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BRIEF REPORT

Detection of bovine leukemia virus and identification of its genotype in Mongolian cattle

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Abstract Epidemiological studies have indicated that bovine leukemia virus (BLV) infection is globally distributed. However, no information regarding the disease and genetic diversity of the virus in the cattle of Mongolia is currently available. In this study, the prevalence of BLV was assessed using PCR, and the genetic diversity was analyzed through DNA sequencing. Of the 517 samples tested, 20 positives were identified. Phylogenetic analysis showed that six, one, and four isolates were classified into genotype 4, 7, and 1, respectively. Most isolates were clustered with isolates from Eastern Europe and Russia. This study is the first to investigate the BLV genotype in Mongolia.

Bovine leukemia virus (BLV) belongs to the genus *Deltaretrovirus* within the family *Retroviridae*. It is closely related to human T-lymphotropic virus type 1 and is a causative agent of enzootic bovine leucosis (EBL) [1]. BLV-infected animals can be characterized into three disease stages, namely, aleukemic (AL), persistent lymphocytosis (PL), and leukemia or lymphoma [2]. Approximately 30 % of infected animals progress to PL, characterized by a polyclonal expansion of B cells, whereas only 0.1–10 % develop malignant lymphosarcoma. A long duration is typically required between these disease stages as BLV modulates the immune system of the host [3]. BLV is transmitted vertically or horizontally through the transfer of infected cells via several potential routes. Our previous studies showed one of the major routes of horizontal transmission to be through bites of blood-feeding insects [4], and BLV-infected pregnant cattle containing a viral load show a high risk for vertical transmission [5]. In particular, infected cattle with high viral loads or PL are considered a major source of infection within a herd [4–6]. Thus, the elimination of infected cattle showing a high risk is important for the control of this infection.

BLV infection can affect cells of both the innate and adaptive immune systems and lead to an increased susceptibility to other infections [7]. Increased prevalence of BLV within dairy herds was found to be associated with decreased milk production and longevity of cows [8, 9]. Furthermore, the annual economic loss to the dairy industry in the USA due to BLV infection was estimated at $525 million during 2003 [10]. In Western Europe, BLV infection has been successfully eradicated, and the European commission has officially declared most of its member states to be free of EBL [11–14]. Likewise, attempts to eradicate BLV infection in Australia and New Zealand have been successful. In contrast, several epidemiological studies have indicated that BLV infection is globally distributed, with high prevalence in the USA [10], Japan [15, 16], Canada [17], Russia [18], Iran [19], the Philippines [20], and South American countries [21, 22] and has resulted in major economic losses due to decreased cattle production and export. However, no reports regarding BLV infections in Mongolian cattle are currently available.

Eight BLV genotypes have been identified on the BLV *env* sequences from several geographical areas globally [23, 24]. Phylogenetic analysis based on the partial

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Table 1 BLV detection in Mongolian cattle

Province/city	District	Farm ID	Breed name	Simple number	Positive samples	Genotype
Tuv	Bornuur	A	Holstein	6	2	4
			Simmental	3	1	1
		B	Holstein	11		
		C	Holstein	15	5	1,4
			Simmental	1		
		D	Holstein	9	3	4
			Simmental	3	1	4
		E	Holstein	2	1	4
			Simmental	11		
		F	Holstein	1	1	1
			Simmetal	5		
			Alatau	9		
		G	Holstein	8		
			Simmental	12		
	Lun	A	Mongolian native	31		
		B	Mongolian native	20		
		C	Mongolian native	22		
		D	Mongolian native	24		
Arkhangai	Bulgan	A	Yak	15		
		B	Yak	9		
		C	Yak	12		
		D	Yak	9		
		E	Yak	10		
		F	Yak	10		
		G	Yak	7		
	Tsenkher	A	Mongolian native	20		
Ulaanbaatar	Songinokhairkhan	A	Holstein	4		
			Simmetal	1	1	1
			Alatau	12	1	ND
		B	Holstein	7		
			Simmetal	3		
			Alatau	1		
		C	Holstein	9		
			Simmetal	5		
			Alatau	5		
		D	Holstein	12		
			Alatau	6		
		E	Holstein	9		
			Alatau	6		
		F	Holstein	18		
			Alatau	2		
		G	Yak	28		
			Holstein	10	1	1
			Simmental	8		
			Alatau	6		

Table 1 continued

Province/city	District	Farm ID	Breed name	Simple number	Positive samples	Genotype
		H	Holstein	**22**		
			Simmental	2		
			Alatau	7	2	7
		I	Holstein	14		
			Simmental	1		
			Alatau	3		
		J	Holstein	1		
			Simmental	5		
			Alatau	5		
		K	Holstein	2		
			Simmental	1	1	4
			Alatau	17		
Total (%)				517	20 (3.9 %)	

ND Not determined

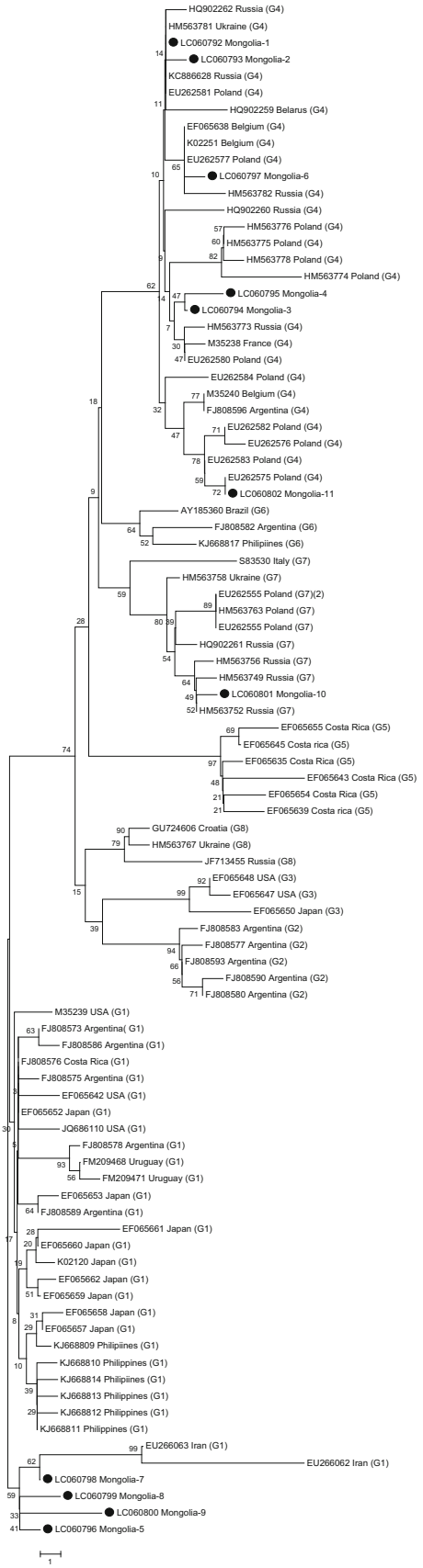
sequences of BLV *env* has indicated that genotype 1 and 3 are mostly found in the USA and Japan. In contrast, genotypes 2, 5, and 6 have been isolated exclusively from South American countries, whereas the genotypes 4 and 7 were most commonly isolated in Russia and Eastern European countries. Additionally, the newly identified genotype 8 has been identified in Russia and the Eastern European counties [25]. However, information concerning the distribution of the BLV genotypes in Mongolia is not available. Therefore, the main purpose of the present study was to identify the genotypes and distribution of BLV infection in the cattle population of Mongolia.

A total of 517 blood samples were collected from dairy cattle, native cattle, and yaks in the Songinokhairkhan district of Ulaanbaatar city, Bulgan and Tsenkher sum of Arkhangai province and, Bornuur and Lun sum of Tuv province in Mongolia during 2014. The blood samples were stored at 4 °C until DNA extraction. Genomic DNA was extracted from the blood samples using a Genomic DNA Purification Kit (Promega, Madison, WI, USA) according the instructions of the manufacturer. Total DNA was diluted in 100 µL of conservation buffer and stored at −30 °C for further use.

BLV infection was tested using a nested PCR assay targeting the *LTR* gene (198 bp) [26], and the viral genotype of the positive samples were further identified using nested PCR targeting *env* (444 bp) as described previously [27].

To detect BLV, nested PCR targeting *LTR* was conducted using rTaq polymerase (Takara Bio Inc., Shiga, Japan) as described previously [26]. A total of 1 µL (100 ng) of DNA sample was used as a template for the initial PCR, and 1.5 µL of the first PCR products were reamplified. The amplified PCR products were confirmed using a MUPID-exU Electrophoresis System (Takara Bio Inc.,) on a 2.0 % agarose gel and visualized under an ultraviolet light printgraph AE-6905CF (Atto, Tokyo, Japan). The β-globin gene was amplified as an internal control to confirm the presence of DNA in the template.

BLV genotyping was conducted as described previously with slight modifications [27]. Briefly, 1 µL (100 ng) of a DNA sample was added to 49 µL of a reaction mixture that consisted of 0.2 µL of Ex Taq polymerase (Takara Bio Inc.,), 4 µL of dNTPs, 2 µL of 10 pM each forward and reverse primer, 5 µL of Ex taq x buffer, and 35.8 µL of double-distilled water. PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 94 °C for 9 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C and 70 °C for the first and second amplification, respectively, for 30 s, extension at 72 °C for 1 min, and a final synthesis at 72 °C for 4 min in a GeneAmp PCR System 9700 cycler (Applied Biosystems, USA). Gel electrophoresis was conducted as described above. Positive samples were subjected to sequencing analysis. PCR products were extracted using a FastGene gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The extracted PCR products were ligated into pGEM-T Easy Vector (Promega), and the plasmid was introduced into *Escherichia coli* strain DH5α (Takara Bio Inc.,), which was plated on Luria-Bertani (LB) agar (Invitrogen, Carlsbad, CA, USA) and cultured in LB broth (Invitrogen). The plasmid DNAs from the positive clones were extracted from the LB culture using a FastGene Plasmid Mini Kit (Nippon Genetics). Amplification of the plasmids for sequencing was performed using a GeneAmp PCR System 9700 (Applied Biosystems). The quality of the plasmids was assessed using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).



◀**Fig. 1** Neighbor-joining phylogenetic tree based on a 444-bp nucleotide sequence of *env* from 11 typical bovine leukemia virus (BLV) strains in Mongolia and BLV strains isolated from several other countries. Mongolian BLV strains are indicated by filled circles. The genotype and geographical origin of each isolate is indicated. The tree was constructed by the neighbor-joining method with 1,000 bootstrap replications

Finally, the nucleotide sequences of the amplified plasmids were determined using a CEQ8000 DNA analysis system (Beckman Coulter, Fullerton, CA, USA).

All identified pathogens were initially analyzed using the Bio-Edit program and BLAST application. Phylogenetic trees were constructed using sequences obtained from the GenBank database. Percentage similarities of the amino acid sequences were computed using the MEGA 6 program.

In the present study, out of the 517 samples tested, 20 were positive for BLV. The infection rate was 2.6 % and 14.6 % in cattle from Songinokhairkhan district of Ulaanbaatar city and Bornuur sum of Tuv province, respectively, and both sites were in areas of intensive cattle farming (Table 1). The distance between these two areas was approximately 60 km and cross-infection between these sampling areas is possible. In addition, the infection rate was variable over the sampling sites, and BLV infection was isolated in five and seven farms from the Songinokhairkhan district of Ulaanbaatar city and Bornuur sum of Tuv province, respectively. The cattle that tested positive were all dairy breeds and, 13, 3 and 4 individuals were belonged to the Holstein, Alatau, and Simmental breeds, respectively. In contrast, no cases of infection were isolated in native Mongolian cattle and yaks. Among the cattle that tested positive, only one individual was under 1 year old, whereas all remaining individuals were 4–17 years old.

Out of 20 LTR-positive samples, 19 samples contained the amplified *env* gene. Out of 19 sequences, 11 independent sequences were identified, and these showed 96.4 %–100 % sequence identity to each other and 95.7 %–100 % identity to viral sequences from several other countries. Eleven isolates were classified into genotype 4, one into genotype 7, and a further four into genotype 1 (Fig. 1). Mongolian isolates of genotypes 4 and 7 were clustered together with isolates from Russia and the Eastern European countries. Genetic analysis showed that the Mongolian isolate LC060792, which belongs to the genotype 4, was 100 % identical to isolates from Russia (KC886628), Ukraine (HM563781), and Poland (EU262581). Another a isolate was found to be 100 % identical to a genotype 4 isolate from Poland (EU262575). In addition, one remaining group belonging to the genotype 1 was clustered with isolates from several counties; however, it was closely related to Iranian isolates.

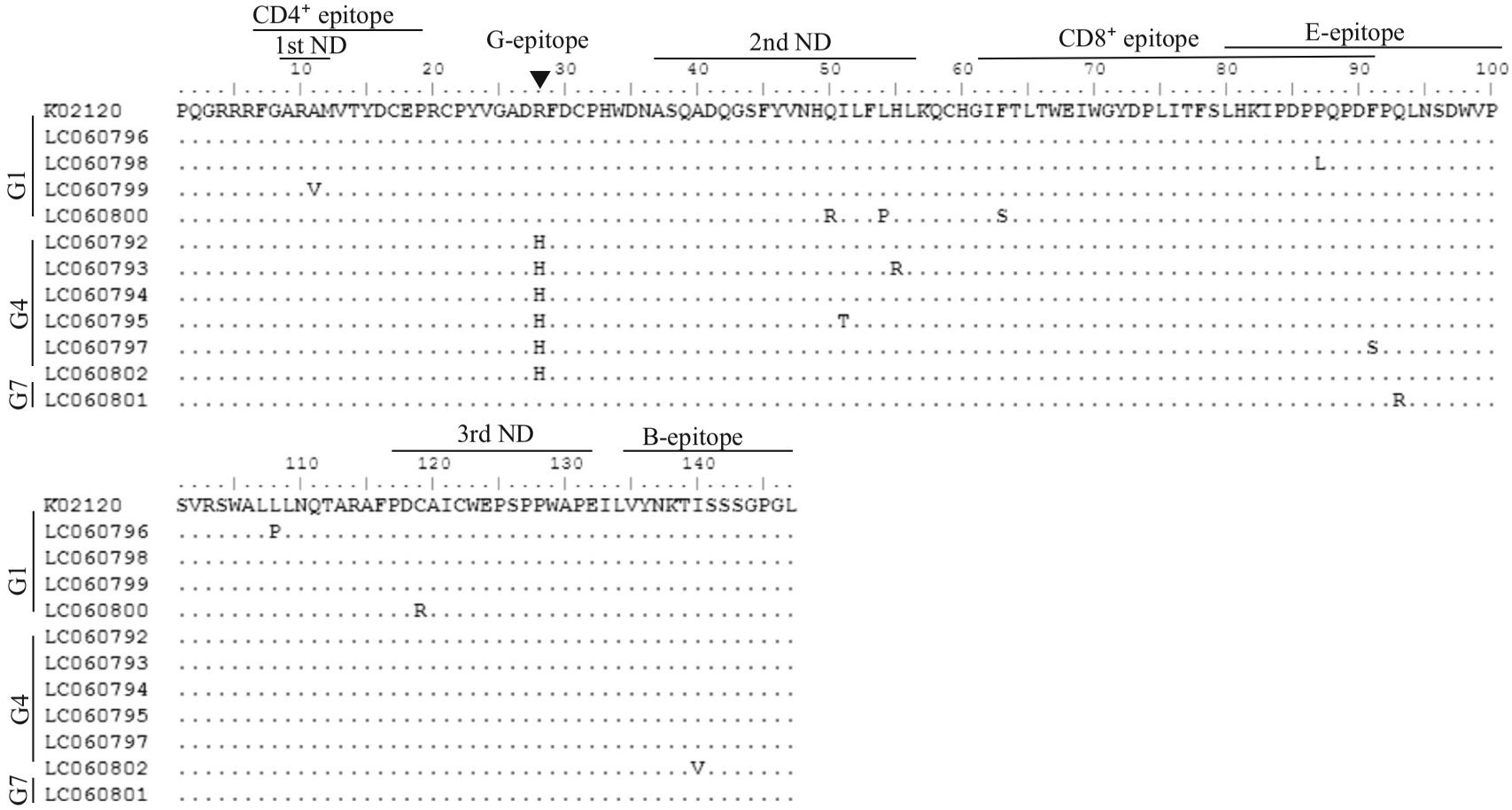


Fig. 2 Amino acid sequence alignment of partial sequences of Mongolian isolates based on the *env* region of bovine leukemia virus (BLV). One typical BLV isolate from each identical sample was submitted to GenBank. Unique nucleotide substitutions are indicated by numbers and filled triangles. The third neutralizing domain (ND) and other epitopes are shown at the top of the alignment. The genotype (G1, G4, or G7) is indicated by a black bar at the far left of the figure. Japanese isolate K02120 was used as a reference in this study

Eleven divergent nucleotide sequences were aligned with that of the Japanese K02120 strain as a reference sequence [28]. All of the Mongolian BLV strains that clustered in genotype 1 showed one common substitution at nucleotide position 127, and all BLV strains that clustered in genotype 4 showed eight common substitutions at nucleotide positions 84, 130, 205, 246, 274, 277, 281, and 337. By contrast, all Mongolian BLV strains belonging to genotypes 1, 4, and 7 showed two common substitutions at nucleotide positions 85 and 121.

All of the divergent predicted amino acid sequences of the *env* regions of Mongolian BLV strains were aligned with that of K02120. This region includes a portion of the first neutralizing domain (ND) (amino acid positions 8–12), the second ND (amino acid positions 38–57), and the third ND (amino acid position 117–132), as well as a portion of the $CD4^+$ T-cell epitope (amino acid positions 8–19), the $CD8^+$ T-cell epitope (amino acid positions 61–89), and the viral G (amino acid position 28), E (amino acid positions 80–100), and B (amino acid positions 134–150) epitopes [24]. Although most of the deduced amino acid sequences showed a high sequence similarity to that of the Japanese K02120 strain, 13 different amino acid substitutions were identified among the Mongolian BLV strains (Fig. 2). Of these substitutions, seven belonged specifically to genotype 1 strains, and five belonged to genotype 4 strains. By contrast, only one amino acid substitution was found in the genotype 7 strain. The substitutions were mainly found in antigenic determinants, such as the $CD4^+$ and $CD8^+$ T-cell epitopes, the neutralizing domains, and the G, E, and B epitopes.

In conclusion, Out of 517 samples, the average rate of BLV infection was 3.9 % and infections were only found in dairy breed cattle. No infections were found in Mongolian native cattle and yaks. Therefore, the BLV prevalence in Mongolian cattle is lower than in several other countries such as Turkey 11 % [29], Japan 35.2 % [16], Iran 29.9 % [19], the Philippines 4.8–9.7 % [20], the USA 46 % [14], Canada 30.9 % [17], Colombia 19.8 %, [22], Argentina 85 % [21], and Russia 28.5–36.1 % [18].

In addition, 11 divergent Mongolian BLV isolates were detected through genetic analysis of *env* genes. Four Mongolian isolates were found to belong to genotype 1, six to genotypes 4, and one to genotype 7, respectively. Interestingly, Mongolian isolates belonging to genotype 4 and 7 were the same and of a similar lineage to BLV isolates from Russia. These findings suggest that BLV infection in Mongolian dairy cattle was probably introduced into Mongolia from Russia by imported dairy cattle for establishment and progression of intensive cattle farming. Historically, Mongolian dairy cattle were imported from the former Soviet Union. In contrast, the Mongolian isolates belonging to genotype 1 were independently clustered with Iranian isolates. Further study is required to determine whether the Mongolian genotype 1 originated in Mongolia.

In an amino acid sequence alignment of Mongolian isolates, substitutions were mainly found in antigenic determinants, such as the $CD4^+$ and $CD8^+$ T-cell epitopes, the second neutralizing domain, and the G, B, and E epitopes. However, whether the Mongolian isolates have different pathogenic properties compared to other isolates remains unclear.

The current observations are preliminary, and the BLV infection rates require further confirmation using a larger number of samples from several provinces in Mongolia. The implementation of systematic control measures against infectious agents such as BLV in livestock should be maintained in Mongolia.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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