Arch Virol (2017) 162:1051–1056
DOI 10.1007/s00705-016-3205-0

BRIEF REPORT

Clinical and subclinical bovine leukemia virus infection in a dairy cattle herd in Zambia

Girja S. Pandey[1] · Edgar Simulundu[1] · Danstan Mwiinga[1] · Kenny L. Samui[1] · Aaron S. Mweene[1] · Masahiro Kajihara[2] · Alfred Mangani[3] · Racheal Mwenda[3] · Joseph Ndebe[1] · Satoru Konnai[4] · Ayato Takada[1,2,5]

Received: 31 October 2016 / Accepted: 11 December 2016 / Published online: 26 December 2016

Abstract Bovine leukemia virus (BLV) causes enzootic bovine leucosis (EBL) and is responsible for substantial economic losses in cattle globally. However, information in Africa on the disease is limited. Here, based on clinical, hematological, pathological and molecular analyses, two clinical cases of EBL were confirmed in a dairy cattle herd in Zambia. In contrast, proviral DNA was detected by PCR in five apparently healthy cows from the same herd, suggesting subclinical BLV infection. Phylogenetic analysis of the *env* gene showed that the identified BLV clustered with Eurasian genotype 4 strains. This is the first report of confirmed EBL in Zambia.

G. S. Pandey and E. Simulundu contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-016-3205-0) contains supplementary material, which is available to authorized users.

✉ Girja S. Pandey
pandeygs@gmail.com

[1] Department of Disease Control, School of Veterinary Medicine, University of Zambia, PO Box 32379, Lusaka, Zambia

[2] Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

[3] Department of Paraclinical Studies, School of Veterinary Medicine, University of Zambia, PO Box 32379, Lusaka, Zambia

[4] Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

[5] Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo, Japan

Bovine leukemia virus (BLV), along with the closely related human T-cell leukemia virus type 1 (HTLV-1), belongs to the genus *Deltaretrovirus*, family *Retroviridae* [1]. Although the natural host of BLV is domestic cattle, this pathogen is considered a model for understanding leukemogenesis and development of novel therapies for HTLV-induced disease in humans [2, 3]. While previous epidemiological studies suggest that consumption of raw milk from BLV-infected cattle may not increase the frequency of leukemia in humans [3], there is need for caution in regarding BLV as a pathogen that is exclusively confined to animals, as recent reports indicate that BLV could be associated with breast cancer in women [4, 5].

BLV causes enzootic bovine leucosis (EBL), a disease characterized by neoplastic proliferation of B lymphocytes [6]. Although the majority of BLV-infected cattle remain asymptomatic, persistent lymphocytosis, which is associated with non-malignant polyclonal expansion of B-cells, occurs in about 30% of infected cattle, with less than 10% developing malignant lymphoma [7, 8]. BLV transmission is primarily through blood of infected cattle, particularly during iatrogenic procedures (e.g., use of infected needles) [9]. Hematophagus flies are also possible contributors in the spread and transmission of BLV [10].

Despite its global distribution, BLV infection is underdiagnosed and usually perceived as of minor economic impact. However, BLV infections can cause substantial economic losses. For instance, in 2003 in the USA, annual economic losses to the dairy industry attributed to BLV infection were approximated at $525 million [11]. Thus, with the aim of reducing economic losses and spread of the disease through trade in animals, some European countries have implemented control measures that have led to the successful eradication of BLV infection in cattle [12, 13]. However, for most countries, the prospects of eradicating

BLV in cattle are curtailed by high prevalence rates as well as the huge economic cost of such control measures.

In Africa, BLV infection in cattle has been reported in several countries [14–20]. While these studies suggest that BLV infections could be widespread in Africa, studies focusing on genetic characterization and classification of BLV circulating in cattle on the continent are very limited. Thus far, molecular characterization of the BLV *env* glycoprotein (gp51) gene sequences obtained globally has revealed the existence of at least ten genotypes [21–23]. In terms of distribution, genotypes 1 and 4 have been detected on several other continents, while genotypes 2, 5, 6 and 9 have been found mostly in South American countries. Meanwhile, genotypes 7 and 8 have been identified in Russia and Eastern European countries. The recently identified genotype 10 was found in Thailand [22]. In Africa, the BLV genotypic distribution is unknown.

In Zambia, serological evidence of BLV infection was found in 5.0% of the animals in traditional cattle herds by agar gel immunodiffusion test [19]. Moreover, bovine lymphosarcoma was also histopathologically diagnosed and described [20]. Although these studies were very limited, their results suggest that BLV could be an important infectious disease of cattle in Zambia. The present study describes diagnostic investigations of EBL in a dairy cattle herd in Zambia.

In March 2015, at a dairy farm in Central Province of Zambia, a three-year-old Holstein Friesian cow in lactation was observed to be slowly losing weight. It also exhibited marked reduction in milk production, lymphadenopathy, constipation, and a temperature of 39.0 °C. Blood and lymph node biopsy samples were obtained for hematological evaluation and East Coast fever diagnosis on 25 April, 2015. By April 2015, the cow's condition deteriorated into severe emaciation, and it was slaughtered upon recommendation at an abattoir on 20 May, 2015. A postmortem examination was conducted.

On 6 July, 2016, a second case (with symptoms that were similar to but more severe than those in the first cow), also involving a three-year-old Holstein Friesian cow in lactation (Supplementary Fig. S1a) was noticed in the same herd. Blood was collected for hematology and polymerase chain reaction (PCR) diagnosis. The animal died on 18 August, 2016, and a detailed postmortem examination was done at the farm along with collection of organ samples showing lesions for histopathology and PCR diagnosis. Thin blood smears were prepared and stained with Giemsa, and a differential cell count was done, including evaluation of other blood parameters. Mesenteric lymph nodes and kidneys were collected and tested for the presence of BLV proviral DNA by PCR. Blood from five apparently healthy cows aged 7-10 years from the same herd was also collected from the jugular vein in EDTA for hematological and PCR evaluation. At the time of diagnostic investigations, the affected farm had 1020 lactating Holstein Friesian cows.

Hematological evaluation of both symptomatic cows demonstrated severe anemia, an increased total leukocyte count, an increased absolute total lymphocyte count, and lymphocytosis (Table 1 and Supplementary Fig. S2a). Moreover, cells resembling neoplastic lymphocytes were observed in blood smears of both clinical cases and one

Table 1 Hematological evaluation of Holstein Friesian cows on a dairy farm in Zambia

Case/cow no.[a]	TWBC ($\times 10^3$/µl) NR: 4-12	ATL $\times 10^3$/µl NR: 2.5-7.5	TRBC $\times 10^6$ /µl NR: 5-10	PCV % NR: 24-46	Hb g/dl NR: 8-15	MCV Fl NR: 40-60	MCHC g/dl NR: 30-36	MCH pg NR: 11-17	BN % NR: 0-2	SN % NR: 15-45	L % NR: 45-75	E % NR: 0-20	M % NR: 2-7	B % NR: 0-2
1	11.4	11.1	4.5	19	6.3	42.2	33.2	14.0	0	2	98	0	0	0
2	12.2	11.4	5.1	20	6.7	39.2	33.5	11.8	0	5	94	1	0	0
3	7.3	4.5	6.1	25	8.3	40.9	33.2	13.6	0	36	62	2	0	0
4	8.4	5.2	6.8	28	9.3	41.2	33.2	13.7	0	33	63	4	0	0
5	9.0	6.9	7.3	30	10.0	41.1	33.3	13.7	0	20	77	3	0	0
6	7.2	3.2	6.0	25	8.3	41.7	33.2	13.8	1	46	45	8	0	0
7	8.7	5.5	7.0	30	10.0	42.9	33.3	14.3	3	30	64	2	1	0

[a] Cases 1 and 2 represent cows that showed clinical signs of EBL, while cases 3-7 were apparently healthy cows. Abbreviations: NR, normal range; TWBC, total white blood cell count; ATL, absolute total lymphocyte count; TRBC, total red blood cell count; PCV, packed cell volume; Hb, hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; BN, band neutrophil; SN, segmented neutrophil; L, lymphocyte; E, eosinophil; M, monocyte; B, basophil

apparently healthy cow (Supplementary Fig. S2a). No blood parasites were seen either in blood or lymph node smears. Hematological examination of blood collected from five apparently healthy cows gave values that were within the normal range, although the total white blood cell count, absolute total lymphocyte count, and lymphocyte percentage in cows 3 and 4 were elevated (Table 1). Moreover, cow 5 showed an above-normal lymphocyte percentage of 77.

Postmortem examination revealed similar lesions in both clinical cases, although the lesions were more pronounced and involved many organs in the second case. Gross and histopathological findings are described in Supplementary Fig. S1 and S2. Generally, gross and histopathological examination of internal organs revealed lymphoproliferative masses and lymphoma characterized by diffuse proliferation of neoplastic lymphocytes with obliteration of most normal tissues, respectively. Notably, the large and small intestine had minute to large white nodules on the serosal surface that did not appear to have perforated the mucosa (Supplementary Fig. S1e).

For molecular analysis, DNA was extracted from all blood samples and tissues (lymph node and kidney) using a QIAamp® DNA Mini Kit (QIAGEN). The primer pair *env*5032/*env*5608r, which amplifies a 598-bp fragment of the BLV *env* gene was used in the test [24]. PCR was performed using a Takara Ex Taq HS kit (Takara Bio Inc.) following the manufacturer's recommendations. Amplified PCR products were visualized after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. PCR products of the expected size for the BLV *env* gene were amplified in all the tested samples (Supplementary Fig. S3).

Amplified PCR products were sequenced, and all the samples produced the same sequence, which was deposited in GenBank under accession number LC193462. Phylogenetic analysis was conducted as described previously [25].

Phylogenetic analysis showed that the virus identified in Zambia clustered with genotype 4 strains found in Eurasia (Fig. 1). Sequence alignment of representative genotype 4 viruses revealed that the Zambian BLV possessed a unique amino acid substitution, Q39R, in the second neutralizing domain (ND) (Fig. 2). Also, within the zinc-binding peptide region, an I51T substitution was observed in the predicted *env* protein sequence of the virus detected in Zambia.

In this study, clinical findings on two suspected cases of EBL were consistent with previous descriptions, particularly with regard to predominant signs such as progressive weight loss, generalized lymphadenopathy, decreased milk production, and diarrhea or constipation [26, 27]. Hematological parameters, especially the absolute total lymphocyte counts of symptomatic cases supported the diagnosis of EBL/persistent lymphocytosis. The results were comparable to those reported for BLV-positive cattle

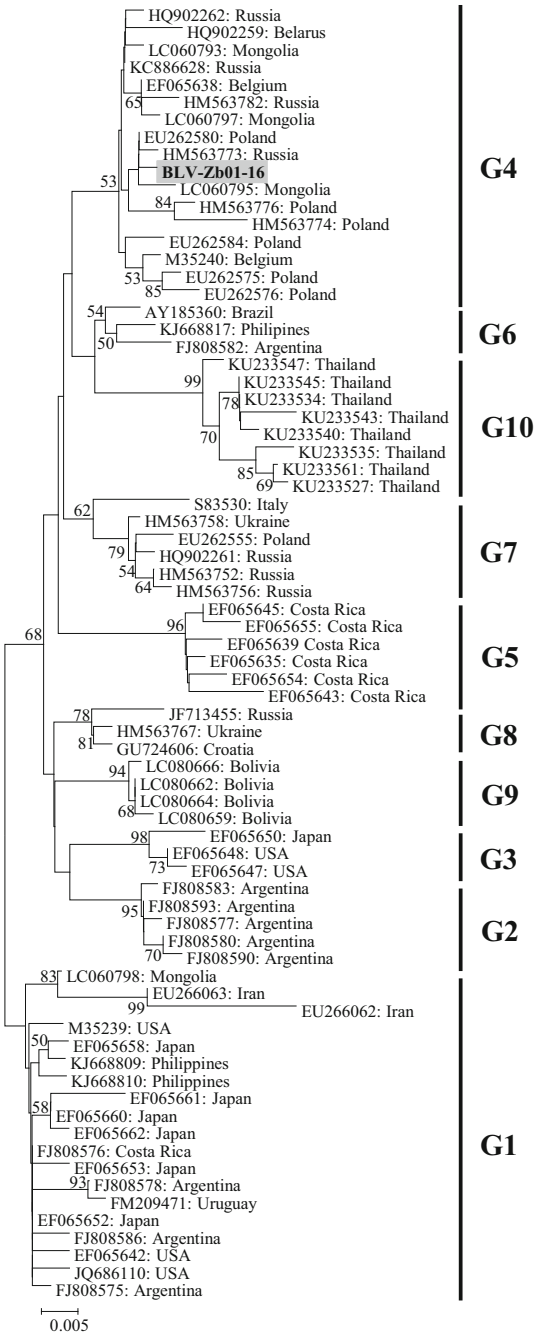

Fig. 1 Phylogenetic relationships of nucleotide sequences of the *env* gene of BLV detected in a dairy cattle herd in Zambia. Phylogenetic analysis was conducted based on 400 bp of *env* gene sequences of BLVs. The BLV sequences included in the analysis are shown by GenBank accession numbers and country of origin. The BLV identified in the present study is in bold and shaded in grey. Numbers at branch nodes indicate bootstrap values ≥50%. The genotypes (G1-G10) are indicated by vertical lines. Scale bar, number of substitutions per site

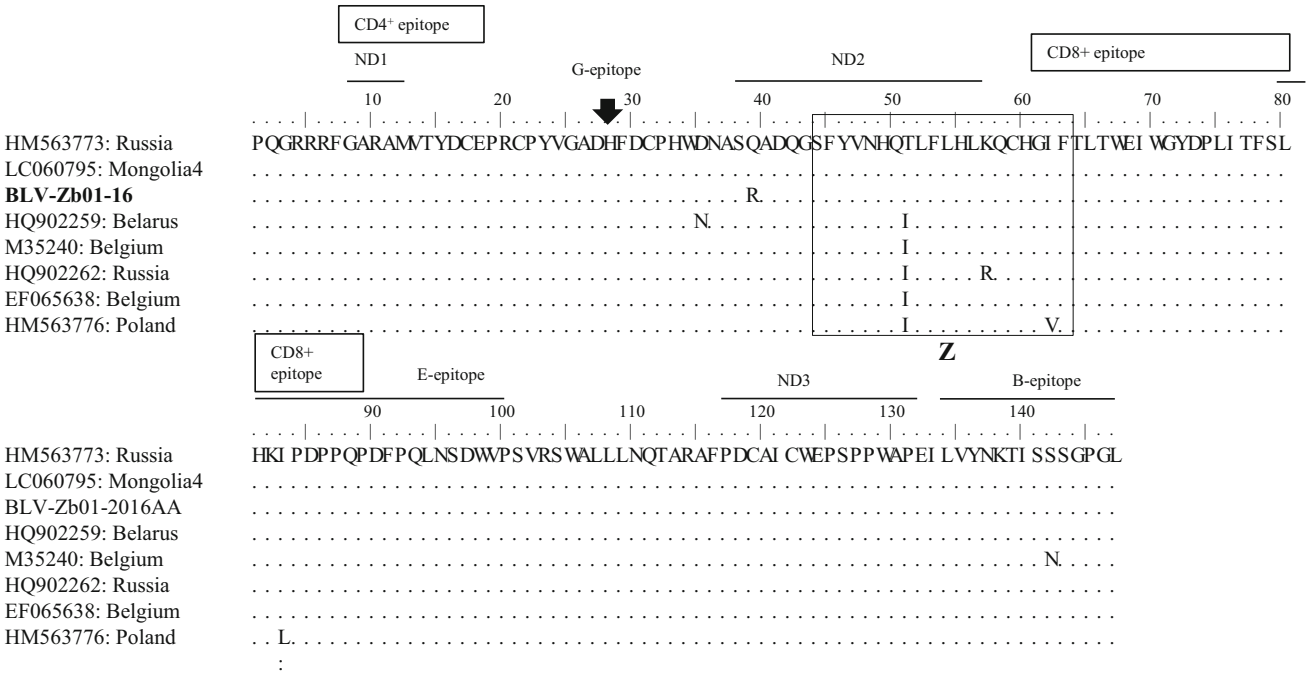

Fig. 2 Amino acid sequence alignment of partial sequence of the *env* protein of the Zambian BLV compared to genotype 4 viruses detected in Eurasia. The virus analyzed in this study is in boldface. The BLV sequences included in the alignment are shown by GenBank accession numbers and countries of origin. Antigenic determinants (epitopes) are indicated either by rectangles, solid lines, or a downward arrow at the top of the alignment. The black frame on the alignment labeled "**Z**" refers to the zinc-binding peptide region. Black dots indicate amino acid sequence identity to the Russian BLV strain (accession no. HM563773)

elsewhere [28]. Although hematological values were normal among the five apparently healthy cows that were examined, the percentage of lymphocytes was elevated in some cows. Along with the detection of BLV proviral DNA by PCR in all of the asymptomatic animals, these data may indicate that the animals were in a 'carrier state' and that the disease could be in progression.

Pathological findings (Supplementary Fig. S1 and S2) were indicative of malignant lymphoma development, which occurs in less than 10% of BLV-infected cattle. Although BLV-induced lymphomas mostly occur in older animals, finding such lesions in visceral organs of young cows suggests that these animals may have acquired the infection quite early in life, probably at birth, through colostrum [29]. Considering that BLV becomes established in dairy herds before the first lactation [30], there is need to conduct a detailed serological survey of BLV infection in the affected herd so that infected cattle could be separated and eliminated from the herd in order to break the chain of transmission to young susceptible animals. While the occurrence of lymphomas in the internal organs of the cows examined was not unusual, the presence of multiple small whitish nodules on the serosal surface of the large intestine may represent an uncommon manifestation of the disease.

The detection of the BLV *env* gene in all the tested animals by PCR seems to suggest that BLV could be well established in the affected herd. There is therefore a need to introduce mandatory testing for BLV infection in dairy and possibly beef cattle for prevention and control of such infectious diseases in the country.

Phylogenetic analysis of the Zambian BLV *env* gene sequence showed a close relationship to genotype 4 viruses of Eurasian origin, indicating that the virus may have been imported into the country with exotic cattle breeds. Although the country of origin of dairy cattle in the tested herd could not be ascertained, exotic cattle in Zambia are usually imported from Europe and South Africa. Incidentally, BLV infection is widespread among dairy cattle in South Africa [17, 18]. However, molecular epidemiological linkages could not be made with regard to the transboundary spread of BLV within the region due to the lack of sequence data from African countries. Nevertheless, it has been suggested that the detection of BLV genotype 4 strains on multiple continents can be explained by extensive cattle trading [23]; hence, the need for introduction of mandatory screening of all cattle imported into Zambia cannot be overemphasized.

Amino acid sequence alignment of partial sequences of the *env* protein of the BLV detected in Zambia revealed

two substitutions (Fig. 2). While the Q-to-R substitution has not been reported previously until now and its impact on the biological features of the virus remain unknown, the I-to-T substitution has been reported and is thought, along with other substitutions, to influence epitope-H-specific antibody recognition [23, 31].

For the first time, this study has provided tangible evidence of the presence of EBL in Zambia. From a public health perspective, considering that BLV could be associated with breast cancer in women [4, 5], consumption of pasteurized or boiled milk should be promoted in Zambia. Moreover, awareness of BLV infection must be raised among all stakeholders, particularly among farmers and veterinarians, to mitigate economic losses and spread of the infection.

Acknowledgements This work was supported by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and the Japan Agency for Medical Research and Development (AMED)/ Japan International Cooperation Agency (JICA) within the framework of the Science and Technology Research Partnership for Sustainable Development (SATREPS).

Compliance with ethical standards

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

References

1. King AMQ, Adams J, Carstens EB, Lefkowitz EJ (2012) Virus taxonomy: classification and nomenclature of viruses: ninth report of the ICTV. Elsevier/Academic Press, Amsterdam, p 1344
2. Aida Y, Murakami H, Takahashi M, Takeshima SN (2013) Mechanisms of pathogenesis induced by bovine leukemia virus as a model for human T-cell leukemia virus. Front Microbiol 4:328
3. Gillet N, Florins A, Boxus M, Burteau C, Nigro A, Vandermeers F, Balon H, Bouzar AB, Defoiche J, Burny A, Reichert M, Kettmann R, Willems L (2007) Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. Retrovirology 4:18
4. Buehring GC, Shen HM, Jensen HM, Choi KY, Sun DJ, Nuovo G (2014) Bovine leukemia virus DNA in human breast tissue. Emerg Infect Dis 20:772–782
5. Buehring GC, Shen HM, Jensen HM, Jin DL, Hudes M, Block G (2015) Exposure to bovine leukemia virus is associated with breast cancer: a case-control study. PLoS One 10(9):e0134304
6. Burny A, Cleuter Y, Kettmann R, Mammerickx M, Marbaix G, Portetelle D, Van den Broeke A, Willems L, Thomas R (1987) Bovine leukaemia: facts and hypothesis derived from the study of an infectious cancer. Cancer Surv 6:139–159
7. Mirsky ML, Olmstead CA, Da Y, Lewin HA (1996) The prevalence of proviral bovine leukemia virus in peripheral blood mononuclear cells at two subclinical stages of infection. J Virol 70:2178–2183
8. Ghysdael J, Bruck C, Kettmann R, Burny A (1984) Bovine Leukemia virus. Curr Top Microbiol Immunol 112:1–19
9. Hopkins SG, DiGiacomo RF (1997) Natural transmission of bovine leukemia virus in dairy and beef cattle. Vet Clin N Am Food Anim Pract 13(1):107–128
10. Buxton BA, Hinkle NC, Schultz RD (1985) Role of insects in the transmission of bovine leukosis virus: potential for transmission by stable flies, horn flies, and tabanids. Am J Vet Res 46(1):123–126
11. Ott SL, Johnson R, Wells SJ (2003) Association between bovine leukosis virus seroprevalence and herd-level productivity on US dairy farms. Prev Vet Med 61:249–262
12. Nuotio L, Rusanen H, Sihvonen L, Neuvonen E (2003) Eradication of enzootic bovine leukosis from Finland. Prev Vet Med 59:43–49
13. Acaite J, Tamosiunas V, Lukauskas K, Milius J, Pieskus J (2007) The eradication experience of enzootic bovine leukosis from Lithuania. Prev Vet Med 82:83–89
14. Mushi EZ, Wibberley G, Kupe DC (1990) Antibodies to enzootic bovine viral leukosis in Botswana. Trop Anim Health Prod 22(2):126
15. Zaghawa A, Beier D, Abd El-Rahim IH, Karim I, El-ballal S, Conraths FJ, Marquardt O (2002) An outbreak of enzootic bovine leukosis in upper Egypt: clinical, laboratory and molecular-epidemiological studies. J Vet Med B Infect Dis Vet Public Health 49(3):123–129
16. Walrand F, Fumoux F, Roelants G, Parodi AL, Levy D (1986) Incidence of bovine leukemia virus specific antibodies in West African cattle. Int J Cancer 37(4):619–621
17. Ndou RV, Sejesho F, Dzoma BM, Motsei LE, Nyirenda M, Bakunzi FR (2011) A serosurvey of the prevalence of enzootic bovine leukosis in the Mafikeng area of the North West Province of South Africa. J Hum Ecol 36(1):53–55
18. Morris SD, Myburgh JG, Van Vuuren M, Van der Vyver F (1996) Serological survey to determine the prevalence of bovine leukaemia virus antibodies in dairy cattle on selected farms in the Gauteng and Mpumalanga provinces. J S Afr Vet Assoc 67(3):146–147
19. Meas S, Nakayama M, Usui T, Nakazato Y, Yasuda J, Ohashi K, Onuma M (2004) Evidence for bovine immunodeficiency virus infection in cattle in Zambia. Jpn J Vet Res 52(1):3–8
20. Pandey GS, Sharma RN, Chizyuka HGB (1986) Study of bovine neoplasms in Zambia. Bull Anim Health Prod Afr 32:289–291
21. Polat M, Takeshima SN, Hosomichi K, Kim J, Miyasaka T, Yamada K, Arainga M, Murakami T, Matsumoto Y, de la Barra DV, Panei CJ, González ET, Kanemaki M, Onuma M, Giovambattista G, Aida Y (2016) A new genotype of bovine leukemia virus in South America identified by NGS-based whole genome sequencing and molecular evolutionary genetic analysis. Retrovirology 13(1):4
22. Lee E, Kim EJ, Ratthanophart J, Vitoonpong R, Kim BH, Cho IS, Song JY, Lee KK, Shin YK (2016) Molecular epidemiological and serological studies of bovine leukemia virus (BLV) infection in Thailand cattle. Infect Genet Evol 41:245–254
23. Rola-Luszczak M, Pluta A, Olech M, Donnik I, Petropavlovskiy M, Gerilovych A, Vinogradova I, Choudhury B, Kuzmak J (2013) The molecular characterization of bovine leukaemia virus isolates from Eastern Europe and Siberia and its impact on phylogeny. PLoS One 8(3):e58705
24. Fechner H, Blankenstein P, Looman AC, Elwert J, Geue L, Albrecht C, Kurg A, Beier D, Marquardt O, Ebner D (1997) Provirus variants of the bovine leukemia virus and their relation to the serological status of naturally infected cattle. Virology 237:261–269
25. Ndashe K, Simulundu E, Hang'ombe BM, Moonga L, Ogawa H, Takada A, Mweene AS (2016) Molecular characterization of infectious bursal disease viruses detected in vaccinated commercial broiler flocks in Lusaka, Zambia. Arch Virol 161(3):513–519
26. Reed VI (1981) Enzootic bovine leukosis. Can Vet J 22:95–102
27. Thompson KG, Johnstone AC, Hilbink F (1993) Enzootic bovine leukosis in New Zealand—a case report and update. N Z Vet J 41(4):190–194

28. Khudhair YI, Hasso SA, Yaseen NY, Al-Shammari AM (2016) Serological and molecular detection of bovine leukemia virus in cattle in Iraq. Emerg Microbes Infect 5:e56
29. Gutiérrez G, Alvarez I, Merlini R, Rondelli F, Trono K (2014) Dynamics of perinatal bovine leukemia virus infection. BMC Vet Res 10:82
30. Merlini R, Gutiérrez G, Alvarez I, Jaworski JP, Carignano H, Poli M, Willems L, Trono K (2016) Bovine leukemia virus becomes established in dairy herds before the first lactation. Arch Virol 161(11):3215–3217
31. Johnston ER, Albritton LM, Radke K (2002) Envelope proteins containing single amino acid substitutions support a structural model of the receptor binding domain of bovine leukemia virus surface protein. J Virol 76:10861–10872