV is secreted through milk, saliva, nasal, blood, and lachrymal secretions forming indirect sources of infection for animals which share feeding and watering troughs. LSD was firstly reported in sub-Saharan Africa. Currently, LSD has been registered in the large domesticated ruminants in Asia, Europe, and the Middle East (Calistri *et al*., 2020). The disease is considered to be a global emerging threat due to its capacity for quick transboundary spread (Tuppurainen, 2018).

The CaPV genus comprises the sheeppox virus (SPPV), goatpox virus (GTPV), and LSDV. LSDV is an enveloped, double-stranded DNA virus of about 151 kb which includes 156 putative genes. Although SPPV, GTPV, and LSDV share high antigenic similarity and genetic identity, nine LSDV genes are disrupted in GTPV and SPPV. Notably, they are known to be virulent and host range functions. They are a gene unique to LSDV (LSDV 132), and genes similar to those coding for interleukin-1 receptor, two copies of myxoma virus M003.2 and M004.1 genes, and vaccinia virus F11L, N2L, and K7L genes (Tulman *et al*., 2001; Tulman *et al*., 2002). The

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absence of these genes in SPPV and GTPV suggests a significant role for them in the bovine host range.

The LSDV genome seems to be conserved. Indeed, LSDV isolates in Africa showed only minor genomic differences from the first description in Zambia in 1929 (Tuppurainen *et al*., 2011). Compared between the attenuated vaccine strain and two field LSDV isolates in South Africa, a total of 438 amino acid substitutions were found. However, they were mainly in the terminal regions (Kara *et al.,* 2003). Furthermore, the LSDV strains in the Middle East and Europe have little variability compared to the current African LSDV isolates (Alkhamis & VanderWaal, 2016; Tuppurainen, 2018). The previous research indicated that the Greek LSDVs shared 100% nucleotide identity with the LSD/Sivas/0114/TUR isolated in Turkey based on GPCR and RPO30 genes (Agianniotaki *et al*., 2017). However, an increased variability of LSDV in recent years has been reported. It is noted that the LSDV field strains in Russia from 2017 to 2019 showed vaccine-like profiles (Kononov *et al*., 2019; Sprygin *et al*., 2020). Some of these LSDV variants had a 12-nucleotide insertion in the G-protein-coupled chemokine receptor (GPCR) gene, like vaccine strains. GPCR gene was previously suggested to be one of the most variable genes in the CaPVs (Tulman *et al*., 2001; Tulman *et al.,* 2002). This gene was confirmed to be a suitable target for genetic discrimination between members of the *Capripoxvirus* genus (Le Goff *et al*., 2009). Others reported a 27-nucleotide deletion in the open reading frame (ORF) 126 similar to the LSDV Neethling vaccine strain. Furthermore, the LSDV strains with the 27-nucleotide insertion in the EEV glycoprotein gene were reported in the outbreaks in China. The question is whether the dynamics of LSDV variants have any relation to recurrent outbreaks and reappearance of the disease in various parts of the world. To point out this question, more information is needed on the biodiversity of the typical field isolates causing LSD. The aim of the study was to investigate the recent LSD outbreak and determine LSDV characterization obtained in different regions in Vietnam in 2020.

MATERIAL AND METHODS

Sample collection

Following the report of a local outbreak by a small farm holder and a local qualified veterinarian two skin scab samples from cattle, obtained in the study, were collected from severely diseased cattle in Bac Giang and Lang Son provinces in 2020. The two outbreaks in Bac Giang and Lang Son provinces are about 100 km apart (Figure 1). The samples were collected by Department of Microbiology – Infectious Diseases, Faculty of Veterinary Medicine, Vietnam National University of Agriculture with approval from the Committee on Animal Research and Ethics (CARE) of the Vietnam National University of Agriculture. The samples were homogenated and triturated to obtain a 10% suspension in 1x phosphate-buffered saline (PBS 1x) and stored at -20 ^{o}C for further studies.

DNA extraction and polymerase chain reaction (PCR)

Total DNA was extracted from the scab t issue homogenized supernatant using Patho Gen-spin™ DNA/RNA Extraction Kit (iNtRON, Seoul, Korea) following the manufacturer's instructions.

PCR was performed to confirm the presence of the LSDV genome using primers amplifying 192 bp of the P32 gene (Ireland & Binepal, 1998). Briefly, PCR was conducted in a 25 µl reaction mixture: 2 µl DNA (50 ng/ul), 12.5 µl of GoTaq® Green Master Mix (Promega, Madison, USA), 1 µl each of forward and reverse primers at a final concentration of 0.4 pmol/µl (Table 1), and 8.5 µl nuclease-free water. Reactions were performed according to the following thermal conditions: 95°C for five minutes, 40 cycles of 95°C for 30 seconds, 52°C – 55°C for 30 seconds depending on each primer set, 72°C for 40 seconds, and a final extension of 72°C for 10 minutes.

Nucleotide sequencing and phylogenetic analyses

For further genetic characterization and phylogenetic analysis of the LSDV, RNA polymerase subunit (RPO30) and GPCR genes were amplified by PCR using the five sets of primers with the condition as mentioned above (Gelaye *et al*., 2015). The PCR products were separated in electrophoresis on 1.5% agarose gels and purified by GeneClean® II Kit (MP Biomedicals, CA, USA). Nucleotide sequencing was performed by 1st BASE, Malaysia.

Nucleotide and deduced amino acid (aa) sequences were aligned and analyzed using CLUSTAL W, Ver. 1.4 in the BioEdit Package, Ver. 7.2 software (Hall, 1999). The homology of nucleotide and aa sequences was determined using GENETYX Ver. 10 software (GENETYX Corp., Tokyo, Japan) and compared with other sequences available in GenBank identified by The Nucleotide Basic Local Alignment Search Tool (BLASTn)

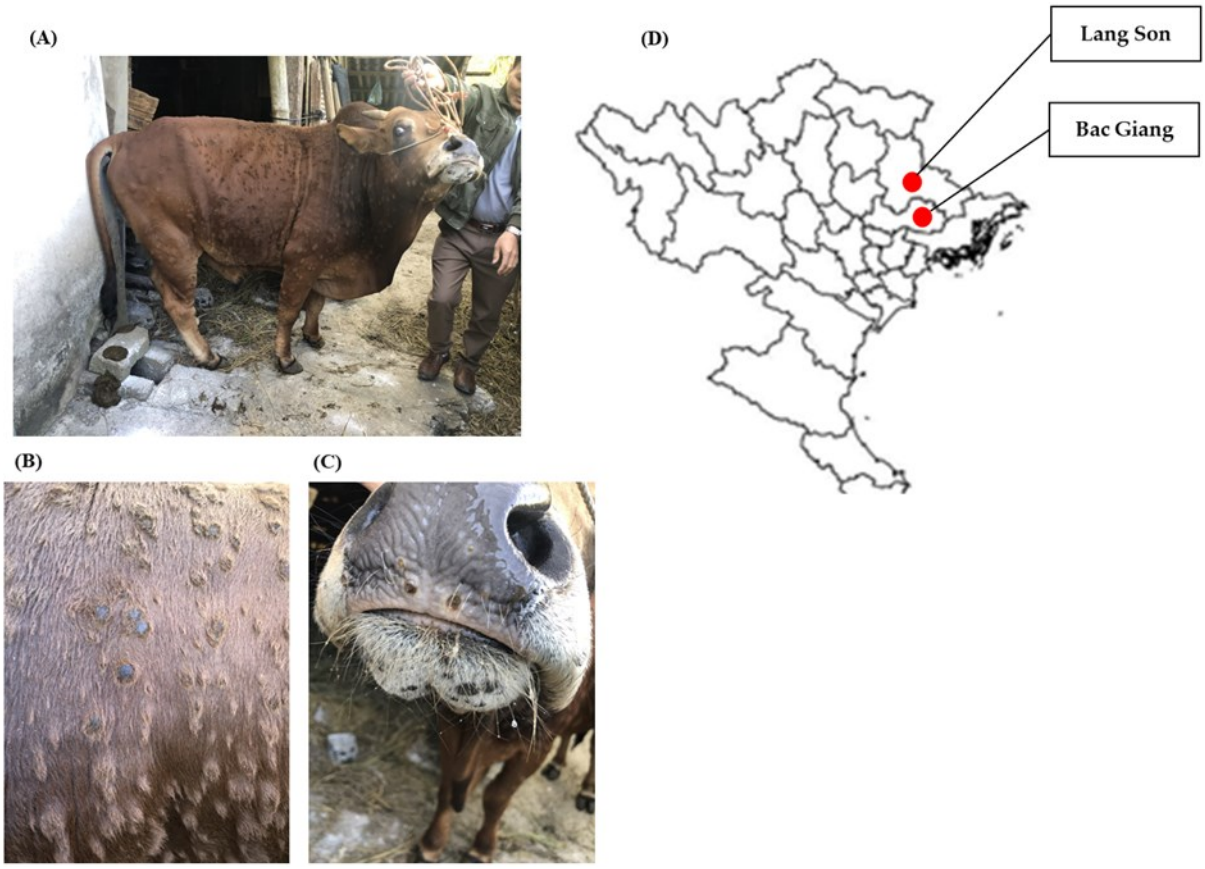

Figure 1. Skin lesions characteristics of lumpy skin disease in 2 cattle in Vietnam. The generalized circumscribed active nodular skin lesions covering the entire body are visible. (A, C) the clinical signs were observed in the suspected cattle from Lang Son province; (B) the clinical signs were observed in the suspected cattle in Bac Giang province; (D) Geographical locations of sample areas in the North of Vietnam (red circles).

Table 1. Primers used in this study

Purpose	Name	Nucleotide sequence (5'–3')	PCR product (bp)	Tm
Detection	P32-1F	TTTCCTGATTTTTCTTACTAT	192	52
	P32-1R	AAATTATATACG TAAATAAC		
Sequencing	CpGPCR-OL1F	TGAAAAATTAATCCATTCTTCTAAACA	617	55
	CpGPCR-OL1R	TCATGTATTTTATAACGATAATGCAAA		
	CpGPCR-OL2F	TTAGCGGTATAATCATTCCAAATA	603	55
	CpGPCR-OL2R	GCGATGATTATGATGATTATGAAGTG		
	CpGPCR-OL3F	CACAATTATATTTCCAAATAATCCAA	684	55
	CpGPCR-OL3R	TGTACATGTGTAATTTTAATGTTCGTA		
	CpRPO30-OL1F	CAGCTGTTTGTTTACATTTGATTTTT	554	55
	CpRPO30-OL1R	TCGTATAGAAACAAGCCTTTAATAGA		
	CpRPO30-OL2F	TTTGAACACATTTTATTCCAAAAAG	520	55
	CpRPO30-OL2R	AACCTACATGCATAAACAGAAGC		

homology searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A maximum likelihood method with 1,000 bootstrap replicates using the Hasegawa-Kishino-Yano and Tamura 3-parameter models was used to establish phylogenetic trees based on the GPCR and RPO30 gene sequences of the two current Vietnamese LSDVs obtained and other sequences available in GenBank using MEGA6 software (Tamura *et al*., 2013).

Recombination events and selection profiles of LSDV gene sequences

Putative recombination involving LSDV genes was conducted by a recombination detection program (RDP) version Beta 4.97 and SimPlot program (version 3.5.1) (Lole *et al*., 1999; Martin *et al*., 2017). Evolution selection profiles were elaborated using Datamonkey (http://www.datamonkey.org/) by following the Fast Unconstrained Bayesian AppRoximation (FUBAR) method (Murrell *et al.,* 2013).

RESULTS AND DISCUSSION

Outbreak investigation

The two suspected cattle infected with LSDV showed fever (40 – 41°C), depression, loss of appetite, nasal and ocular discharges, salivation, and circumscribed nodules with different sizes on the skin (Fig.1).

Molecular detection of LSDV and sequencing of the RPO30 and GPCR genes

The viral DNA of LSDV was detected in the two samples by PCR using primers targeting 192 bp of the P32 gene. Afterwards, two fragments for the RPO30 gene and three for the GPCR gene were successfully amplified and sequenced. The full-length of RP030 and GPCR gene sequences of the two Vietnamese LSDV obtained in this study (LSDV/Vietnam/BG1-21 (BG1-21) and LSDV/Vietnam/LS1-21 (LS1-21)) were deposited into the GenBank under accession numbers MZ666000 through MZ666003.

The RPO30 gene of the two obtained LSDV was bioinformatically compared with other sequences available in the GenBank. They shared the highest nucleotide identity (E value: 0.0) with five previous Vietnamese strains (MZ577073 – MZ5770762) and previous Chinese strains (MN598007 and MW355944). In addition, the GPCR gene sequences of the two current LSDVs shared 100% nucleotide identity with the reported Vietnamese strains (MZ577074–MZ577076) and China/GD01/2020 strain (MW355944).

Phylogenetic analysis of LSDV

Phylogenetic analyses of the full-length RPO30 and GPCR genes were performed for the two current LSDV strains (BG1-21 and LS1-21) and the LSDV strains deposited in the GenBank. The results clearly indicated that the two current Vietnamese LSDVs are clustered together (Figs. 2 & 3). In detail, the phylogenetic tree based on the full-length RPO30 gene sequences (606 nucleotides) showed that the two obtained Vietnamese LDSV strains clustered closely with the previous Vietnamese LSDVs (MZ577073 – MZ577076) isolated in 2020 and Chinese strains reported in 2019 and 2020 (MW355944, MN518933, and MN598007). They belonged to a different group with other LSDV sequences in the phylogenetic tree (Fig.2). With regards to the GPCR gene, the phylogenetic also revealed that the obtained LSDV strains were closely related to the previous Vietnamese isolated in 2020 (MZ577074 – MZ577076) and the China/GD01/2020 strain (MW355944) (Fig.3).

Amino acid (aa) substitutions in the Vietnamese LSDV

With regards to the sequences of the full-length RPO30 genes, the length of the sequences in two LSDVs obtained was composed of 606 nucleotides. The deduced aa sequences of RPO30 protein revealed that BG1-21 strain had no change with other available in GenBank unlike the two aa substitutions that were found in the LS1-21 isolate at S98F and L103F, respectively (Table 2).

As for the GPCR genes, the current LSDV strains demonstrated an ORF of 1,146 nucleotides and are identical to each other. Interestingly, the multiple sequence alignments of GPCR gene revealed that the current LSDV strains showed the absence of the same 12-nucleotide deletion found in a historical field strain from Kenya and in currently circulating field strains from Bangladesh and China. In addition, the deduced aa sequences of GPCR proteins displayed aa residues at four LSDVs specific signatures (A^{11}, T^{12}, T^{34}, S^{99}, and P^{199}) and the insertion of four aa's ^{30}TILS^{33} in the two LSDVs obtained in this study. When comparing with the available LSDVs in the GenBank, there were no sequence changes in the current LSDVs in this study. In particular, they showed three aa residues at positions 76, 127, and 268 different from those of the vaccine strains (Table 2).

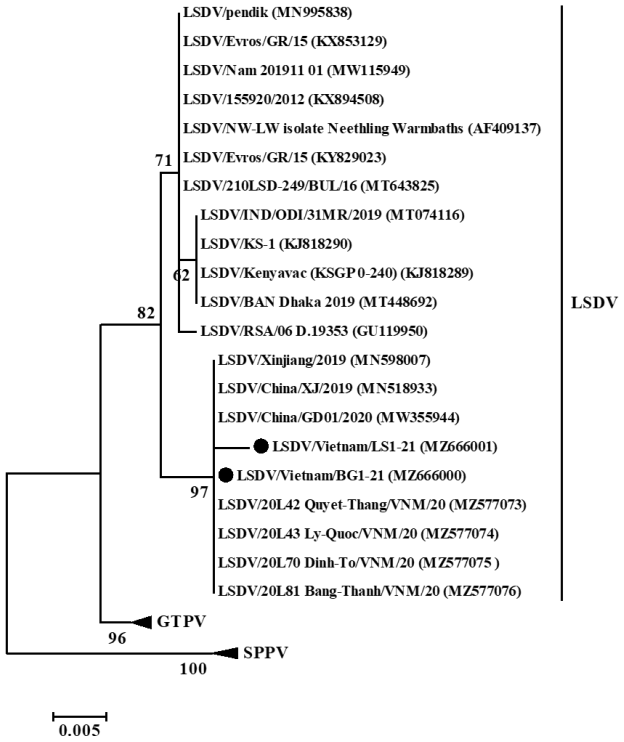

Figure 2. Phylogenetic tree of the full-length sequence of RPO30 gene from the two current Vietnamses strains compared with the sequences from other LSDV strains downloaded from GenBank. The tree was constructed using the maximum likelihood method (1,000 bootstrap replicates) in MEGA6 software. Bootstrap values are shown at the nodes. The sequence determined in this study is marked with a black dot.

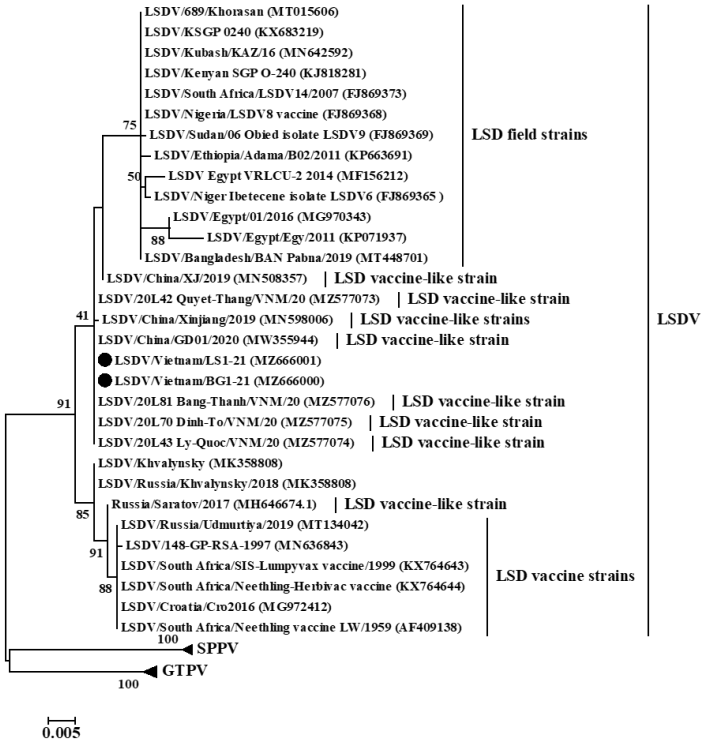

Figure 3. Phylogenetic tree of the full-length sequence of GPCR genes from the two current Vietnamses strains compared with the sequences from other LSDV strains downloaded from GenBank. The tree was constructed using the maximum likelihood method (1,000 bootstrap replicates) in MEGA6 software. Bootstrap values are shown at the nodes. The sequence determined in this study is marked with a black dot.

Table 2. Amino acid substitutions in the RPO30 and GPCR proteins of the current LSDV isolates and others LSDV strains

LSDV strain ID	GenBank accession no.	RPO30 protein			GPCR protein	
		76	127	268	98	103
Consensus[a]		S	M	T	S	L
LSDV/Vietnam/BG1-21	MZ666000[c], MZ666002[d]	.[e]	.	.	.	.
LSDV/Vietnam/LS1-21	MZ666001[c], MZ666003[d]	.	.	.	F	F
Reported Vienamese strains	MZ577074 – MZ577076	.	.	.	.	.
LSDV/China/Xinjiang/2019	MN598006	.	.	.	.	.
LSDV/China/GD01/2020	MW355944	.	.	.	.	.
Vaccine strains[b]	KX764643, KX764644, AF409138	N	I	I	.	.

[a]: The consensus amino acid sequence was derived from 50 LSDV strains from GenBank
[b]: indicates South/Africa/SIS-Lumpyvax vaccine/1999 (KX764643), South Africa/Neethling-Herbivac vaccine (KX764644), and South Africa/Neethling vaccine LW/1959 (AF409138)
[c]: indicates nucleotide sequence of RPO30 gene
[d]: indicates nucleotide sequence of GPCR gene
[e]: same as above sequence

Recombination events and selection profiles among LSDV sequences

Recombination analyses performed for the full-length RPO30 and GPCR gene sequences of the current and previous Vietnamese strains, the highest similar homology strains (Chinese strains), and vaccine strains did not demonstrate any putative recombination events.

Furthermore, only one site in the RPO30 (nucleotide position at 372) and GPCR (nucleotide position at 194) genes of the two LSDV obtained in this study were considered to be under negative selection (with a posterior probability of negative selection at a site equal to or more than 0.9) (Table 3).

Regarding the P32 gene, it had been used for molecular diagnosis of CaPVs due to its highly conserved among members of the CaPV genus. In particular, the P32 gene contains the most significant antigenic determinant present in all species of CaPV thus, it can be used to differentiate them (El-Kholy *et al*., 2008; Hosamani *et al*., 2004; Ireland & Binepal, 1998; Rashid *et al*., 2017). In this study, conventional PCR using primers targeting a 192 bp region of the LSDV P32 gene was conducted to detect the presence of LSDV in the sample from suspected cattle (Ireland & Binepal, 1998). As a result, two skin nodule samples positively amplified the viral DNA. This finding of the present study indicated continuous circulation of LSDV in the Bac Giang and Lang Son provinces of Northern Vietnam.

Upon phylogenetic analyses of both full-length RPO30 and GPCR sequences, the previous and current Vietnamese LSDVs were within the subgroup together with the Chinese

Table 3. Substituted nucleotide positions as negative selection in the RPO30 and GPCR gene sequences

Gene	Nucleotide position	α	β	β - α	Prob[α > β]	Prob[α<β]
RPO30	372	31.326	3.851	-27.475	0.905	0.060
GPCR	194	31.259	1.879	-29.380	0.944	0.039

α: indicates posterior synonymous substitution rate at a site; ß: indicates posterior non-synonymous substitution rate at a site; α > ß: negative selection; α < ß: positive selection; α = ß: neutral selection; Prob[α > ß] ≥ 0.9: posterior probability of negative selection at a site; Prob[α < ß] ≥ 0.9: posterior probability of positive selection at a site

LSDVs strains in 2019 (China/Xinjiang/2019 strain (MN598007)) and 2020 (China/GD01/2020 strain (MW355944)). This finding is consistent with that of a previous study. It is indicated that the Vietnamese LSDVs were closely related to the LSDV field isolates China/GD01/2020 (MW355944) (Fig.2 and 3) (Tran *et al*., 2021). Geographically, Vietnam shares a long border with China. Some possible factors seem to be related to the spread of diseases across the border such as informal trade of animals, movement of contaminated materials, vector movement from the neighboring countries, and illegal cattle-related products via international borders or pastoral communities near the border, etc. (FAO, 2020; Ochwo *et al*., 2020).

Another study also mentioned that LSDV could spread across a long distance (several hundred kilometers from initial outbreak sites within a short period) (Gupta *et al*., 2020; Liu *et al*., 2020; Sprygin, 2018). It is reported that although animal movements from Egypt to Israel were restricted, the infection spread to Israel, 80 to 200 km away through the air movement of biting insects (Gupta *et al*., 2020). In Vietnam, LSD was first reported in mid-October 2020, coinciding with the peak activity of the vectors in the north of Vietnam, in a farm about 120 km from Hanoi (to the South) and to the border of China (to the North). The two family-owned cattle farms in this study had the same characteristics as most of the small households in the mountainous areas of northern Vietnam by the free grazing style such as the shed near the kitchen area, raised together with chickens and ducks, grazing-land together, and sharing water from ditches. This helps explain why LSD widely spreading quickly since it was first recorded.

In China, the first LSD outbreak was determined on August 3, 2019 (Lu *et al*., 2019). Since then, LSD outbreaks have been detected in western and eastern China and also in Taiwan Island outside Mainland China. From these above reasons, it is strongly suggested that the current and previous Vietnamese LSDVs could have been introduced from China. However, the exact factors related to LSDV introduction into Vietnam and its wide spread still need to be clarified through further study.

It is noted that the obtained LSDVs in this study differs from vaccine strains based on RPO30 and GPCR gene comparison. The current Vietnamese LSDVs showed the insertion of 12-nucleotide in GPCR gene. This characteristic was also found in the GPCR gene of the historical field Kenyan LSDV (KSGP-0240), Bangladesh LSDV (BAN Pabna/2019), LSDVs from China (Xinjiang/2019 and GD/01/2020. It is supposed that there is a common exotic source for LSDV introduction in Vietnam. Furthermore, the presence of the 12-nucleotide insertion was considered one of the characteristics to make these different from the commonly circulating field LSDV encountered in Africa, Europe, and the Middle East (Agianniotaki *et al*., 2017; Gelaye *et al*., 2015; Le Goff *et al*., 2009; Şevik *et al*., 2016; Sprygin *et al*., 2018). For instance, the important difference in the GPCR gene (12 nucleotide deletion) between the Greek field LSDV isolates and the vaccine strains was also reported (Agianniotaki *et al.,* 2017).

To understand the genetic properties of the current Vietnamese LSDVs, the deduced aa sequences of RPO30 and GPCR proteins were conducted. Compared with the vaccine strains, two aa substitutions were firstly observed in the RPO30 of the LS1-21. Additionally, the obtained LSDVs in this study showed three aa substitutions in the GPCR proteins (Table 2). However, the role of these substitutions is unclear. Further studies should be conducted to evaluate these substitutions.

Recently, recombination has been found among LSDVs (Sprygin *et al*., 2018; Sprygin *et al*., 2020; Wang *et al*., 2021). As described previously, recombination vaccine-like LSDVs have been reported in Russia, Kazakhstan, and China. The vaccine-like LSDVs were likely generated by at least one field strain and one vaccine LSDV strain. With regard to recombination among Vietnamese LSDVs, no recombination was found to have occurred among them based on analysis of full-length RPO30 and GPCR sequences. It was thus suspected, that the recombinant variant was not introduced into Vietnam. As reported, it is indicated that the previous Vietnamese LSDVs were considered recombination LSDV strains based on the complete genome sequences (Tran *et al*., 2021). On the other hand, the characteristic of the current Vietnamese strains shares the highest homology in RPO30 and GPCR genes with the previous Vietnamese and the contemporary recombinant LSDV strains from China (Wang *et al*., 2021). Therefore, it is necessary to determine the LSDV recombination of further Vietnamese LSDVs based on the complete genome sequences.

In summary, the current study has provided insights into the genetic and characterization of the full-length of RPO30 and GPCR genes of LSDV strains circulating in cattle in Vietnam. The essential information regarding the genetic characteristics of circulating LSDV may be useful for further strategies of LSDV diagnosis and control.

ACKNOWLEDGEMENT

We would like to thank the local veterinarian for their excellent technical assisstance.

STATEMENT ON COMPETING INTEREST

The authors has no competing of interest to declare.

AUTHOR'S CONTRIBUTION

TTHG, investigation, methodology, data analysis, and manuscript writing; MTN, investigation, methodology, and manuscript wirting, DHA, NVG, investigation, data analysis, HTML, project administration, supervision, and manuscript writing.

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