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Research article

Survey of bovine leukemia virus genotypes from Thai swamp buffaloes raised in central and eastern Thailand

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Abstract

Importance of the work: Bovine leukemia virus (BLV) is a known etiologic agent of enzootic bovine leukosis in cattle and has also been observed in buffaloes. The genotyping of BLV based on the *gp51-env* gene has been conducted exclusively in cattle, but there are no studies in buffaloes.

Objectives: To identify the genotypes of BLV among Thai buffaloes raised in central and eastern Thailand.

Materials & Methods: Polymerase chain reaction (PCR) and BLV-specific primers were used to perform proviral DNA detection of BLV. Then, the genotype was detected from nucleotide sequences of the *gp51-env* gene based on phylogenetic analysis.

Results: Nested PCR showed that the occurrence of the BLV provirus in the Thai-buffalo blood samples was 23.1% (42/182). A phylogenetic tree assay showed that the 24 partial nucleotide sequences of the Thai swamp-buffalo BLV *gp51-env* gene belonged to genotype1. Evolutionary trees obtained from reference-cattle BLV nucleotide sequences selected based on similarity to a representative of Thai swamp-buffalo BLV nucleotide sequences (OB BLV6) were used to internally validate genotype 1. The buffalo-circulating BLV and bovine-circulating BLV were shown to be part of a distinct clade.

Main finding: The evolutionary phylogenetic tree showed that all the samples were genotype 1, but there were significant differences between buffalo-circulating BLV and bovine-circulating BLV. Hence, some features may diverge, at least in the *gp51-env* genes.

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Introduction

Bovine leukemia virus (BLV) is a known etiologic agent of enzootic bovine leukosis in cattle (Kettmann et al., 1976; Burny et al., 1988). Natural infections have been observed in cattle and buffaloes (Mammerickx et al., 1981; Office International des Epizooties, 2008). BLV is a member of the *Deltaretrovirus* genus and the *Retroviridae* family. An outer envelope and an inner core encompass the particles of the BLV in virus particles, whereas the viral genome is known to consist of two single-stranded RNA molecules (Copeland et al., 1983; Sagata et al., 1985). The viral genome comprises genes that encode the following proteins: Gag, RNA-dependent DNA polymerase and viral envelope protein (Env); furthermore, the viral genome encodes certain nonstructural proteins such as R3, G4, Tax and Rex (Copeland et al., 1983).

BLV persistence and replication within infected cells, leading to the development of leukemia, involve a complex interplay of viral integration, genetic variation, immune-response modulation and clonal expansion of infected cells. The diversity that influences the generation of the Gp51-Env glycoprotein is reflected in the classification of the genotypes (Felmer et al., 2005; Rodriguez et al., 2009; Matsumura et al., 2011; Yu et al., 2019). The extracellular Gp51-Env glycoprotein is essential for viral entry into host cells via the binding receptor and syncytium formation (Olaya-Galán et al., 2022). In addition, the Gp51-Env glycoprotein is a known target of neutralizing antibodies due to its surface location (Bruck et al., 1982; Mamoun et al., 1990; Callebaut et al., 1993). To survive in the host body, the virus must evade the immune response; thus, the virus needs to change its nucleotide sequence at the *gp51-env* gene. Consequently, the virus must undergo appropriate changes that allow it to be used as a molecular clock. Therefore, *gp51-env* gene sequence analysis is widely used to determine BLV phylogenetic analysis. There are 11 diverse genotypes of cattle classified in the *gp51-env* gene, which were determined through phylogenetic analysis (Rodriguez et al., 2009; Yu et al., 2019). The BLV genotype distribution has revealed two distinct forms of detection: global (such as genotypes 1, 4 and 6) and specific study area detection (such as genotype 10 that was discovered in the Southeast Asian countries of Thailand, Myanmar and Vietnam) (Balić et al., 2012; Lee et al., 2016; Yu et al., 2019; Le et al., 2023).

A limited number of published studies have used molecular techniques to investigate viral genetics in buffaloes. The prevalence of long terminal repeats (LTRs) of the viral genome found in the swamp and river buffaloes of the Philippines was 27.6% (123/445) based on performing nested polymerase chain reaction (nested PCR; Mingala et al., 2009). After examining a portion of the *gp51-env* gene of the bovine leukemia virus, all samples collected in a study involving Brazilian buffaloes were negative (de Oliveira et al., 2016). Researchers using quantitative PCR to identify the *tax* gene, reported the prevalence of Columbian buffaloes to be 19.7% (12/61), and the *tax* gene was determined to be diverse (Olaya-Galán et al., 2022).

Buffaloes are economically important domestic animals in Thailand (Bunmee et al., 2018). Thailand's buffalo population has increased because of the nation's support of commercial buffalo farming. Until the present, there has been a lack of data from studies on BLV in swamp buffaloes in Thailand, particularly regarding the genotype of BLV. Therefore, the aim of the present study was to survey the infection rate and characterize the genotype of BLVs based on any differences in the partial *gp51-env* gene of BLVs in Thai swamp buffaloes.

Materials and Methods

Sample collection

In total, 182 blood samples collected from swamp buffaloes were included in this study. The blood samples were collected from each buffalo using venipuncture in the coccygeal or jugular vein, withdrawing at least 5 mL/sample. Blood was collected in tubes containing ethylenediaminetetraacetic acid and stored at -20°C.

All animals originated from farms in the central region of Thailand (Pathum Thani, Lopburi, Saraburi, Bangkok, Nonthaburi, Samut Sakhon and Nakhon Nayok provinces), as well as from the eastern region (Prachinburi, Chachoengsao and Chonburi provinces). Samples of buffaloes from 18 farms were tested in the study. The subjects were female buffaloes aged 3–15 yr within the mature age range. Generally, the animals appeared healthy upon both distant and close inspection. These animals were raised on breeder farms in herds of 5–400 buffaloes, predominantly fed on roughage, supplemented with concentrated feed. The buffaloes had completed deworming and vaccinations for foot-and-mouth disease and hemorrhagic septicemia. Blood samples were collected during health checks prior to breeding cases. These farms had been enrolled in a reproductive system and health checkup program that was carried out between January 2020 and December 2021. The blood-collection process was administered under the supervision of Dr. Thuchadaporn Chaikhun-Marcou, the Large Animal Clinic, Department of Large Animals, Faculty of Veterinary Medicine, Mahanakorn University of Technology, Thailand.

Ethics statements

In this study, the process used to collect blood samples from the swamp buffaloes complied with all relevant animal welfare rules (reference number ACUC MUT-2023/1). The collection process was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Mahanakorn University of Technology, Thailand.

Nested polymerase chain reaction detection of proviral DNA in bovine leukemia virus

Nested PCR was performed to measure the infection rates of BLV infection among the swamp buffaloes. The names of the two pairs of primers used were: BLV-env-1 and BLV-env-2 primers, and BLV-env-3 and BLV-env-4 (Table 1). Initially, this study used a Biofact Genomic DNA Prep extraction kit (Seoul; Republic of Korea) to extract the genomic DNA from the swamp-buffalo samples. The extraction protocol required 200 µL/sample of buffalo blood, according to the company's extraction protocol. Genomic DNA was collected at -20°C and retained at this temperature until it was used.

Table 1 List of polymerase chain reaction primers used in this study

Primer	Sequence (5'-3')	Position*
BLV-env-1	5'-TCTGTGCCAAGTCTCCCAGATA-3'	5032–5053
BLV-env-2	5'-AACAACAACCTCTGGGAAGGG-3'	5629–5608
BLV-env-3	5'-CCCACAAGGGCGGCGCCGGTTT-3'	5099–5121
BLV-env-4	5'-GCGAGGCCGGGTCCAGAGCTGG-3'	5142–5534

*Accession number K02120 was used as reference strain for primer binding sites in this study.

The first round of PCR was performed in the presence of a PCR component with Taq polymerase (Toyobo; Osaka, Japan) and the BLV-env-1 and BLV-env-2 primers (Fechner et al., 1996). The temperature profiles in the first PCR round were: an initiation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s; an annealing step at 58°C for 30 s; an extension step at 72°C for 1 min; and a final step at 72°C for 7 min. The second PCR round used the PCR products obtained from the first round, specifically BLV-env-3 and BLV-env-4. The process also used the same temperature profile as in the first PCR round.

Genotyping based on nucleotide sequencing and phylogenetic analysis

This study's randomized selection of 24 samples detected proviral DNA for the *gp51-env* gene. The blood samples that gave positive results for the genotyping study were randomly drawn. In total, 24 nucleotide sequences of the *gp51-env* gene were obtained by examining the chromatogram generated by a pair of primers identified as BLV-env-1 and BLV-env-2. The nucleotide sequence (383 bp in length overall and representing the partial position of the *gp51-env* gene in this research study) was found in the 5152–5534 bp region of K02120. Prior to transmission to the distributing corporation, a PCR clean-up method (Bioneer; Seoul, Republic of Korea) was used to purify the 24 chosen PCR products. All nucleotide sequences were provided by Marcogen (Republic of Korea), a distributor of nucleotide sequencing materials. An internally overlapping chromatogram of the nucleotide sequence was used to perform phylogenetic analysis. In total, 24 Thai swamp-buffalo BLV nucleotide sequences were used to create a phylogenetic tree, with reference BLV nucleotide sequences being submitted to GenBank. The maximum-likelihood approach with 1,000 bootstraps, along with the Tamura-Nei model implemented in the MEGA7 and MEGA11 software (Kumar et al., 2016; Tamura et al., 2021), was used to create a phylogenetic tree using at least two reference BLV nucleotide sequences that were surrogates of each genotype. All 24 Thai swamp-buffalo BLV nucleotide sequences were deposited in GenBank under the accession numbers LC739411–LC739434.

Results and Discussions

Bovine enzootic leukosis is a disease caused by BLV. Buffaloes are known to be one of the natural hosts of this disease, as has been established by the Office International des Epizooties. However, there has been only limited information using molecular techniques published pertaining to BLV in buffaloes, particularly the genotyping of BLV based on the *gp51-env* gene. The present study described the BLV present in Thai swamp buffaloes and the infection rate of BLV determined based on nested PCR and established BLV genotyping based on the *gp51-env* gene as evidence of BLV virus infection. Although this study was conducted in central and eastern Thailand, there is evidence that swamp buffaloes located in other areas could also be infected with BLV (Wang et al., 1991; Meas et al., 2000; Mingala et al., 2009; Olaya-Galán et al., 2022)

Infection rate of bovine leukemia virus among Thai swamp buffaloes

Nested PCR with specific primers targeting the *gp51-env* gene was used to detect the infection rate of proviral DNA in the blood samples of swamp buffaloes. A molecular technique to investigate the *gp51-env* gene BLV is an effective method for identifying a symptomatic BLV infection, and it is popular because in this case, the results from the buffaloes can be

compared with the rates of infections in cattle. The present study identified the BLV infection rate was 23.1% (42/182) among the tested swamp buffaloes. Other published molecular studies on swamp and riverine water buffaloes performed blood sample analysis using different regions of the genome of BLVs to detect specific primers. Mingala et al. (2009) used primers specific to the LTR region in Filipino water buffaloes and recorded a BLV infection rate of 27.6% (123/445), whereas Olaya-Galán et al. (2022) used a region of the tax gene of the virus in Colombian buffaloes and recorded a rate of 19.7% (12/61). From the results of all these studies involving buffalo, it may not be possible to clearly compare the degrees of prevalence or the infection rates between studies. An infection rate must involve any characteristics that would affect the results of primer affinity in those studies, such as those associated with the relevant primer binding sites (Khapilina et al., 2021). The infection rate and degree of prevalence obtained from cattle and swamp buffaloes raised in central and eastern Thailand were also evaluated. Using a commercially available enzyme-linked immunosorbent assay test kit to detect seroprevalence in replacement heifers, the BLV infection rate was 32.5% (32/80) (Rukkwamsuk and Rungruang, 2008). The molecular prevalence has been reported as 26.2% (195/744) in cattle studies throughout the nation (Lee et al., 2016), whereas the prevalence was 34.2% (41/120) in studies involving dairy cows in Chiang Mai, Thailand (Saekhow and Chaisri, 2017). These reports indicated the infection status in cattle. The environments for raising cows and buffaloes in Thailand are similar; therefore, both cattle and swamp buffaloes may be exposed to similar disease risk factors such as insect vectors and animal manipulations. The present study results indicated that not only the Thai cattle but also Thai swamp buffaloes in the present study area were infected with BLV.

Genotyping of Thai-buffalo bovine leukemia virus based on phylogenetic analysis

A set of primers (BLV-env-1 and BLV-env-2) was used to re-amplify the proviral genome from the 24 Thai swamp-buffalo blood samples. A phylogenetic analysis was recreated through the use of the primers obtained from the 24 Thai swamp-buffalo BLV nucleotide sequences. A representative nucleotide sequence obtained from 11 BLV reference nucleotide sequences per genotype was also used. On the phylogenetic tree, a genotype of the Thai-buffalo BLV belonged to genotype 1 (Fig. 1). This study involved the BLV nucleotide's separation from cattle genotype 1 when phylogenetic analysis was performed on the Thai-buffalo BLV nucleotide sequences.

There are two notable features of the genotype distribution: genotypes 1, 4 and 6 are distributed globally and the discovered genotypes were associated with certain specific continents—for example, genotype 8 with Europe and genotype 10 with Southeast Asia (Balić et al., 2012; Lee et al., 2016; Polat et al., 2016; Yu et al., 2019; Moe et al., 2020; Le et al., 2023). The outcomes of our investigation demonstrated that genotype 1 (Fig. 1) was not oly detectable among buffaloes but also distributed in cattle throughout the world. The results of the present study may explain the existence of the virus in buffaloes because the virus binds to cell receptors and modifies individual amino acids in structural and nonstructural proteins of each virus genotype (Copeland et al., 1983; Polat et al., 2016; Olaya-Galán et al., 2022). A simulation of the predicted interactions between the AP3D1 proteins of cattle, sheep and buffalo with the BLV-Gp51-Env protein revealed conserved amino acids in the binding regions of the proteins (Olaya-Galán et al., 2022). It is possible that genotype 1 may successfully be replicated in cattle around the globe.

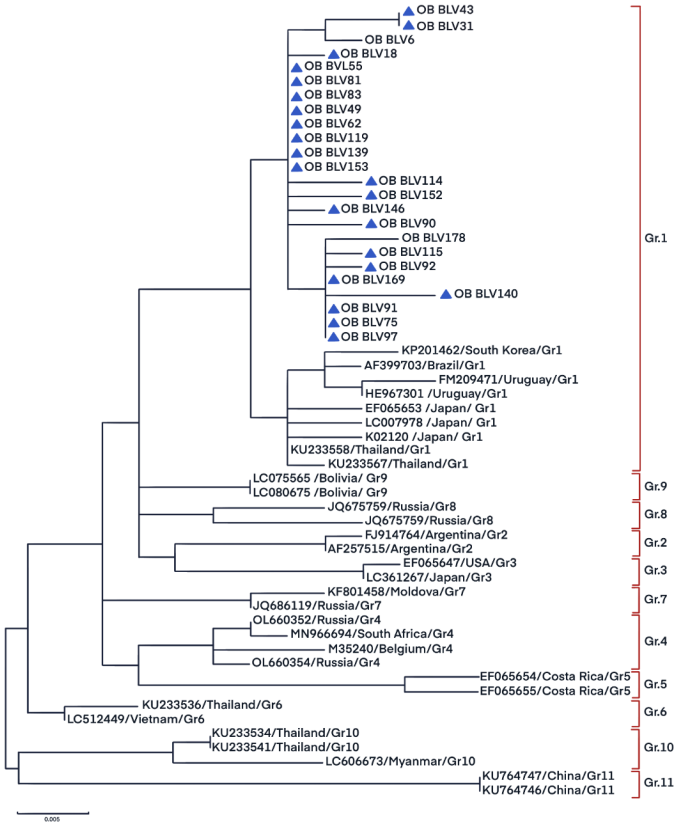

Fig. 1 Phylogenetic tree established based on phylogenetic analysis using 383 base pairs of nucleotide sequences obtained from 24 Thai swamp buffalo BLV nucleotide sequences representative of each genotype. The reference nucleotide sequence had 35 nucleotide sequences, while the nucleotide sequence of genotype 1 had 20 nucleotide sequences. Each taxon included representative nucleotide sequences along with relevant accession numbers, countries of study and relevant genotypes

Accordingly, further investigations are needed to determine if genotype 1 will indeed successfully replicate in buffaloes, which would most likely be a condition of the likelihood that the studied continents already carry the BLV genotype found in cattle.

The purpose of examining the evolution via Thai swamp-buffalo BLV nucleotide sequences and a nucleotide sequence of reference strains is to clarify that the virus belongs to swamp buffaloes due to a separate clade within genotype 1. A specific nucleotide sequence, namely OB BLV6, was applied for the nucleotide identities contained in the GenBank database via the BLAST program. The results indicated five nucleotide sequences closely related to OB BLV6, with nucleotide sequence identities of 99.2% for LC733294 (Japan) and 98.6% for MK780741 (Pakistan), LC512445 (Vietnam), MN167078 (Taiwan) and LC361264 (Japan). Subsequently, a phylogenic tree was established and evolution studies were conducted that used the nucleotide sequences of G1 in the GenBank database and 24 Thai swamp-buffalo BLV nucleotide sequences of the *gp51-env* genes. The present results revealed that the virus was split into two clades among Thai swamp buffaloes and the reference nucleotide sequences (Fig. 2). These present findings indicated that with only modest variations in partial *gp51-env* genes, the characteristics of isolation from Thai swamp buffaloes could vary from those of cattle.

As shown in Figs. 1 and 2, all the 24 nucleotide sequences revealed the genotype 1 clade, which was different from the reference nucleotide sequence clade that had been previously reported as genotype 1. In addition, for internal validation of genotype 1 based on evolutionary trees, the present study used OB BLV6 as a representative of BLV in Thai BLV nucleotide sequences to search GenBank for similar nucleotide sequences within genotype 1. Accordingly, the OB BLV6 nucleotides in Thai swamp buffaloes and the LC733294 nucleotides in Japanese cows showed 99.2% similarity. Considering Fig. 2, even though the virus had the exact same origin, both the buffalo-circulating BLV and the bovine-circulating BLV were allocated as a distinct clade. The present study determined that even though it is the same genotype, viruses may possess some characteristics that enable them to survive in different hosts. This is evidence that the virus may be different, at least in the *gp51-env* genes, wherein it may diverge. Furthermore, the present results could be used for detailed studies of BLVs in buffaloes, such as those involving virus isolation and the relevant immune response. Since this virus has not been fully described in the published literature, it has not yet been studied in detail.

Although this virus has been observed in swamp buffaloes as natural hosts, its association with the viral genotype has not yet been identified according to the *gp51-env* genotype through phylogenetic analysis. In accordance with the findings of the present study, genotype 1 of BLV was detected and allocated in a distinct clade that was separate from cattle BLV nucleotide sequences and Thai swamp-buffalo BLV nucleotide sequences. The outcomes of the present study revealed that the virus found in buffaloes (at least in Thai swamp buffaloes) may possess certain features that are different from those observed in cattle. Therefore, the properties of the viruses found in buffaloes could be used in further detailed BLV studies.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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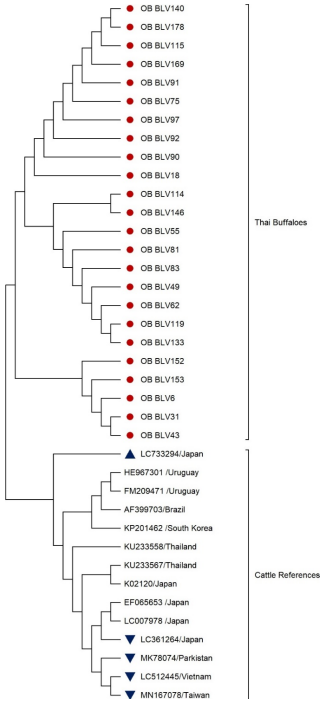

Fig. 2 Phylogenetic tree depicting use of 383 base pairs of nucleotide sequences obtained from 24 Thai swamp buffalo BLV nucleotide sequences (presented as red-filled circles), along with appropriate reference BLV nucleotide sequences of genotype 1. The reference BLV nucleotide sequences of a genotype 1 obtained by similarity of nucleotide sequences using OB BLV6 assays obtained from the established Genbank database, among the five nucleotide sequences similar to OB BLV6, the upright blue triangle is the most similar to OB BLV6, while the remaining BLV nucleotide sequences were inverted blue triangles

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