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PRESENCE OF BOVINE LEUKEMIA VIRUS IN COLOSTRUM SAMPLES AND ITS POTENTIAL TO INFECT NEWBORN CALVES

PRESENCIA DEL VIRUS DE LA LEUCOSIS BOVINA EN MUESTRAS DE CALOSTRO Y SU POTENCIAL PARA INFECTAR TERNEROS

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ABSTRACT

Bovine leukemia virus (BLV) is a retroviral agent that primarily affects dairy cattle. Colostrum is the main source of immunoglobulins for calves; however, it is also a vehicle for the vertical transmission of BLV. The aim of this work was to determine the presence of BLV in colostrum and its potential to infect newborn calves. Seven mothers and their seven daughter calves were evaluated. Blood, colostrum, and milk samples were taken. An ELISA was performed to detect BLV in blood, colostrum and milk serums. Furthermore, a PCR test (*env* gene) was used for BLV detection in blood and colostrum. An identity analysis was used (phylogeny) to verify the origin of the sequences obtained from the DNA of mothers, calves, and colostrum. Six of the seven cows were positive for BLV by ELISA, while five of them were positive by PCR. None of the calves was positive by PCR at birth. However, the BLV provirus was found in two calves on day 15 and in one calf on day 30. The identity of the sequences was confirmed as BLV. Colostrum and milk are vehicles for the vertical transmission of BLV, which explains that three of the seven calves evaluated showed BLV infection during the early postpartum period.

Key words: phylogeny, viral infection, vertical transmission.

RESUMEN

El virus de la leucemia bovina (BLV) es un agente retroviral que afecta principalmente al ganado lechero. El calostro es la principal fuente de inmunoglobulinas para terneros. Sin embargo, también es un vehículo para la transmisión vertical del BLV. El objetivo de este trabajo fue determinar la presencia de BLV en muestras de calostro y su potencial para infectar terneros recién nacidos. Se evaluó un grupo de siete madres y sus siete terneras hijas. Se tomaron muestras de sangre, calostro y

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leche. Se realizó una prueba ELISA para determinar la presencia de anticuerpos anti-BLV en sueros de sangre, calostro y leche. Además, se realizó la técnica de PCR (gen *env*) en muestras de sangre de las madres y terneras, y en muestras de calostro. Se realizó un análisis de identidad (filogenia) para verificar el origen de las secuencias obtenidas del ADN de las madres, las terneras y el calostro. Seis de las siete vacas fueron positivas para BLV por ELISA, y cinco de ellas fueron positivas por PCR. Ninguno de los terneros fue positivo por PCR al nacer. Sin embargo, se encontró ADN proviral en dos terneras el día 15 y en otra ternera el día 30. Se confirmó que la identidad de todas las secuencias obtenidas era del BLV. El calostro y la leche son vehículos para la transmisión vertical de BLV, lo que explica que tres de las terneras evaluadas mostraron infección por BLV durante el período posparto temprano.

Palabras clave: filogenia, infección viral, transmisión vertical.

INTRODUCTION

Bovine leukemia virus (BLV) is a retrovirus that affects the immune system of cattle. Once acquired, infection is lifelong because the provirus is inserted into the genome of B lymphocytes (Safari et al., 2020). Moreover, 30% to 70% of clinically healthy animals develop persistent lymphocytosis (PL) and remain so until they die (Farias et al., 2018). On the other hand, between 0.1% and 10% of the cattle show the tumorous form of the disease when infected for more than three years (Dees et al., 1996; OIE, 2018). BLV does not produce chronic viremia, and in 70% of the cases, the virus uses a polyclonal expansion of B cells as a mechanism of viral replication (Hulo et al., 2011). BLV is widely distributed in the world. e.g., the molecular prevalence for BLV in Colombia is 44% of infection in Holstein cows (Úsuga-Monroy et al., 2015), while lower molecular prevalence is observed in Chile (27.9%), Bolivia (30.7%) and Peru (42.3%). Countries such as Paraguay and Argentina present prevalence higher than those found in Colombia (54.7% and 77.4%, respectively) (Polat, et al., 2017).

Dairy herds infected with BLV show a reduction in milk production ranging from 2.5 to 7.67% compared to the average production of the herd (Emanuelsson et al., 1992; Ott et al., 2003; Úsuga-Monroy et al., 2018a), and an increase in productive losses as cows become susceptible to other infectious etiology diseases such as mastitis or metritis (Kakinuma et al., 2014). BLV is transmitted horizontally through inadequate iatrogenic practices during dehorning (Darlington et al., 1985; Ruíz et al., 2018), multiple-use needles for vaccination, and the reuse of gloves on several animals during palpation (Divers et al., 1995; Ortega et al., 2016). On the other hand, vertical transmission of BLV can occur through mother-child interaction, with an intrauterine infection of 26% (Lassauzet et al., 1991; Meas et al., 2002), particularly during the last six months of gestation in mothers infected with BLV. In fact, perinatal transmission plays a key role in the spread of BLV, especially through the birth canal (Schwartz and Lévy, 1994; Mekata et al., 2014).

Colostrum and milk can also be a vehicle for the vertical transmission of BLV because these may contain infected cells, especially if the mothers are co-infected with bovine immunodeficiency virus (BIV) (Meas et al., 2002; Brujeni et al., 2010). Colostrum is a natural food for calves produced by the mammary gland of their mother during the first days after birth (Desjardins-Morrissette et al., 2018). Infections by pathogens such as bacteria or viruses constantly affect dairy systems, and therefore, the proper development of the immune system is essential. In this sense, colostrum is a source of nutrients and antimicrobial factors such as lysozymes or lactoferrin, which protect the calf from infections during the first week of life (Furman-Fratczak et al., 2011; Moretti et al., 2020). However, antibodies or immunoglobulins are responsible for generating passive immunity during the first days of calves (Hurley et al., 2011; Agianniotaki et al., 2018).

BLV has tropism towards mammary gland cells, so epithelial cells express BLV antigens when they are infected (Buehring et al., 1994; Buehring et al., 2014). Although colostrum is an essential nourishment for newborn calves because it is the main source of immunoglobulins, the excretion of exosomes containing BLV proteins through milk (Yamada et al., 2013) or of cells infected with the virus indicates that both colostrum and milk can be an important vehicle for the transmission of viral particles. Accordingly, the aim of this work was to determine the presence of bovine leukemia virus in colostrum, and its potential to infect newborn calves.

MATERIALS AND METHODS

Study area and animals

This work is descriptive. Samples of blood, colostrum and milk were taken from seven

cows and their seven daughters during a 45-day monitoring period. All the cows belong to the Holstein breed and pertain to the Paysandú herd of Universidad Nacional de Colombia, Medellín campus, located in Santa Elena (6°12'20.5" N; 75°30'20.4" W) - 16 km from Medellín, Department of Antioquia, Colombia. The selected cows were maintained under an intensive dairy management system, and subjected to the same sanitary control. In the herd, calves are manually fed in the morning and afternoon with milk from any milking cow, milk samples were used for serological tests only. For all the samples (blood, colostrum or milk), the level of antibodies was determined through an indirect ELISA, while molecular detection of the viral envelope gene (*env*) was performed using a PCR only for blood and colostrum samples. The Research Ethics Committee of the Universidad Nacional de Colombia, Medellín campus, approved this study (CEMED-022, July13, 2015).

Blood samples

Blood samples were taken as follows: on the day of parturition (day 0), a blood sample was taken from the mother and the newborn calf prior to colostrum ingestion. On days 15, 30 and 45, blood samples were taken only from calves. Two blood tubes (4 mL) were collected in heparinized syringes per animal by coccygeal venipuncture. The samples were homogenized by inversion and kept refrigerated at 4°C.

Serum extraction from blood samples

Blood samples were transferred to 15 mL conical tubes and centrifuged at 3,000 rpm at 4°C. for 4 minutes. The blood serum was collected in a 1.5 mL tube and stored at -20°C until use.

DNA extraction from buffy coat

The buffy coat was recovered after blood samples were transferred to 15 mL tubes and centrifuged (at 3,000 rpm at 4°C for 4 minutes). DNA was extracted using the salting out technique (Miller et al., 1988), resuspended in TE 1X pH 8.0 buffer (1 M Tris HCl and 0.5 M EDTA) and stored at 4°C until analysis. DNA quality and quantity were determined using a spectrophotometer (Thermo Scientific, NanoDrop, Waltham, Massachusetts, United States) and on 1% agarose gel.

Colostrum samples

Colostrum samples were collected on the parturition day (day 0) directly from the udder of each mother. After washing and disinfecting the udder, a sample of 50 mL of colostrum as a pool of four quarters was collected. The samples were deposited in a 50 mL conical tube with bronopol ($C_3H_6BrNO_4$) as an antimicrobial preservative, homogenized by inversion and kept refrigerated at 4°C.

Serum extraction from colostrum samples

Colostrum samples were centrifuged at 1,500 g for 10 minutes to remove the fatty layer. The clarified fraction or serum was collected in a 1.5 mL tube and stored at -20°C until use.

DNA extraction from colostrum samples

The cell button was recovered after the colostrum samples were centrifuged (1,500 g for 10 minutes). The cell button was washed with 50 µL of PBS, and the Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used for DNA extraction according to the manufacturer's instructions**.**

Milk samples

Milk samples were taken as follows: on days 15, 30 and 45, a sample was taken from a milk bucket used for feeding the calves. A sample of 50 mL of milk as a bucket pool was collected. The samples were deposited in a 50 mL conical tube with bronopol ($C_3H_6BrNO_4$) as an antimicrobial preservative, homogenized by inversion and kept refrigerated at 4°C. As calves in the herd are manually fed in the morning and afternoon with milk from any milking cow,milk samples were used for serological tests only.

Serum extraction from milk samples

Milk samples were centrifuged at 1,500 g for 10 minutes to remove the fatty layer. The clarified fraction or serum was collected in a 1.5 mL tube and stored at -20°C until use.

ELISA test for blood, colostrum or milk serum

ELISA was performed using a commercial kit (Svanovir, BLV gp51-Ab Svanova, Uppsala, Sweden) according to the manufacturer's instructions. The optical density of the controls and samples was measured at 450 nm in an absorbance microplate reader (BioTek, ELx800, Nogarazza, Province of Vicenza, Italia).

Molecular analysis by PCR

Detection of the proviral genome of BLV by nested PCR was established using DNA of blood (buffy coat) and colostrum (cell button) samples of newborn calves and their mothers. A region of the viral *env* gene was amplified to obtain a 444 bp fragment using the oligonucleotides previously reported (Beier et al., 2001). The first round of PCR was performed in a final volume of 25 µL with 150 ng of DNA,

3.0 µL of 10 mM of each oligonucleotide *env* 5023 (5′-TCTGTGCCAAGTCTCCCAGATA-3′) and *env* 5608 (5′-AACAACAACCTCTGGGGAGGGT-3′), 0.4 mM of dNTPs, 1X buffer PCR (ThermoScientific), 3 mM of $MgCl_2$, and 1U Taq Polymerase DNA. The second was done in a final volume of 30 µL and the same concentrations of the other reagents. The first amplification PCR product was used as DNA template (5 µL) and *env* 5099 (5′-CCCACAAGGGCGGCGCCGGTTT-3′) and *env* 5521 (5′-GCGAGGCCGGGTCCAGAGCTGG-3′) oligonucleotides were used. Both PCR reactions were performed in a thermocycler under the following conditions: 5 minutes at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 60°C, 1 minute at 72°C, and 5 minutes at 72°C. As a negative control, the PCR reactions were run without a template DNA; and as a positive control, the PCR reactions were run with DNA of a cow that tested positive in a previous study (Úsuga-Monroy et al., 2015). The product of the second reaction was visualized on 2% agarose gel in a gel documentation system (BIORAD, Gel Doc XR+, Hercules, California, United States)

Identity analysis of BLV sequences in blood and colostrum samples

A commercial company (Macrogen, Seoul, Korea) sequenced six PCR products. The isolates were obtained from three blood samples of BLV positive calves and three colostrum samples of their mothers. PCR products were sent to the company in a 1.5 mL tube without purification at a concentration of 100 ng/µL in a final volume of 30 µL. Once the results were obtained, the nucleotide sequences of the samples were compared with 25 partial sequences of the viral *env* gene registered in GenBank. These were representative of the nine BLV genotypes and represented different geographic regions, including several South American countries. Sequences were manually aligned in the software MEGA V7. A phylogenetic analysis was performed using the Maximum Likelihood analysis. The Kimura 2-parameter model with Gamma distribution (K2+G) was the best substitution model based on the Bayesian Information Criterion (BIC). Bootstrap values were determined with 1,000 replicates, and only values greater than 70% were considered as significant. Mean nucleotide distances among colostrum and blood isolates from colostrum and calves, were estimated by the K2+G model in MEGA V7.

Data analysis

Calves and colostrum were classified as positive for BLV provirus when a positive result was obtained by PCR. Blood, colostrum, and milk serums were classified as positive or negative according to their positive percentage value (PP). Blood serum with a PP < 15 was considered negative, and positive when the PP value was ≥ 15. Colostrum or milk serum with a PP < 10 was considered negative, and positive when the PP value was ≥ 10. The positive percentage value was established as:

$$[PP = (OD_{corrected}\ \text{sample}/OD_{corrected}\ \text{positive control}) \times 100]$$

OD = optical density

RESULTS AND DISCUSSION

The present study revealed that six of the seven colostrum samples collected from mothers on the parturition day (day 0) had antibodies against the viral gp51 protein. At birth, none of the female calves evaluated had antibodies against the viral gp51 protein. However, measurements obtained on days 15, 30 and 45 showed that six of the seven calves showed antibodies circulating in the blood. Two calves (calves 2 and 3) were positive by PCR on day 15, while calf 4 was positive on day 30 (Table 1). On the other hand, all bucket milk samples tested BLV positive by ELISA on days 15, 30, and 45.

The presence of immunoglobulins in colostrum protects calves against BLV infection. A study conducted by Nagy et al. (2007) reported no presence of proviruses in the calves of BLV positive mothers fed with colostrum, but they were detected in 4 of the 12 calves that were deprived of colostrum. It is important to note that colostrum and milk are also vehicles for the transmission of many zoonotic diseases such as bovine tuberculosis or zoonotic diphtheria, and diseases that directly affect calves, such as paratuberculosis, salmonellosis, mycoplasma, leptospira, bovine viral diarrhea and bovine leukemia (Dhanashekar et al., 2012).

The antibody transfer from the mother's bloodstream into the colostrum occurs naturally to protect calves from external pathogens. Because cattle present sindesmocorial placentation, it is not possible to transfer antibodies or other proteins from the mother to the fetus in this phase of the placental tissue (Baruta et al., 2011), and therefore colostrum is the sole source of immunoglobulins for calves. The circulating antibodies in the blood of calves 1, 5 and 7 could be obtained through the consumption of colostrum and are part of passive immunity. However, the antibodies in calves 2, 3 and 4 could be generated by the immune response against BLV infection, because

Table 1. Molecular and serological presence of BLV in blood, colostrum and milk serum from seven Holstein calves and their mothers using two assessment methods (ELISA and PCR). The samples were taken 45 days after parturition (follow-up).

		ELISA			PCR	
Animal	Day	Blood serum	Colostrum serum	Milk serum	Blood DNA	Colostrum DNA
Dam 1	0	BLV+	BLV+		BLV+	BLV-
Calf 1	0	BLV-			BLV-	
	15	BLV+		BLV+	BLV-	
	30	BLV+		BLV+	BLV-	
	45	BLV+		BLV+	BLV-	
Dam 2	0	BLV+	BLV+		BLV+	BLV+
Calf 2	0	BLV-			BLV-	
	15	BLV+		BLV+	BLV+	
	30	BLV+		BLV+	BLV+	
	45	BLV+		BLV+	BLV+	
Dam 3	0	BLV+	BLV+		BLV+	BLV+
Calf 3	0	BLV-			BLV-	
	15	BLV+		BLV+	BLV+	
	30	BLV+		BLV+	BLV+	
	45	BLV+		BLV+	BLV+	
Dam 4	0	BLV+	BLV+		BLV+	BLV+
Calf 4	0	BLV-			BLV-	
	15	BLV+		BLV+	BLV-	
	30	BLV+		BLV+	BLV+	
	45	BLV+		BLV+	BLV+	
Dam 5	0	BLV+	BLV+		BLV-	BLV-
Calf 5	0	BLV-			BLV-	
	15	BLV+		BLV+	BLV-	
	30	BLV+		BLV+	BLV-	
	45	BLV+		BLV+	BLV-	
Dam 6	0	BLV-	BLV-		BLV-	BLV-
Calf 6	0	BLV-			BLV-	
	15	BLV-		BLV+	BLV-	
	30	BLV-		BLV+	BLV-	
	45	BLV-		BLV+	BLV-	
Dam 7	0	BLV+	BLV+		BLV+	BLV-
Calf 7	0	BLV-		BLV+	BLV-	
	15	BLV+		BLV+	BLV-	
	30	BLV+		BLV+	BLV-	
	45	BLV+		BLV+	BLV-	

BLV+: positive by PCR or ELISA; BLV-: negative by PCR or ELISA.

these calves were PCR positive for BLV infection (Konishi et al., 2018).

Although mother 6 did not transmit antibodies against BLV to calf 6, this was not infected with the virus and remained healthy during the study period. The mother of calf 6 had no antibodies in blood and colostrum against BLV and was negative for BLV by PCR. Because of that, mother 6 could not excrete the virus or transmit it through colostrum. Therefore, calf 6 was not infected when consuming colostrum or milk. However, as the evaluation period was short,

it is possible that the virus did not have time to replicate in calf 6, and so the proviral genome was not detected in the molecular analysis. On the other hand, it is important to consider some there are some genetic factors that regulate resistance to viral infections (Hayashi et al., 2017) or pro-inflammatory cytokines (Konnai et al., 2005), which may have conferred resistance to viral spread in calf 6.

The proviral BLV genome was not identified in calf 1, calf 5 and calf 7, although these calves presented antibodies in blood from day 15. The antibodies against BLV were obtained through the ingestion of colostrum from the mother, for this reason immunoglobulins are important as a means of protection against multiple pathogens, including BLV (Nagy et al., 2007). However, proviral BLV genome was found in DNA samples from calf 2, calf 3 and calf 4. Although these calves presented antibodies in blood from day 15, the immune system was not efficient against BLV infection (Ruiz et al., 2018).

All the sequences obtained from DNA in colostrum and calf's blood were identical nucleotidically (100%) using the Kimura 2-parameter model with Gamma distribution (K2+G). The analysis involved 6 nucleotide sequences and the codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Estimates of evolutionary divergence between sequences isolated from colostrum 1, 2 or 3 and calf 1, 2 or 3 do not show divergence.

The isolates from colostrum and calf samples were in the same cluster due to their identical nucleotide sequence. Sequence analyses were conducted by Maximum Likelihood within the same branch in the phylogenetic tree based on all the isolates from three calves and three colostrum samples. The isolates obtained from blood of calves and colostrum were classified within BLV genotype 1 (Fig. 1). The presence of genotype 1 in the herd is supported by phylogenetic approaches, with genotype 1 being the most frequent worldwide (Inoue et al., 2011). In Colombia, genotype 1 has been reported in dairies of the provinces of Nariño (Benavides et al., 2017) and Antioquia (Úsuga-Monroy et al., 2018b). The data obtained in the present study show a possible vertical transmission through colostrum because calves were infected by the same BLV genotype present in their mothers. BLV envelope gene is highly conserved; the 444pb fragment of the *env* gene is the most widely used for phylogenetic analyses (Camargos et al., 2002; Felmer et al., 2005; Moratorio et al., 2010; Polat et al., 2017; Lee et al., 2016). However, other regions such as the polymerase gene could be used as markers for genotyping blood and colostrum isolates (Heenemann et al., 2012).

BLV enters the host organism through infected cells by direct contact between body fluids such as blood, saliva, milk, or colostrum (Gillet et al., 2007; Haghparast et al., 2012; Yuan et al., 2015). During the primary infection, BLV infects B cells; once integrated, BLV provirus expresses the necessary proteins to replicate and generate new viral particles. This process concludes after one to four weeks. After infection, the presence of the BLV provirus was identified in three blood samples from the calves within a short time in the present study. During a persistent infection, BLV uses the mitosis of B cells to generate new cells with the integrated provirus, and this process lasts from several months to years (Gutiérrez et al., 2014). B cells infected with BLV increase their cell division rate and have the ability to form syncytia, which is a good indicator of viral replication capacity. Studies have shown that the N-terminus of the envelope protein gp51 has three conformational epitopes, F, G, and H (Bruck et al., 1982; Lee et al., 2015). These epitopes have an important role in viral infectivity and syncytia formation (Bai et al., 2015). During the first hours of life, calves are more susceptible to BLV infection, because cells of their gastrointestinal tract are more sensitive to allow for the passage of proteins through cellular light. However, apart from immunoglobulins, free viral particles or B cells infected with BLV (from colostrum) also pass.

CONCLUSIONS

The colostrum of BLV positive mothers may contain infected B lymphocytes or free viral particles with infective capacity, becoming a potential source of BLV transmission. This would explain that three of the calves showed the BLV provirus integrated into their genome during the early postpartum period. Six of the seven cows tested positive for BLV by ELISA, and five of the seven cows were positive by PCR on the parturition day. At birth, none of the calves had antibodies against BLV; these were acquired through the consumption of colostrum and were present during the rest of the evaluation days (15, 30, 45). Two of the seven calves were positive by DNA molecular detection of the proviral *env* gene on day 15, and one calf was positive on day 30. This is the first report on the presence of BLV DNA in colostrum samples in Holstein cows from Colombian herds. The results obtained confirm that colostrum is a potential vehicle in the vertical transmission of BLV infection. Further research is

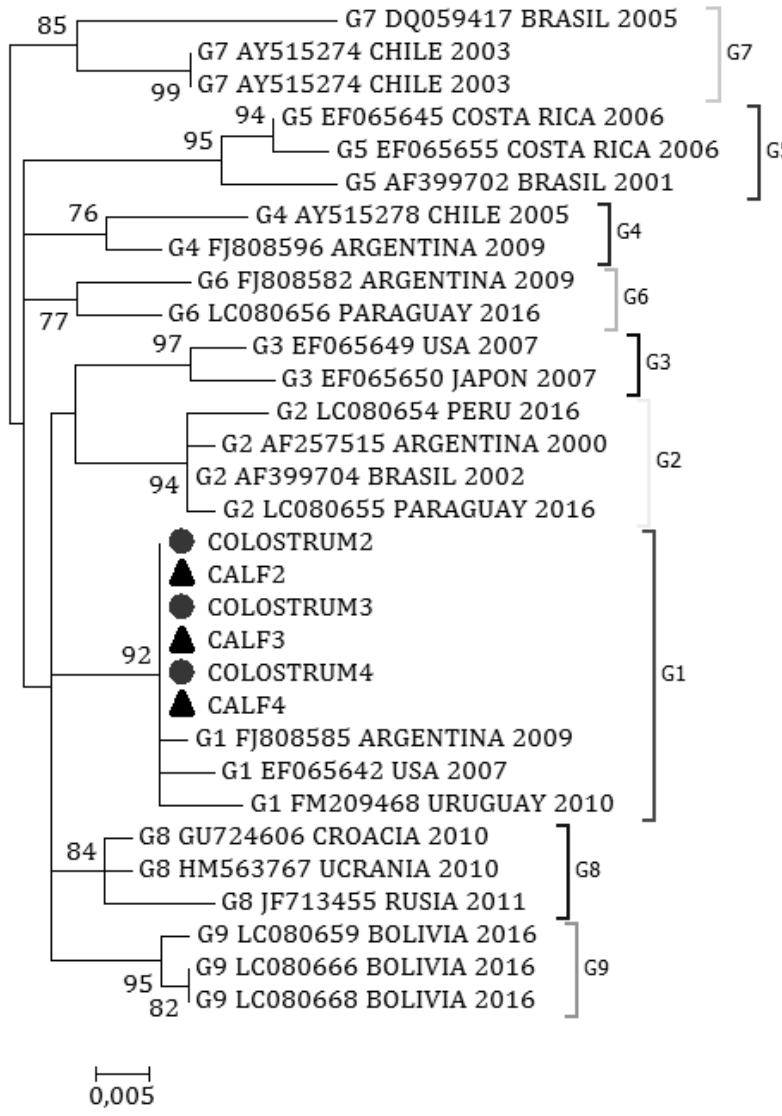

Fig. 1. Phylogenetic tree by Maximum Likelihood using the Kimura 2-parameter model with Gamma distribution (K2+G) as substitution models. Bootstrap values are indicated as data from 1,000 replicate (Bar 0.005 substitutions per site) based on partial sequences of 400 nucleotides of BLV *env* gene. Isolates from blood of calves are shown as a triangle (▲) and colostrum are shown as a circle (●). The isolates were compared with 25 GenBank sequences reported for the *env* gene. Genotype, access numbers, and country of origin of the sequences are indicated in the figure.

required to determine the exact role of colostrum and milk in BLV spread.

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