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Research Paper

Prevalence and molecular epidemiology of bovine leukemia virus in Colombian cattle

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ABSTRACT

Bovine leukemia virus (BLV) is one of the five agents considered most significant for cattle. It is important to determine the prevalence and molecular epidemiology of BLV throughout the country in order to gain a more thorough understanding of the current situation of BLV and to reveal the possibility of masked genotypes that the primers used by OIE are unable to identify. Blood samples were collected at random from 289 cows distributed in 75 farms across the country. PCR amplification of *env, gag* and *tax* gene segments was performed. The obtained amplicons were sequenced and then subjected to phylogenetic analyses. A total of 62% of the cows present at 92% of the farms were BLV-positive for *gag* fragment. Genotype 1 was exclusively detected by *env* gene segment when analyzed using previously reported primers. However, *tax* gene analysis revealed circulation of genotype 6 variants, which were also detected based on *env* gene analysis with newly designed primers. These results indicate that current genotyping approaches based on partial *env* sequencing may bias BLV genetic variability approaches and underestimate the diversity of the detected BLV genotypes. This report is one of the first molecular and epidemiological studies of BLV conducted in Colombia, which contributes to the global epidemiology of the virus; it also highlights the substantial impact of BLV on the country's livestock and thus is a useful resource for farmers and government entities.

1. Introduction

Viruses are one of the main causes of health problems, of which bovine leukemia virus (BLV) is one of the five agents considered most significant for cattle. Colombia had previously reported a seroprevalence of animals of 42.7%, a figure that is consistent with worldwide reports, which range between 5 and 90% (Lee et al., 2016; Murakamia et al., 2011; Ortiz et al., 2016; Polat et al., 2016).

BLV belongs to *Retroviridae* family and Deltaretrovirus genus with two copies of a single-strand positive-RNA with a length of 8714 nucleotides. Its genome contains 8 open reading frames with 3 gene segments (*gag, pol, env*) encoding the structural proteins and enzymes necessary for viral replication, a pX region encoding the Tax and Rex auxiliary proteins, which perform regulatory functions, and two long terminal regions (LTRs) at the terminal ends of the genome (Lee et al., 2016; Ochirkhuu et al., 2016; Polat et al., 2017b, 2016; Rosewick et al.,

Abbreviations: BLV, bovine leukemia virus; ORFs, open reading frames; LTRs, long terminal regions; gp51, extracellular portion envelope protein; gp30, transmembrane region envelope protein; RFLP, restriction fragment length polymorphism; OIE, World Organization for Animal Health; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AIC, Akaike information criterion

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Table 1
Primers used for amplification*, sequencing* and construction of plasmid DNA┼ for the BLV *gag, tax* and *env* gene segments. NA (not applicable).

Region and position on the genome	Primer sequence 5`- 3` (Forward and reverse)	PCR product (bp)	Annealing Temperature (°C)	Minimum detectable concentration (ng/ul)	Reference
*gag**┼	AACACTACGACTTGCAATCC	385	59.3	2.3	(Buehring et al., 2014)
1068–1453	GGTTCCTTAGGACTCCGTCG				
*tax**┼	CTTCGGGATCCATTACCTGA	373	56.5	0.042	
7197–7570	GCTCGAAGGGGGAAAGTGAA				
*env**	CCCACAAGGGCGGCGCCGGTTT	509	62.8	159×10^{-9}	(Fechner et al., 1997)
5107–5636	AACAACAACCTCTGGGAAGGGT				
*env**	TGTCCCTAGGAAAYCAAC	750	56	159×10^{-4}	Current study
4938–5688	AGATTAACCAGGGAGATAGG				
env┼	ATGAGATGCTCCCTGTCCCTAG	801	57.6	NA	(Corredor et al., 2018)
4925–5726	ACGTCTGACCCGGGTAGG				

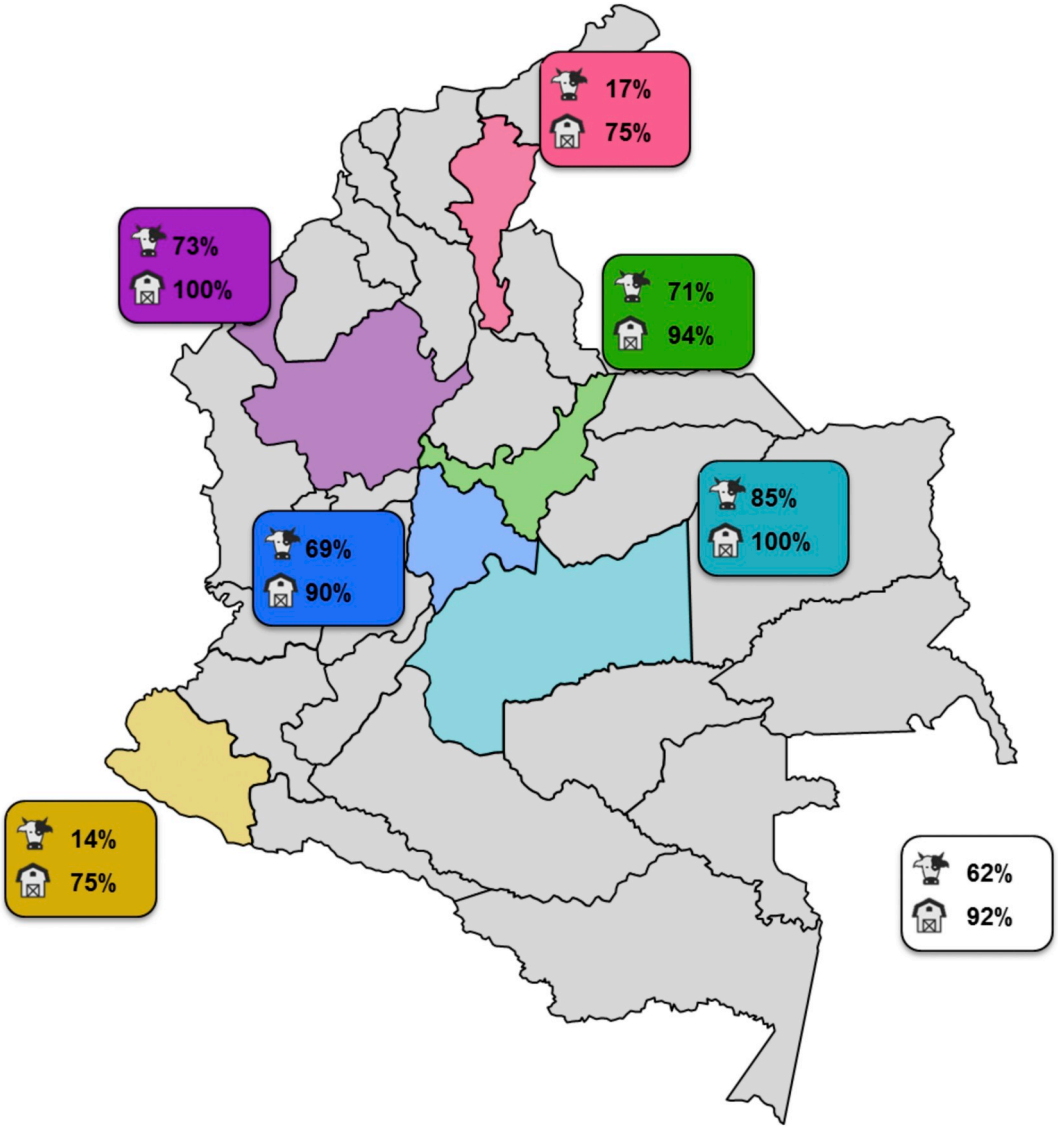

Fig. 1. Prevalence of BLV by detection of the *gag* and *tax* gene segments in the Colombian cattle population. Map of Colombia showing prevalence per animal and per farm, distributed in six regions of Colombia: Cundinamarca 69 and 90% (dark blue), Boyacá 71 and 94% (green), Antioquia 73 and 100% (purple), Meta 85 and 100% (turquoise), Nariño 14 and 75% (yellow) and Cesar 17 and 75% (pink), respectively. The overall prevalence of BLV in Colombia was 62% per animal and 92% per farm. The Colombian map was prepared by the authors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2013). The *gag* gene consists of 1183 nucleotides and encodes the capsid protein p24-CA, the *env* gene has a length of 1547 nucleotides and encodes the envelope glycoproteins, which are comprised by the extracellular portion (gp51) and the gp30 transmembrane region (gp30) (Corredor et al., 2018; Polat et al., 2016). gp51 is essential for recognition and entry of the virus into the host cell and is one of the most immunogenic viral proteins. The pX region has 3304 nucleotides and encodes the Tax protein, which fulfills regulatory functions

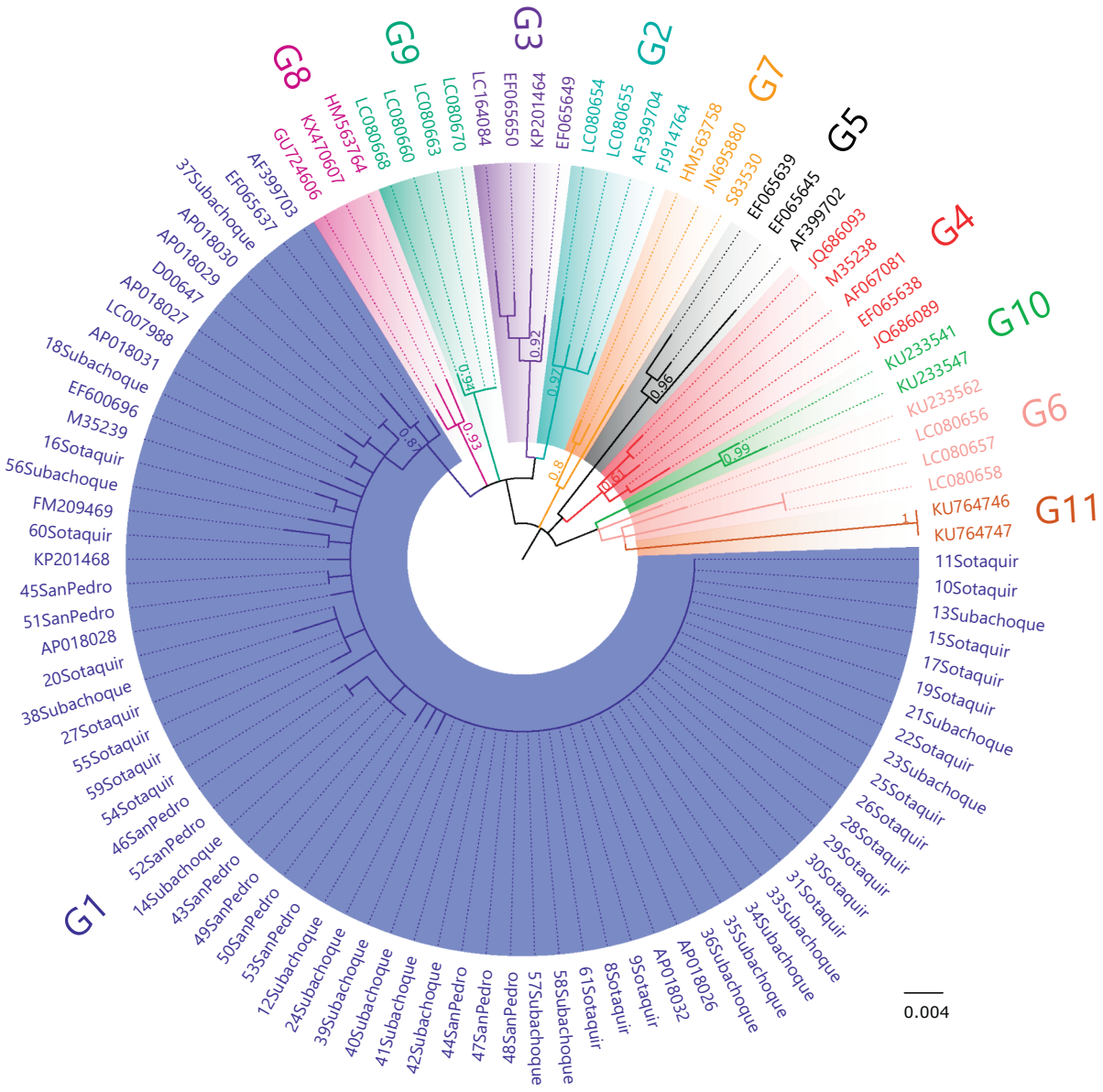

Fig. 2. Maximum-likelihood phylogenetic tree analysis of the *env* gene of BLV circulating in Colombia. The ML method based on the General Time Reversible model maximum composite likelihood (MCL) approach was used, and the topology with a superior log likelihood value was selected. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.3005)). The rate variation model allowed some sites to be evolutionarily invariable ([+*I*], 39.13% sites). The analysis involved 100 nucleotide sequences. A total of 423 positions were included in the final dataset.

(Rosewick et al., 2013).

At present, 11 BLV genotypes have been reported in different regions of the world (Ababneh et al., 2012; Bazzucchi et al., 2019; Gautam et al., 2018; Lee et al., 2016; Ochirkhuu et al., 2016; Polat et al., 2017b, 2016; Yu et al., 2019). Frequently, BLV genotype detection is based on a 444 bp segment amplification, as indicated in the World Organization for Animal Health (OIE) manual for viral diagnosis (Fechner et al., 1997; OIE, 2018). Both in this document and in several others, Fechner primers are used, and they indicate that genotype 1 is the most prevalent worldwide (Lee et al., 2005; Polat et al., 2017b). However, the topology of some trees displays different clusters in the same genotype, leading to the question of whether the amplification of the 444 bp segment of the *env* region efficiently identifies all the current genotypes, or instead mimics the presence of other circulating genotypes (Buehring et al., 2014; Felmer et al., 2005; Ochirkhuu et al., 2016).

In Colombia there are very few studies on the seroprevalences of this virus and even less on prevalence and genotype. Consequently, the aim of this study was to identify the circulating genotype of BLV throughout the country in order to gain a more thorough understanding of the current situation of BLV and to reveal the possibility of masked genotypes that the primers used by OIE are unable to identify.

2. Materials and methods

2.1. Sample collection and DNA extraction

The samples were randomly collected, taking into consideration the areas with greatest bovine cattle production in the country, based on the bovine census carried out by the Colombian Agricultural Institute (ICA) in 2014, which counted 22,574,780 heads of cattle distributed among 495,072 properties. The number of samples to be studied was determined based on the sampling formula to estimate a proportion using the WinEpiscope tool available online (http://www.winepi.net/).

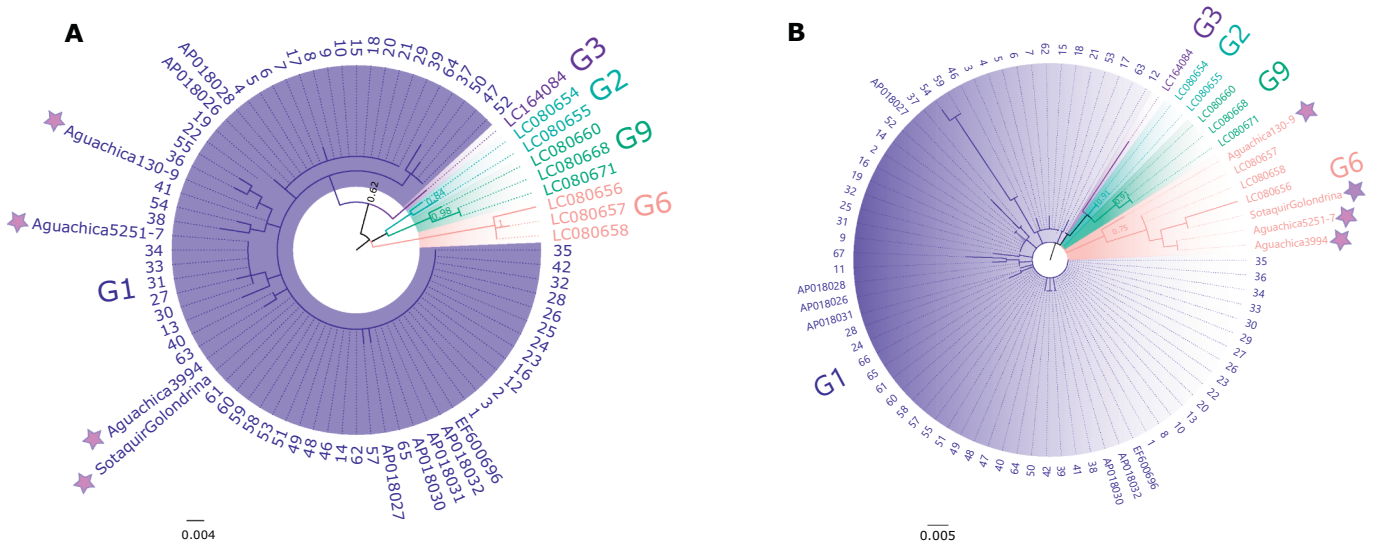

Fig. 3. Maximum-likelihood phylogenetic tree analysis of the *gag* and *tax* genes of BLV circulating in Colombia. The evolutionary history was inferred using the ML method based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; (+G, parameter = 0.1621) for *gag* and (+G, parameter = 0.1749) for *tax*)). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 43.30% sites for *gag* and [+I], 42.83% sites for *tax*). A total of 306 and 314 positions were included in the final dataset for *gag* and *tax*, respectively. Fig. 3A, phylogenetic tree of the *gag* gene segments with 80 sequences: 64 Colombian and 16 reference sequences. Note that all Colombian samples are grouped into genotype 1, including the four G6 samples. Fig. 3B, phylogenetic tree constructed with the *tax* gene segments with 83 sequences: 67 Colombian and 16 reference sequence segments. Note that four Colombian samples are included in the genotype 6 cluster and are shown with an * both in *gag* and *tax* trees.

The sample size (n) was estimated using the 42% prevalence reported by (Ortiz et al., 2016), which yielded an n value of 289. Blood samples were taken from the coccygeal vein of healthy cows distributed throughout the country in 6 different regions, between 2015 and 2016. Mononuclear cells were separated from blood samples using density gradient centrifugation with LymphoSep (MP®); after recovering the buffy coat, total DNA was extracted in order to look for proviral DNA with the High Pure PCR Template Preparation Kit (Roche®).

2.2. PCR sensitivity tests

Previously amplified amplicons belonging to the segments of *gag* (381 bp) and *tax* (396 bp) genes of BLV were purified with a PCR Wizard kit (Promega®), followed by the insertion of each of them into the pELMO vector (Ramos et al., 2017) and transforming them into *E. coli* TOP10 cells (Invitrogen®). The plasmid DNA was used as an amplification template in the sensitivity tests. Serial dilutions of each plasmid were performed with initial concentrations of 230 ng/µl and 411 ng/µl for *gag* and *tax*, respectively. Subsequently, the respective PCR assays were performed at each dilution level to determine the detection limit of the technique corresponding to the maximum amplified dilution. For the *env* region, a segment of 801 bp was amplified as described above, but the sequence was cloned into the pEXP5-CT/TOPO (Invitrogen) vector with an initial concentration of 159 ng/µl.

2.3. Detection of BLV gene segments

Initially, the constitutive gene bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. It was amplified in order to ensure that the DNA was long enough to be able to contain the virus segments. The primers (5´CCT TCA TTG ACC TTC ACT ACA TGG TCT A 3′ and 5´ GCT GTA GCC AAA TTC ATT GTC GTA CCA 3′) were those reported by (Buehring et al., 2014) and the amplified segment was 857pb long. Multiple PCR was performed to amplify both the GAPDH and *gag* gene segments simultaneously. Afterwards, individual PCRs were performed to amplify the *gag, tax* and *env* genes. The PCR conditions and the primers used are found on the Table 1. For all the PCR assays described below, the PCR master mix (Roche®) was prepared according to the manufacturer's instructions. Plasmid DNA containing each gene segment (*gag, tax,* and *env*) was used as a positive control.

Once positive samples for BLV were identified by *gag* gene amplification, PCR was carried out to detect a 373 bp segment of the *tax* region and another PCR was done in order to detect the circulating genotype with a segment of 530 bp of the *env* gene. The newly designed pair of primers for *env* overlap with the region that was first amplified using Fechner's set of primers, because the Fechner primer amplified the region between nt 5107 and nt 5636, whereas the new set of primers amplified the segment between nt 4938 and nt 5688.

The amplicons obtained from the PCRs were purified with a High Pure PCR Product Purification kit (Roche®) according to the manufacturer's instructions. Subsequently, sequencing was performed by the Sanger sequencing service of Macrogen Korea, the coverage for each sequence obtained was 4× for *env* gene and 2× for *gag* and *tax* gene. The sequences obtained in this study were deposited in the GenBank database with accession numbers MH041897 to MH042017, MH057402 to MH057465 and MH057466 to MH057532 for *env, gag* and *tax*, respectively.

2.4. Phylogenetic analysis

In order to identify the circulating genotype of BLV, the sequences obtained in this study were compared to complete BLV genome sequences available in GenBank. 64 *gag* and 67 *tax* sequences from this study were compared to the 16 complete sequences of BLV reported in the GenBank. These 16 sequences are from different genotypes. For the *env* gene, in the first PCR with Fechner primers, 53 Colombian sequences were compared to 49 partial sequences reported in the Genbank, and for the second PCR with the new primers, 121 Colombian sequences were compared to 49 partial sequences, which included the first 11 BLV genotypes described. These GenBank sequences were randomly selected, but from different regions around the world.

The combined multiple alignment of all Colombian sequences was performed with the ClustalW program implemented in Mega 7.

Once aligned, the best evolutionary model that described our sequence data was assessed using the “Find Model” interface in the Mega7

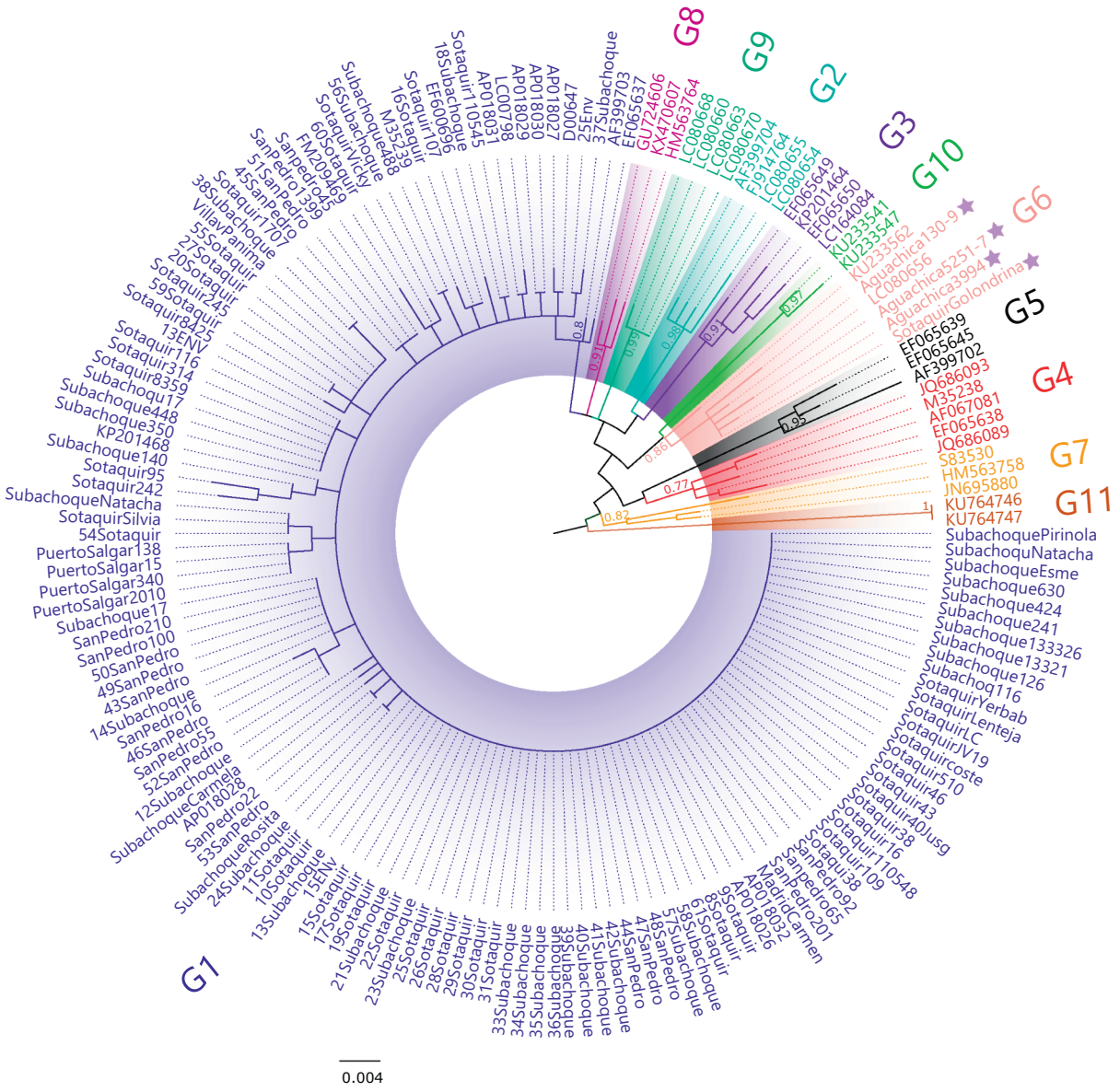

Fig. 4. Maximum-likelihood phylogenetic tree analysis of the *env* gene of BLV circulating in Colombia. The evolutionary history was inferred using the ML method based on the Tamura-Nei model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3588)). The analysis involved 168 nucleotide sequences, 124 Colombian and 44 reference sequences from Genbank. Genotype 6 samples are shown with an *.

package based on the Akaike information criterion (AIC). Using this model, maximum likelihood trees were constructed using the MEGA 7.0 software. As a measure of the robustness of each node, the bootstrapping method (1000 pseudo-replicates) was employed. The phylogenetic trees were edited using the FigTree program v1.4.1, which is available online (http://tree.bio.ed.ac.uk/software/figtree/).

For the *env* sequences, phylogenetic inference was also performed by maximum likelihood analysis using the RAxML program. Phylogenetic tree inference using maximum likelihood/rapid bootstrapping was run on XSEDE (RAxML - HPC2 on XSEDE (8.2.10) (Stamatakis, 2014) with 1000 bootstrap replicates using the MrBayes program (Huelsenbeck et al., 2015). The phylogenetic inference was performed in a Bayesian framework with GTR + G, two runs and three chains.

3. Results

3.1. Prevalence of BLV

In order to determine the prevalence of BLV in Colombia, *gag* and *tax* segments were amplified. All samples were positive for the GADPH gene and 179 out of 289 samples were positive for *gag* and *tax* segments. Consequently, the observed prevalence of BLV based on both gene segments was 62% per animal and 92% per farm. Across the country, this prevalence was distributed by department as follows: Cundinamarca 69 and 90%, Boyacá 71 and 94%, Antioquia 73 and 100%, Meta 85 and 100%, Nariño 14 and 75% and Cesar 17 and 75% (Fig. 1).

3.2. Viral genotype

To determine the circulating genotypes in Colombia, phylogenetic

analyses were performed. The *env* region of the BLV genome was amplified, but only 53 samples could be sequenced and phylogenetically analyzed, as shown in the phylogenetic tree in Fig. 2, where all these samples were grouped within the genotype 1 clade, with a support bootstrap value of 87% and the phylogenetic inference was performed in a Bayesian framework to confirm the ML analyses (data not shown).

For the *gag* and *tax* segment, amplification was achieved in the same 179 samples and the corresponding sequence was obtained only in 131 of them for both segments.

Even though *gag* and *tax* sequences are not used for genotyping, these sequences were studied, and for *gag* segment all the Colombian samples were grouped in the same clade, which corresponded to genotype 1 (Fig. 3A). However, for the *tax* region, four of the sequences (marked as Sotaquira Golondrina, Aguachica130–9, Aguachica 5251–7 and Aguachica3934) were clustered separately from the genotype 1 clade, together with genotype 6 representatives (Fig. 3B). These four sequences seemed to have phylogenetic discordances between *tax* and *env* regions. Therefore, a new set of primers was designed, considering the current genetic variability of BLV strains, as deduced by available sequences of all BLV genotypes reported in GenBank. With this new set of primers, a 750 bp region of the *env* gene was amplified, which was 244 nt longer than the sequence amplified by the set of primers currently used for genotyping purposes.

3.3. Evidence of circulation of genotype 6

To analyze a phylogenetic discordance between *env* and *tax* regions, the 131 samples that were positive for *gag* and *tax* segments were amplified with the new set of primers designed for the *env* gene. The results showed that the four samples that were grouped within genotype 6 by *tax* gene were also clustered together with genotype 6 strains when analyzed by this new *env* gene segment. Altogether, the analyses performed indicate the circulation of both BLV genotypes 1 and 6 in Colombia, with 117 out of 121 samples belonging to genotype 1, while the 4 remaining correspond to genotype 6 (Fig. 4).

4. Discussion

Molecular epidemiological studies of BLV worldwide, as well as in Colombia, enable the identification of circulating strains in specific regions, in order to propose and build public policies aimed at controlling and, in the future, eradicating this virus, which in addition to producing problems for cattle, has also been reported as a possible risk factor involved in human's pathologies (Buehring et al., 2017; 2015; Ceriani et al., 2018; Olaya et al., 2016; Schwingel et al., 2019). On the other hand, the variability of genotypes as well as results of structural and functional properties of its envelope proteins provide useful information for research focused on the production of vaccines using specific strains. In the case of Colombia, based on this study's results and previous reports (Benavides et al., 2017; Úsuga-Monroy et al., 2018), it will be appropriate to include conserved epitopes shared between genotypes 1,2,3 and 6. In addition, phylogenetic studies are important for understanding the geographical distribution of the virus, and thus identify conserved and hypervariable regions, including specific mutations that could be related to different levels of virulence or pathogenicity.

Currently, the OIE and others use primers reported by Fechner et al., 1997 (Lee et al., 2005; Polat et al., 2017a) to detect BLV circulating genotypes, and such primers were used initially in this study. However, when carrying out the phylogenetic analysis, all the Colombian isolates were grouped under genotype 1, a result that differs from the sequences obtained from other gene regions (*gag* and *tax*). With the *tax* region, 4 of the positive samples of BLV were grouped under genotype 6; the *tax* region is not usually used for genotyping, since it is considered a polymorphic region in retrovirus. This suggests that the primers reported by Fechner present disadvantages for identifying genotypes of the virus reported after 1996, and thus, a new set of primers was designed in the current study. The new primers were designed by bioinformatic analyses including 69 sequences of the complete genome of the virus available in the GenBank representing the genotypes reported to date, selecting a conserved region from among all the genotypes in the *env* gene. As a result, a fragment of 750 bp was amplified and sequenced, finding that, in fact, the Fechner primers did not enable the identification of other genotypes, and indicating that genotypes 1 and 6 are simultaneously circulating in Colombia (Fig. 4).

Even though the *tax* segment is not typically used for genotyping, its amplification provided good information, which led to extending the *env* segment, with the aim of having the four samples display a change of position in the tree. The finding that the *tax* sequences produce warnings regarding the genotypes should be addressed in future studies, in order to verify the hypothesis that the *tax* gene is useful for genotyping purposes. Even though the Fechner primers were not able to detect G6, when the amplification region is extended over the *env* segment, the resolution of the result was increased, enabling better determination of the genotypes, while ruling out recombination and coinfection effects.

In this study, finding of genotype 1 was expected because this is the most prevalent genotype worldwide. However, evidence of G6 in Colombia is a novel finding. Overall, G6 has been reported in four Asian countries (Philippines, Thailand, Jordan, and India) and five South American countries and in Italy (Argentina, Brazil, Bolivia, Peru and Paraguay) (Bazzucchi et al., 2019; Gautam et al., 2018; Polat et al., 2017a). Different genotypes distributed in the world and the emergence of new genotypes in specific areas, as reported here, suggests that importing and exporting processes in the cattle industry contribute to increased viral prevalence and virus diversity, which in the case of silent diseases such as enzootic bovine leukosis are transferred unnoticed between cattle from different regions. This suggests the necessity of establishing global policies of control and diagnosis.

One of the most relevant findings of this study was the detection of genotype 6 by *tax* region. So far, no other studies have used this ORF for genotyping, and there are few available sequences for this region in databases that are representative of all viral genotypes that would enable more in-depth studies in terms of comparing the relationship between *env* and *tax* genes for the effects of genotyping. Further studies are needed to confirm its application for genotyping, but at least this study's results suggest that this gene, in addition to the *env* region, could be useful for genotyping.

On the other hand, in addition to identifying the circulating genotypes, it is also important to discuss the prevalence reported in this study, which found a positive prevalence of 62% in animals and 92% of farms, which is one of the highest prevalence rates reported in Latin America. In Colombia, the latest report published by Ortiz et al. found a lower seroprevalence (42% per animal and 67% by farm) (Ortiz et al., 2016) compared to this study, using the same cattle population.

One of the main differences between the two studies lies in the diagnostic method. Here, prevalence was determined by PCR tests, which directly detect fragments of the viral genome, whereas the study by Ortiz et al. used the ELISA commercial kit, which detects antibodies in the host (Ortiz et al., 2016). Even if the presence of antibodies could be interpreted as presence of the virus, according to some authors the sensitivity of ELISA kits might be lower than the detection level of a PCR test (Lee et al., 2005; Trono et al., 2001). Now, in terms of antibodies production, it is possible that the amount of antibodies in sera samples could be below the detection limits of ELISA, which implies that false negative samples could be reported, especially in the case of viruses with slow replication rates such as BLV, giving as a result low levels of antibodies in blood due to immune response evasion (Frie and Coussens, 2015; Lee et al., 2005).

Even though there no records available on the importance of the cattle industry in our country and in Latin America, Ritcher et al. found that BLV has substantial effects on mortality, morbidity, premature

birth, culling, stillbirths, abortion and reinfection, which have a significant influence on the monetary level of direct losses (Richter et al., 2017). It would therefore be relevant for governmental entities and policymakers to consider adopting enzootic bovine leukosis as an official control for the disease, to facilitate the diagnosis of infected animals by members of the cattle industry, and furthermore to control the dissemination of the virus through the future promotion of eradication programs in order to minimize the impact that BLV has on cattle and eventually on humans who consume bovine-derived food products (Olaya-Galán et al., 2017).

Studies like this one are very important for science and for One Health approaches, in which interfaces between animals-humans and ecosystems should be considered (Kelly et al., 2016). In the case of BLV, it has been shown that the virus not only is present in cattle but also in other species such as sheep, buffalo, goats, alpacas and humans (Buehring et al., 2014; Mesa et al., 2013; Nekoei et al., 2015; Robinson et al., 2016). Although the effect BLV might have on humans is not yet clear, evidence of its presence has been reported, and it has been proposed as a potential risk factor for breast cancer development, although this hypothesis has been rejected by others (Barez et al., 2015; Gillet and Willems, 2016; Zhang et al., 2016). In addition, due to the fact that BLV is considered one of the main viral agents associated with economic impact in livestock production, which is distributed worldwide (Richter et al., 2017) and seems to have a zoonotic behavior, there are enough arguments to continue searching for vaccine candidates and strategies aimed at controlling viral infection by preventing risk factors within farms as well as the implementation of good livestock production practices for each specific region (Olaya-Galán et al., 2017; Olaya et al., 2016; Ortiz et al., 2016).

5. Conclusions

The epidemiological data provided here demonstrated a higher prevalence of BLV in Colombia compared to the seroprevalence previously reported. In addition, the findings contribute to the epidemiology of the virus by identifying genotype 1 and 6 in Colombia. Furthermore, the new primers that were designed in this study will be available for future studies willing to amplify all the current circulating genotypes, as an update of the proposal of Fechner et al. Finally, although *tax* is not commonly used for genotyping, phylogenetic analyses showed that this gene does contribute to the genotype identification and may be useful for BLV genotyping as well.

Ethics approval and consent to participate

This paper is part of the project called “Búsqueda y relación filogenética del virus de la leucosis bovina (BLV) en tejido mamario humano y en linfocitos de bovinos” financed by Colciencias and approved in virtual session of the ethics committee of Science Faculty of Pontificia Universidad Javeriana (June 9, 2014). For the blood samples, the informed consent was taken orally by two of the researchers, Alfredo Sanchez and Diego Ortiz, who were the veterinarians who visited the farms and spoke with the owners to request the samples. Additionally, through Pontificia Universidad Javeriana, the research team requested authorization for taking these samples from the Institutional Committee for the Care Use of Animals, (CICUAL, by its acronym in Spanish), which approved the veterinarians because of their status as VECOL staff members, who are responsible for taking samples and requesting informed consent from farm owners. The informed consent was taken at each farm where cattle was tested, with no objections noted.

Consent for publication

Not applicable.

Availability of data and materials

All relevant data are included in the paper.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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