The Journal of Veterinary Medical Science

NOTE

Virology

Molecular analysis of bovine leukemia virus in early epidemic phase in Japan using archived formalin fixed paraffin embedded histopathological specimens

Kohei NISHIKAKU[1)], Tatsuo NOGUCHI[1)], Satoshi MURAKAMI[1)], Yasushi TORII[1)] and Tomoko KOBAYASHI[1)]*

[1)]Department of Animal Science, Faculty of Agriculture, Tokyo University of Agriculture, Kanagawa, Japan

ABSTRACT. Bovine leukemia virus (BLV) is an important pathogen associated with enzootic bovine leukosis. In this study, we performed PCR and sequencing analysis to characterize BLVgp51 sequences from formalin-fixed paraffin-embedded (FFPE) specimens made from 1974 to 2000 and successfully obtained BLV proviral genome sequences from 94% of the analyzed samples. Furthermore, from these samples, we reconstructed eight full-length and nearly full-length BLVgp51 sequences. These sequences were classified as BLV genotype 1, implying that genotype1 has already been circulating in Japan since the 1970s. In our results, the proviral DNA was detected in the 1970s, 1980s, and 1990s in the same manner, indicating that the detection of BLV proviral genome depends on storage conditions rather than storage period. The sequences obtained in this study provide direct insights into BLV sequences before 2000, which serves as a good calibrator for inferring ancient BLV diversity.

KEY WORDS: bovine leukemia virus, epidemiology, formalin-fixed paraffin-embedded specimen, enzootic bovine leucosis

J. Vet. Med. Sci.
84(3): 350–357, 2022
doi: 10.1292/jvms.21-0570

Received: 25 October 2021
Accepted: 24 December 2021
Advanced Epub:
18 January 2022

Bovine leukemia virus (BLV) belongs to the genus *Deltaretrovirus* in the family *Retroviridae*. BLV is an important pathogen associated with enzootic bovine leukosis (EBL) [25]. Dairy and beef cattle farms faced significant economic losses due to EBL. Most BLV-infected cattle are asymptomatic, however, approximately 30% of infected cattle develop persistent lymphocytosis and 1%-5% develop malignant B-cell lymphoma [2]. BLV is primarily transmitted by transferring infected cells via blood-contaminated needles or gloves, surgical equipment, colostrum, and blood-sucking insects.

The complete genome of BLV is 8,714 nucleotides. The viral genome includes three major genes, *gag*, *pol*, and *env* [1]. The *env* gene encodes the surface glycoprotein GP51 and the transmembrane glycoprotein GP30, which are both essential for viral infectivity and immune evasion [32]. BLVgp51 gene has been the focus of phylogenetic analysis because of its crucial biological functions and its localization on the viral surface. Previous research has identified at least 10 BLV genotypes based on the genetic diversity of the BLVgp51 sequences [21].

The history of EBL in the world dates back to the 19th century. EBL was first recognized in dairy cattle in Germany in 1871 [18]. Following the initial report, this disease spread westward gradually during the early 20th century [3]. The number of EBL case reports in European countries had increased by the mid-twentieth century [6]. BLV has now spread to over 60 countries and regions around the world, and it has been successfully eradicated from European countries due to the implementation of systematic control and eradication programs [11, 21, 26, 29–31].

The first case of EBL in Japan was reported in 1927 in Iwate Prefecture, Northern Japan [16]. After the first report, the number of bovine leukosis cases has steadily increased. In 2020, 4,197 cases were reported as bovine leukosis according to the animal hygiene statics of Japan. The increase in the number of bovine leukoses is due to the increase in the number of BLV-infected cattle. While many studies have employed a phylogenetic study to examine BLV diversity, the results of these studies came from the analysis of viral gene sequences collected in the first two decades of the 21st century [19–21]. In this study, we performed PCR and sequencing analysis to characterize BLVgp51 sequences from formalin-fixed paraffin-embedded (FFPE) specimens made from

*Correspondence to: Kobayashi, T.: tk205370@nodai.ac.jp, Department of Animal Science, Faculty of Agriculture, Tokyo University of Agriculture, 1737 Funako, Atsugi, Kanagawa 243-0034, Japan

(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

1972 to 2000. We conducted a retrospective molecular study of the early BLV outbreak in Japan using the information on sample collection years. Moreover, the phylodynamic analysis was conducted to infer viral epidemiology, with the identification of the historical fluctuations in population dynamics.

A total of 28 FFPE specimens from 28 EBL cattle individuals were distributed from the archives of Livestock Hygiene Service Center and Meat Inspection Center in Japan Meat Inspection Center in Japan (Hokkaido Abashiri Livestock Hygiene Service Center, Hokkaido Nemuro Livestock Hygiene Service Center, Chiba Prefectural Chuo Livestock Hygiene Service Center, Chiba Prefectural Tousou Meat Inspection Office and Iwate Prefectural Chuo Livestock Hygiene Service Center) (Table 1). Most FFPE specimens were derived from lymph nodes or tumor tissues and preserved at room temperature (generated in the 1970s:10 specimens, in the 1980s: eight specimens, in the 1990s: nine specimens, in 2000: one specimen). These specimens were analyzed using data obtained in part from autopsies or postmortem inspection of slaughtered cattle by veterinary officers. All data or information exclude personally identifiable information, such as farmers' names. To confirm as these specimens were derived from lymphoma, they were sectioned to 5 µm thickness and incubated at 45°C for 2 hr and then stained with hematoxylin-eosin (data not shown).

FFPE specimens were deparaffinized with xylene, ethanol, and phenol-chloroform after being sliced with a surgical blade [28]. Briefly, 1 ml of xylene was added and centrifuged at 10,000 rpm, and the supernatant was removed. These steps were repeated once. Subsequently, 1 ml of 100% ethanol was added and centrifuged at 10,000 rpm, and the supernatant was removed. These steps were repeated once, and the DNA pellet was dried. Next, 675 µl of TE (pH9.0), 75 µl of 10% SDS, and 18.8 µl of Proteinase K (20 mg/µl) were added to the sample and incubated at 48°C for 24–48 hr with shaking (800 rpm). Afterward, 75 µl of 10% SDS and 18.8 µl of Proteinase K (20 mg/µl) were added and incubated for more than 24–48 hr with shaking. The supernatant was transferred into a clean tube without disturbing the pellet after 500 µl of TE-saturated phenol was added and centrifuged at 3,000 rpm. These steps were repeated once. Later, 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added and centrifuged at

Table 1. Sample information and PCR amplification of BLVgp51 of formalin-fixed paraffin-embedded (FFPE) specimens

Location		Collection date	Breed	Organ	PCR amplification								Detection of the BLV proviral genome	Daseignated name
Prefecture	City				A	B	C	D	E	F	G	H		
Hokkaido (n=9)														
	Nemuro	1987	Dairy cattle	Heart	+	-	+	+	+	-	-	-	+	
	Nemuro	1988	Holstein	Lymph nodes[a]	-	-	-	+	+	-	-	+	+	
	Nemuro	1989	ND	Lymph nodes[a]	+	-	-	+	+	-	-	-	+	
	Nemuro	1990	ND	Lymph nodes[a]	-	-	-	+	+	-	-	-	+	
	Nemuro	1993	Holstein	Lymph nodes[a]	-	-	-	+	+	-	-	+	+	
	Abashiri	1995	Holstein	Lymph nodes[a]	+	+	+	+	+	+	+	+	+	Hokkaido-1995
	Shiribeshi	1998	Beaf cattle	Unknown	-	-	-	-	-	-	-	-	-	
	Nemuro	1999	ND	Unknown	-	+	+	+	+	-	-	+	+	
	Abashiri	2000	ND	Heart	+	+	+	+	+	+	+	+	+	Hokkaido-2000
Iwate (n=8)														
	Kuji	1972	Japanese shot horn	Anterior lymph nodes	-	-	-	+	-	-	-	-	+	
	Hachimantai	1973	Holstein	Stomach	-	-	-	+	+	+	-	+	+	
	Hachimantai	1974	Holstein	Lymph nodes[a]	-	-	-	+	+	-	-	+	+	
	Sumita	1974	Japanese black cattle	Heart	+	+	+	+	+	+	+	+	+	Iwate-1974
	Sumita	1975	Japanese black cattle	Abomasum	-	+	-	+	+	-	-		+	
	Kuji	1975	Japanese shot horn	Intestine	-	+	-	+	+	-	-	+	+	
	ND[b]	1975	Japanese black cattle	Unknown	-	+	-	+	+	+	-	+	+	
	Kamaishi	1978	Japanese black cattle	Intestine	-	+	-	+	+	-	-	+	+	
Chiba (n=11)														
	Mutsuzawa	1974	Cross breed	Anterior lymph nodes	-	-	-	+	+	-	-	-	+	
	Ichihara	1974	Holstein	Lymph nodes[a]	-	+	+	+	+	+	+	+	+	Chiba-1974
	Ichihara	1981	Holstein	Iliac lymph nodes	+	+	+	+	+	+	+	+	+	
	Misaki	1984	Holstein	Heart	-	-	-	+	-	-	-	-	+	
	ND	1984	ND	Unknown	-	+	+	+	+	+	+	+	+	Chiba-1984
	ND	1987	ND	Unknown	-	+	+	+	+	+	+	+	+	Chiba-1987
	Sodegaura	1989	Holstein	Lymph nodes[a]	-	-	-	+	+	-	-	-	+	
	Kisarazu	1991	Holstein	Heart	-	-	-	+	+	+	-	-	+	
	Yamada	1992	Holstein	Lymph nodes[a]	-	-	-	+	-	-	-	-	+	
	ND	1996	Dairy cattle	Lymph nodes[a]	+	+	+	+	+	+	+	+	+	Chiba-1996
	ND	1997	Dairy cattle	Lymph nodes[a]	+	+	+	+	+	+	+	+	+	Chiba-1997

a: unspecified; b: no data.

3,000 rpm and the supernatant was transferred into a clean tube (around 400 µl) without disturbing the pellet. These steps were repeated once. Afterward, 16 µl of 5 M NaCl and 1 ml of 100% ethanol were added and incubated overnight for room temperature. Samples were centrifuged at 12,000 rpm for 30 min, and the supernatant was removed. Subsequently, 1 ml of 80% ethanol was added and centrifuged at 12,000 rpm for 30 min and dried up. Finally, 25 µl of nuclease-free water was added and gently mixed the DNA with the solution.

The BLV proviral genome was detected by PCR targeting BLVgp51 (903 bases total). We used eight overlapping primers designed to anneal to highly conserved BLVgp51 gene region (Fig. 1 and Supplementary Table 1). PCR was conducted with Expand High Fidelity PCR systems (Roche, Basel, Switzerland). The reaction mixture contained deoxynucleotide mix (200 µM), each primer (300 nM each), template DNA (<500 ng), 15 mM $MgCl_2$, and Expand High Fidelity enzyme mix (1.9 U/reaction). The reaction parameters were 2 min at 94°C, followed by 45 cycles of 15 sec at 94°C, 30 sec at 55° C and 45 sec at 72°C, and followed by a 7 min extension cycle at 72°C. The PCR products were analyzed by electrophoresis in a 2% agarose gel. The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced (Fasmac Inc., Yokohama, Japan). The Sequencer5.2.4 software was used for editing, alignment, and identification of nucleotide sequences (Gene Codes, Ann Arbor, MI, USA).

The phylogenetic tree was constructed using the maximum likelihood (ML) method with the Kimura 2-parameter model plus gamma distribution (K2+G) in MEGA7 [17]. The eight FFPE sequences obtained from this study with 192 representative BLV sequences with the collection years were retrieved from the GenBank database and aligned using MEGA7 (Supplementary Table 2). The robustness of the tree was assessed by performing 1,000 bootstrap replicates. Regression of root-to-tip genetic distance (inferred from ML trees) against the sampling time was performed by TempEst [24].

The Markov Chain Monte Carlo (MCMC) approach was employed using BEAST v2.4.8 to estimate the time to the most recent common ancestor (tMRCA) and effective population size with the following parameters: Nucleotide substitution model; HKY model + empirical, Clock model; Relaxed clock exponential, Prior; Coalescent exponential population, with 300,000,000 states, logged every 10,000 states, and 10% burn-in [5]. Tracer v1.5 was used to assess the sampling size effectiveness by MCMC (effective sample size (ESS) >200), and TreeAnnotator was used to construct the maximum clade credibility (MCC) tree [27]. Finally, the MCC tree was visualized using FigTree v1.4.4 software [22]. The effective sample size of 48 BLVgp51 sequences from Japan was estimated using the Bayesian skyline plot (BSP) in BEAST 2.4.8 with the following parameters: Nucleotide substitution model; HKY model + empirical, Clock model; Strict clock, Prior; Coalescent Bayesian Skyline, with 300,000,000 states. The BSP was visualized by Tracer v1.5 [23].

A total of 28 FFPE specimens generated before 2000 were analyzed for the BLV proviral sequences from archived samples. Detailed information on the samples is listed in Table 1. Using primer set D, BLVgp51 partial sequences were detected in 27 samples (96%) (amplicon size 148 bp). Moreover, full-length (903 bp) BLVgp51 sequences from five samples and nearly full-length (862 bp) sequences from three samples were successfully reconstructed (full-length: Hokkaido-1995, Hokkaido-2000, Iwate-1974, Chiba-1974, and Chiba-1984. Near full-length: Chiba-1995, Chiba-1996, and Chiba-1997). These results indicated that FFPE specimens, even if stored for 40 years, are useful for obtaining molecular information of viruses. The ML tree of BLVgp51 sequences revealed that these sequences belonged to genotype 1, implying that BLV genotype 1 has already been circulated in Japan since 1974 (Fig. 2). Moreover, there is a positive correlation between genetic divergence and sampling time and the coefficient of correlation with eight FFPE sequences was slightly higher than that without FFPE sequences (R^2=0.399) (Supplementary Fig. 1).

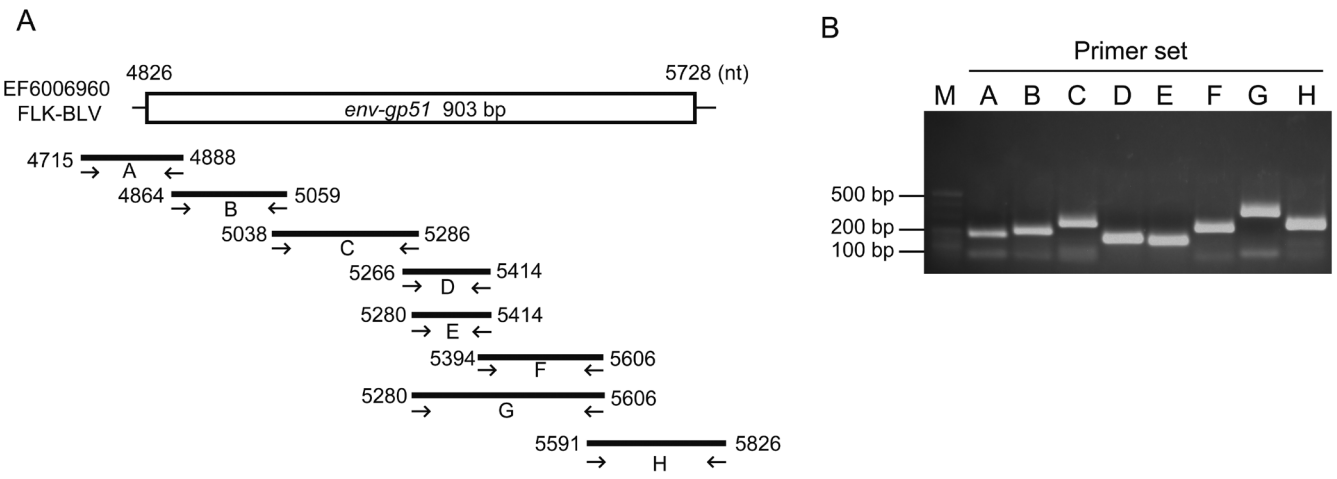

Fig. 1. Strategy for amplification and sequencing of bovine leukemia virus glycoprotein gp51 (BLVgp51) (nucleotide 4,826–5,728, 903 bp). (**A**) A schematic diagram shows the strategy for amplifying and sequencing the BLVgp51 in eight short PCR fragments. BLV genome is shown on top of the figure and the relative position of primers can be seen by the bar under the genome. (**B**) Amplification of BLVgp51 of representative formalin-fixed paraffin-embedded specimen (Hokkaido-1995) by eight sets of primers. Primer sets A to H are designated as in Supplementary Table 1. M: DNA ladder marker.

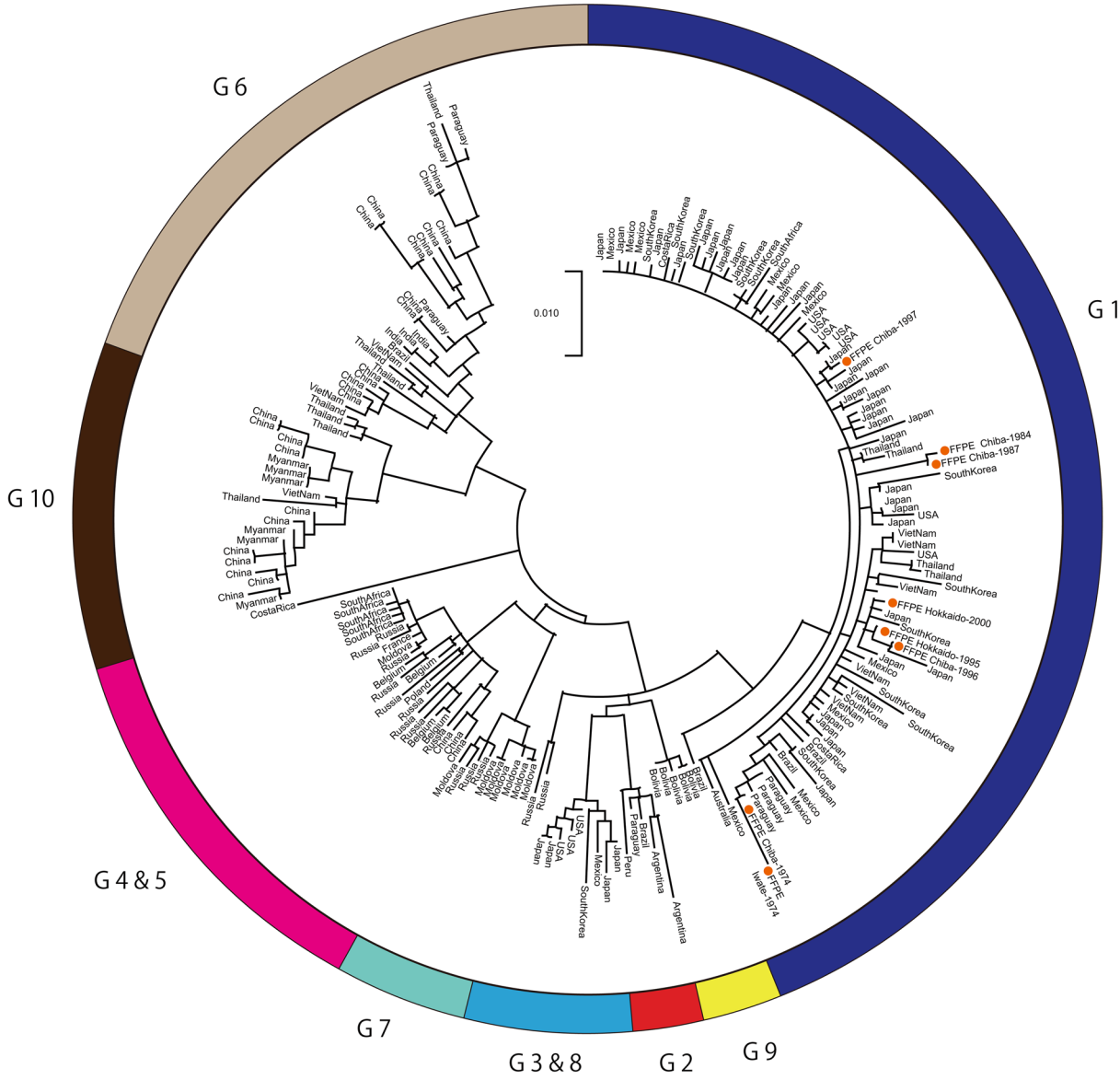

Fig. 2. Maximum likelihood (ML) phylogenetic tree of bovine leukemia virus glycoprotein gp51 (BLVgp51). ML phylogenetic tree of the 200 BLVgp51 sequences including eight sequences from formalin-fixed paraffin-embedded specimen (length 862 bp to 903 bp). These sequences were obtained from the GenBank database with collection date, and representative sequences of each genotype were used for phylogenetic analysis. G: Genotype.

These results indicated that FFPE sequence data are suitable for phylogenetic molecular clock analysis in BEAST.

The MCMC tree based on BLVgp51 sequences revealed that eight FFPE sequences were classified into three micro clades (MC1-MC3) (Fig. 3). MC1 included FFPE sequences collected in the 1970s (Iwate-1974 and Chiba-1974), but no additional sequences from Japan. The other sequences from Latin America strain (Paraguay and Mexico) belong to the same clade. MC2 and MC3 included FFPE sequences collected in the 1980s (Chiba-1984 and Chiba-1987) and 1990s to 2000 (Hokkaido-1995, Chiba-1996, Chiba-1997 and Hokkaido-2000), respectively. MC2 was confirmed in four countries: Japan, Korea, Mexico and South Africa. For MC3, Japan, Korea, and Mexico were confirmed as well as MC2, but Costa Rica, Thailand, and Vietnam were also included (Fig. 4).

The tMRCA of these MCs were inferred to be 1950 for MC1, 1962 for MC2, and 1967 for MC2 (Fig. 4). Moreover, we found that the tMRCA of the root of genotype 1 is 1922 (Fig. 4). The BSP analysis of sequences obtained from Japan indicated that the effective population size started to increase from the 1970s (Fig. 5). These results imply that the invasion of BLV have occurred multiple times from mid-1900s, probably during the period with increased cattle imports from overseas. As there are few BLV sequences reported before 2000, the sequences from FFPE specimens are useful for analyzing the early BLV epidemic phase in Japan.

FFPE specimens provide valuable information for retrospective molecular epidemiological studies [13]. However, DNA

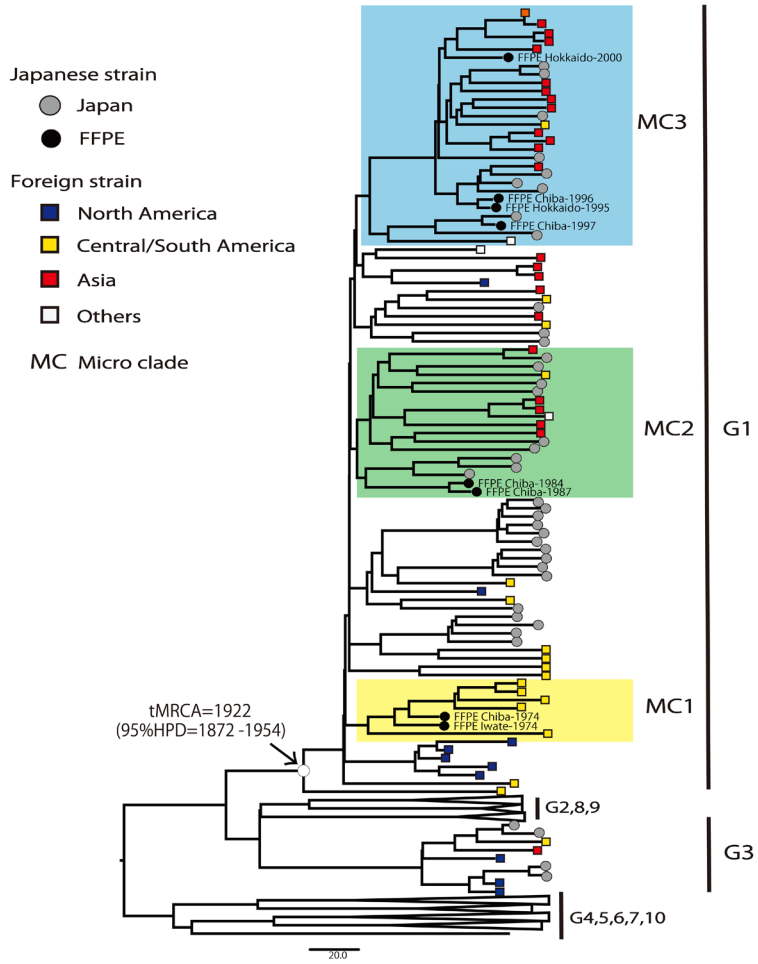

Fig. 3. Time-scaled Maximum clade credibility (MCC) tree of the bovine leukemia virus (BLV) glycoprotein gp51 (gp51). The time-scaled MCC tree of BLV was performed using 200 BLVgp51 sequences (length 862 bp to 903 bp). The phylogenetic and time to the most recent common ancestor (tMRCA) were estimated using relaxed molecular clock by BEAST v2.4.8. The years that the tMRCA were estimated to exist is shown for root of genotype 1. The sequences from formalin-fixed paraffin-embedded specimen and sequences from Japan are indicated by the black circle and gray circle. The foreign strains are indicated by square. MC: micro clade.

extraction and PCR amplification of FFPE specimens are significantly difficult because of DNA fragmentation, chemical modification, and inhibition of enzyme reactions by the effects of oxidation and formalin fixation during long-term storage [8–10]. In a previous study, more than 50% of the samples failed to amplify the target DNA after being stored for approximately 15 years [4]. Concerning BLV analysis, molecular genetic analysis of FFPE specimens of EBL cattle has been reported using FFPE specimens within three years of storage, but no reports have analyzed FFPE specimens stored for longer than that (Unpublished data of Meat Sanitation Inspection Station Miyagi Prefectural Government and Meat Inspection Office, Shimane Prefecture). In this study, we employed the phenol-chloroform approach to extract DNA from FFPE specimens. Today, many commercial kits are available for extracting DNA from FFPE specimens [15]. However, in our preliminary analysis, while relatively new FFPE specimens could analyze using these kits, the phenol-chloroform extraction method applied in this study yielded the most successful PCR amplification for samples that were created several decades ago.

There was a significant difference between primer sets whether the amplified product could be obtained or not. Using primer sets D and E (149 and 135 bp), which set for amplifying relatively short sequences, BLV proviral genome was successfully amplified in 27 (96%) and 25 (89%) samples, respectively. However, using primer sets C and G, which set for amplifying longer sequences (249 and 327 bp), BLV proviral genome was amplified in nine (32%) and ten (36%) samples, respectively. Furthermore, the detection of BLV proviral genome depends on storage conditions rather than storage period, that the proviral DNA was detected in the 1970s, 1980s, and 1990s in the same manner. All FFPE specimens used in this study were stored at room temperature, but in Japan, room temperature and humidity vary by region, so storage conditions also vary by facility. Moreover, reagents or procedures of FFPE preparation may also be different from each facility. Of these samples, the purity of the extracted DNA was high in the FFPE specimens that were quickly fixed in 20% formalin (Chiba-1996, Chiba-1997) and all PCR amplicons were obtained. On the other hand, the purity was low in the FFPE specimens that were severely damaged. These specimens may have been damaged due to incomplete dehydration and paraffin permeation during the FFPE preparation procedure. Incomplete dehydration and paraffin permeation prevented gene detection because the residual formalin in the water caused DNA fragmentation and Protease K inhibition. These results indicate that rapid fixation, sufficient dehydration, and paraffin permeation are important for obtaining high purity DNA. Overall, our results imply that the BLV proviral genome can be detected in specimens archived more than 40 years ago.

Using eight sets of primers for amplification, five full-length (903 bp) and three nearly full-length (862 bp) of BLVgp51 sequences were successfully reconstructed. Phylogenetic analysis indicated that these sequences were classified into genotype 1 (Fig. 3). Studies have reported that BLV genotype 1 is the most prevalent worldwide and reported in major BLV-positive countries, including North and South America, Africa, and Asia [19, 21, 31]. This genotype includes the earliest BLV sequence in Australia (D00647), at the same time it seems to be well established in the Americas and increasingly common in Asia [7, 31, 32]. Our results implied that genotype 1 has already been circulating in Japan since the 1970s. Early evidence of the prevalence of genotype 1 was also reported in the USA where sequences collected before 2000 were available (EF065644, EF065656, etc.). Moreover, sequences in MC1 are relatively close to those sequences from the foreign strain in the MCMC tree, indicating the

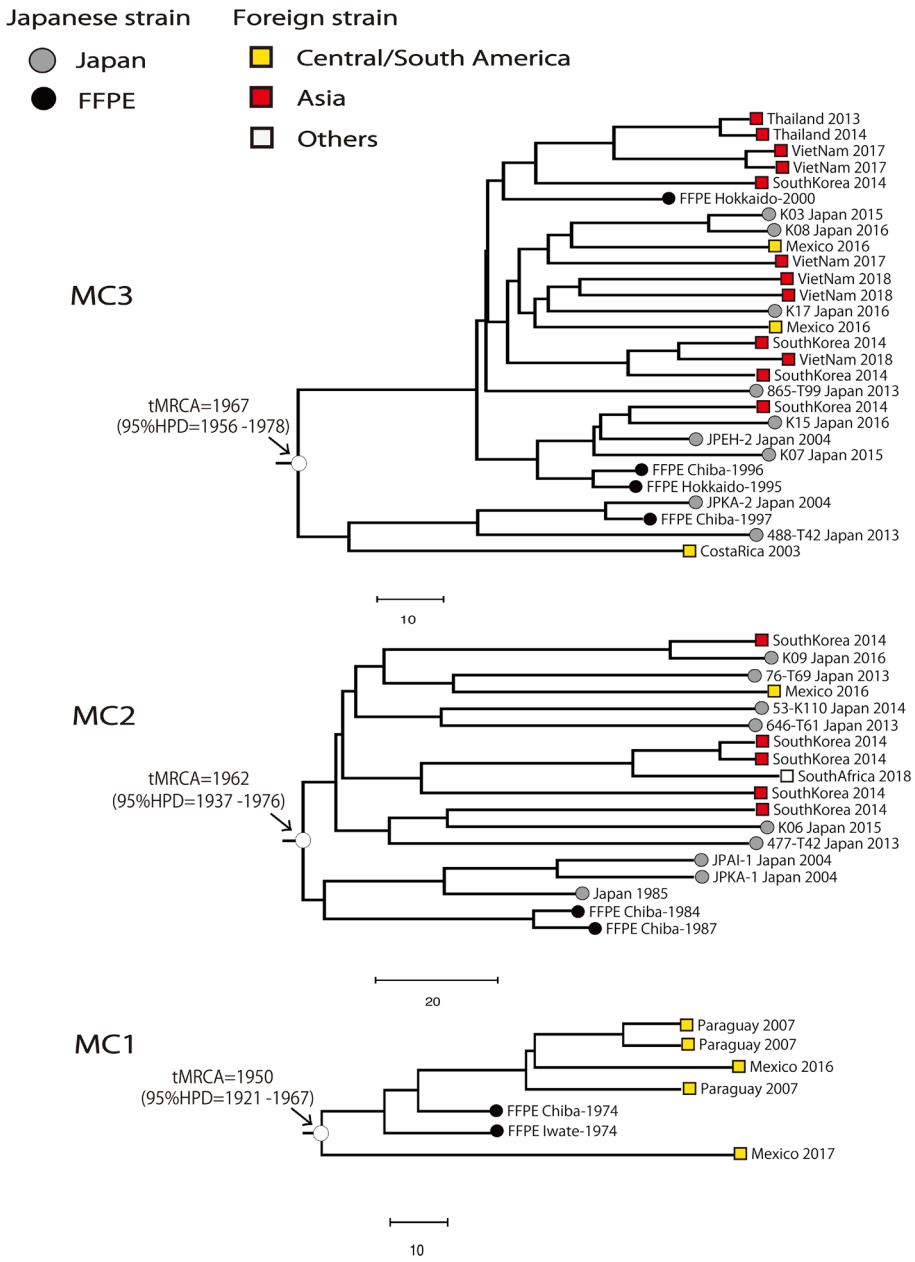

Fig. 4. The detailed time-scaled Maximum clade credibility (MCC) tree of bovine leukemia virus (BLV) genotype 1. The detailed trees of micro clade (MC) 1 to MC3 of the time-scaled MCC tree (Fig. 3) are shown. The root of each MC shows the estimated time to the most recent common ancestor (tMRCA). The country and collection date are indicated for each Taxa. The sequence information used in phylogenetic analysis were listed in Supplementary Table 2.

close relationships of BLV sequences between Japan and the foreign strains. These results further indicate that the introduction of livestock from overseas poses a risk of spreading infectious diseases into Japan.

A previous study reported that, although the infection rate was low, BLV has already spread throughout Japan by 1980 [12]. In the 1970s, the number of live cattle imported to Japan from overseas significantly increased, especially from the USA, Canada, and South Korea according to the animal quarantine statics of Japan. BSP analysis of BLVgp51 sequences obtained from Japan indicated that the effective population size of BLV started to increase from the 1970s (Fig. 5). As four sequences from FFPE specimens generated between 1970 and 1980, these sequences provide important source of information on the diversity of viral sequences in the early phase of the epidemic in Japan. Some FFPE sequences, including the oldest specimens generated in 1974 (Iwate-1974 and Chiba-1974) formed a distinct MC (MC1), which is not mainly circulating in Japan today. MC2 FFPE sequences (Chiba-1984 and Chiba-1987) spread to Japan on distinct introduction events from other clades. Further, as FFPE sequences of MC3 share a relatively new tMRCA with the sequences from foreign countries, these sequences were introduced to Japan more recently.

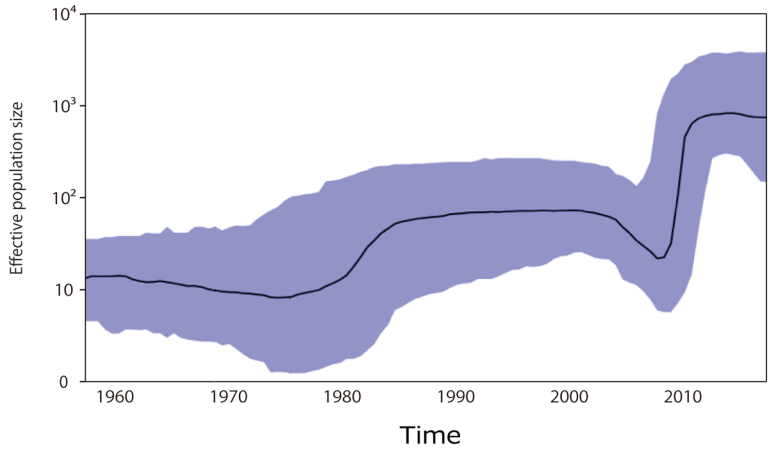

Fig. 5. Bayesian Skyline plots (BSP) of the effective sample size through the time of the bovine leukemia virus glycoprotein gp51 (BLVgp51) sequences from Japan. The posterior median estimate is indicated by the black line, and the blue shaded areas correspond to the 95% high posterior density. The X-axis represents time in years, whereas Y-axis shows the effective population size (Ne). BSP, based on a strict clock coalescent framework analysis was constructed using 48 sequences collected in Japan by BEAST v.2.4.8.

BLV was prevalent within Europe in the 19th century and expanded to the Americas in the 20th century. Then, with the export of cattle from North America, BLV is said to have expanded worldwide from the mid-1900s [14]. In fact, genotypes 1 and 3 emerged in the 20th century and were introduced into Japan in the mid-1900s after spreading worldwide from the USA and Brazil, which is consistent with phylogenetic analysis and previous epidemiological reports.

This study has a practical limitation. The actual sequence of the FFPE specimens could not be obtained for the primer region (70 bp of the sequences). We tried several primers patterns to amplify the whole BLVgp51 sequence in FFPE samples, however, because of the fragmentation of the DNA from FFPE specimens, only eight sets of primers worked for successful PCR amplification in our study (data not shown). These regions in BLVgp51 sequences were highly conserved, and 99.8% of the bases in these regions in the sequences reported in Japan matched the consensus sequence. However, analysis using the obtained sequences might result in underestimating the genetic diversity of the FFPE specimens' sequences. Despite this limitation, this is the first study to employ FFPE specimens to investigate the genetic diversity of the early BLV epidemic in Japan. It should be noted, however, that our knowledge of the genetic diversity of BLV in Japan is still limited. BLV sequences from diverse prefectures and from all pandemic periods to get a better understanding of the evolution and dissemination of BLV.

This study reveals that archived FFPE specimen analyses provide useful information not only for pathological diagnosis but also for molecular analysis. As public Livestock Hygiene Service Centers and Meat Inspection Offices hold a large quantity of past FFPE specimens from a wide variety of species, this study indicates that those specimens may serve as a valuable information source for retrospective livestock analysis.

CONFLICT OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENTS. This study was supported in part by JSPS KAKENHI Grants-in-Aid for Scientific Research C 21K05943 and Livestock Promotional Funds of Japan Racing Association. We would like to express our gratitude to Ms. Ryoko Tanaka for the helpful suggestions and deeply thanks to the staff of each Livestock Hygiene Service Center (Abashiri, Nemuro Shiribeshi and Rumoi in Hokkaido, Chuo in Chiba and Chuo in Iwate) and to the staff of Tousou Meat Inspection Center in Chiba for providing FFPE specimens and information about EBL cattle.

REFERENCES

1. Aida, Y., Murakami, H., Takahashi, M. and Takeshima, S. N. 2013. Mechanisms of pathogenesis induced by bovine leukemia virus as a model for human T-cell leukemia virus. *Front. Microbiol.* **4**: 328. [Medline] [CrossRef]
2. Barez, P. Y., de Brogniez, A., Carpentier, A., Gazon, H., Gillet, N., Gutiérrez, G., Hamaidia, M., Jacques, J. R., Perike, S., Neelature Sriramareddy, S., Renotte, N., Staumont, B., Reichert, M., Trono, K. and Willems, L. 2015. Recent Advances in BLV Research. *Viruses* **7**: 6080–6088. [Medline] [CrossRef]
3. Bendixen, H. 1963. *Leukosis Enzootica Bovis. Diagnostik, Epidemiologi, Bekaempelse* Carl F. Mortensen, Copenhagen.
4. Bolognesi, C., Forcato, C., Buson, G., Fontana, F., Mangano, C., Doffini, A., Sero, V., Lanzellotto, R., Signorini, G., Calanca, A., Sergio, M., Romano, R., Gianni, S., Medoro, G., Giorgini, G., Morreau, H., Barberis, M., Corver, W. E. and Manaresi, N. 2016. Digital sorting of pure cell populations enables unambiguous genetic analysis of heterogeneous formalin-fixed paraffin-embedded tumors by next generation sequencing. *Sci. Rep.* **6**: 20944. [Medline] [CrossRef]
5. Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C. H., Xie, D., Suchard, M. A., Rambaut, A. and Drummond, A. J. 2014. BEAST 2: a

software platform for Bayesian evolutionary analysis. *PLOS Comput. Biol.* **10**: e1003537. [Medline] [CrossRef]
6. Burny, A., Bruck, C., Chantrenne, H., Cleuter, Y., Dekegel, D., Ghysdael, J., Kettmann, R., Leclercq, M., Leunen, J. and Mammerickx, M. 1980. Bovine leukemia virus: Molecular biology and epidemiology. pp. 231–289. In: Viral Oncology (Klein, G, ed.), Raven Press, New York.
7. Coulston, J., Naif, H., Brandon, R., Kumar, S., Khan, S., Daniel, R. C. and Lavin, M. F. 1990. Molecular cloning and sequencing of an Australian isolate of proviral bovine leukaemia virus DNA: comparison with other isolates. *J. Gen. Virol.* **71**: 1737–1746. [Medline] [CrossRef]
8. Dedhia, P., Tarale, S., Dhongde, G., Khadapkar, R. and Das, B. 2007. Evaluation of DNA extraction methods and real time PCR optimization on formalin-fixed paraffin-embedded tissues. *Asian Pac. J. Cancer Prev.* **8**: 55–59. [Medline]
9. Dietrich, D., Uhl, B., Sailer, V., Holmes, E. E., Jung, M., Meller, S. and Kristiansen, G. 2013. Improved PCR performance using template DNA from formalin-fixed and paraffin-embedded tissues by overcoming PCR inhibition. *PLoS One* **8**: e77771. [Medline] [CrossRef]
10. Feldman, M. Y. 1973. Reactions of nucleic acids and nucleoproteins with formaldehyde. *Prog. Nucleic Acid Res. Mol. Biol.* **13**: 1–49. [Medline] [CrossRef]
11. Gautam, S., Mishra, N., Kalaiyarasu, S., Jhade, S. K. and Sood, R. 2018. Molecular characterization of bovine leukaemia virus (BLV) strains reveals existence of genotype 6 in cattle in India with evidence of a new subgenotype. *Transbound. Emerg. Dis.* **65**: 1968–1978. [Medline] [CrossRef]
12. Ito, T. 1987. Statistics on regional prevalence of gp antibody against bovine leukosis virus in Japan. *Bull. Natl. Inst. Anim. Health* **90**: 35–60.
13. Iwamoto, K. S., Mizuno, T., Ito, T., Akiyama, M., Takeichi, N., Mabuchi, K. and Seyama, T. 1996. Feasibility of using decades-old archival tissues in molecular oncology/epidemiology. *Am. J. Pathol.* **149**: 399–406. [Medline]
14. Johnson, R. and Kaneene, J. B. 1992. Bovine leukemia virus and enzootic bovine leukosis. *Vet. Bull.* **62**: 287–312.
15. Kocjan, B. J., Hošnjak, L. and Poljak, M. 2015. Commercially available kits for manual and automatic extraction of nucleic acids from formalin-fixed, paraffin-embedded (FFPE) tissues. *Acta Dermatovenerol. Alp. Panonica Adriat.* **24**: 47–53. [Medline]
16. Kubota, G. 1927. A case report of lymphosarcoma. *Chuoujuikaizasshi* **40**: 375–378.
17. Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**: 1870–1874. [Medline] [CrossRef]
18. Leisering, A. 1871. Hypertrophy der Malpighischen Körperoen der Milz. *Bericht uber das Veterinarwesen im Konigreich* **16**: 15–16 (in German).
19. Marawan, M. A., Mekata, H., Hayashi, T., Sekiguchi, S., Kirino, Y., Horii, Y., Moustafa, A. M., Arnaout, F. K., Galila, E. S. M. and Norimine, J. 2017. Phylogenetic analysis of env gene of bovine leukemia virus strains spread in Miyazaki prefecture, Japan. *J. Vet. Med. Sci.* **79**: 912–916. [Medline] [CrossRef]
20. Mekata, H., Sekiguchi, S., Konnai, S., Kirino, Y., Horii, Y. and Norimine, J. 2015. Horizontal transmission and phylogenetic analysis of bovine leukemia virus in two districts of Miyazaki, Japan. *J. Vet. Med. Sci.* **77**: 1115–1120. [Medline] [CrossRef]
21. Polat, M., Takeshima, S. N. and Aida, Y. 2017. Epidemiology and genetic diversity of bovine leukemia virus. *Virol. J.* **14**: 209. [Medline] [CrossRef]
22. Rambaut, A. 2019. Figtree v1.4.4. http://tree.bio.ed.ac.uk/software/figtree [accessed on Octorber 21, 2021].
23. Rambaut, A. and Drummond, A. 2009. Tracer v1.5.0. http://beast.bio.ed.ac.uk/Tracer [accessed on Octorber 21, 2021].
24. Rambaut, A., Lam, T. T., Max Carvalho, L. and Pybus, O. G. 2016. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol.* **2**: vew007. [Medline] [CrossRef]
25. Rodríguez, S. M., Florins, A., Gillet, N., de Brogniez, A., Sánchez-Alcaraz, M. T., Boxus, M., Boulanger, F., Gutiérrez, G., Trono, K., Alvarez, I., Vagnoni, L. and Willems, L. 2011. Preventive and therapeutic strategies for bovine leukemia virus: lessons for HTLV. *Viruses* **3**: 1210–1248. [Medline] [CrossRef]
26. Ruzina, M. N., Andrianov, B. V., Shaĭkhaev, G. O. and Sulimova, G. E. 2012. [Identification and classification of bovine leukemia virus isolates in Russia and Ukraine based on the pol viral gene polymorphism]. *Genetika* **48**: 855–862. [Medline]
27. Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J. and Rambaut, A. 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol.* **4**: vey016. [Medline] [CrossRef]
28. Tamura, G. 2004. Pathology and Clinical Medicine. pp. 371–375. vol. 22 an extra ed., Bunkodo, Tokyo.
29. Wang, M., Wang, Y., Baloch, A. R., Pan, Y., Xu, F., Tian, L. and Zeng, Q. 2018. Molecular epidemiology and characterization of bovine leukemia virus in domestic yaks (*Bos grunniens*) on the Qinghai-Tibet Plateau, China. *Arch. Virol.* **163**: 659–670. [Medline] [CrossRef]
30. Yang, Y., Kelly, P. J., Bai, J., Zhang, R. and Wang, C. 2016. First molecular characterization of bovine leukemia virus infections in the Caribbean. *PLoS One* **11**: e0168379. [Medline] [CrossRef]
31. Yu, C., Wang, X., Zhou, Y., Wang, Y., Zhang, X. and Zheng, Y. 2019. Genotyping bovine leukemia virus in dairy cattle of Heilongjiang, northeastern China. *BMC Vet. Res.* **15**: 179. [Medline] [CrossRef]
32. Zhao, X. and Buehring, G. C. 2007. Natural genetic variations in bovine leukemia virus envelope gene: possible effects of selection and escape. *Virology* **366**: 150–165. [Medline] [CrossRef]