Bovine leukosis virus detection in Creole Colombian breeds using nested-PCR

Detección del virus de la leucosis bovina en ganado criollo colombiano mediante PCR-anidado

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Abstract

Using 360 DNA samples from eight Creole bovine breeds Blanco Orejinegro (BON), Casanareño (CAS), Costeño con Cuernos (CCC), Chino Santandereano (ChS), Caqueteño (CQT), Hartón del Valle (HV), Romosinuano (RS) and San Martinero (SM), two synthetic Colombian breeds: Lucerna (LUC) and Velásquez (VEL) and two introduced breeds Brahmán (B) and Holstein (H); the presence of Bovine Leukosis Virus (BLV) was evaluated through the amplification of a viral gene region *env* (provirus detection – nested-PCR). The percentage of presence and independence test were calculated (X^2). Presence of BLV was higher in HV breed, followed by ChS (83.3% and 60% respectively); VEL and LUC breeds showed the same percentage (50%). In CAS, CCC and CQT the presence of virus was 26.7%, 23.3% y 16.7% respectively. On the other hand, no virus presence was found in BON, SM and RS. For the introduced breeds the presence of virus was 83.3% for H and 6.7% for B. The average of presence for Creole bovine breeds was lower than introduced breeds. A high and significant dependence was found between the presence of BLV with breed, sex and sampling places. The presence was lower in males than in females and in the northern than the southwestern and central areas of the country.

Key words: Creole cattle, enzootic bovine leukosis, molecular diagnostic.

Resumen

Se evaluó la presencia del virus de la leucosis bovina (VLB) en 360 muestras de ADN de ocho razas bovinas criollas Blanco Orejinegro (BON), Casanareño (CAS), Costeño con Cuernos (CCC), Chino Santandereano (ChS), Caqueteño (CQT), Hartón del Valle (HV), Romosinuano (RS) y San Martinero (SM), dos Razas Sintéticas Colombianas: Lucerna (LUC) y Velásquez (VEL) y dos razas foráneas: Brahmán (B) y Holstein (H). Para la detección del pro-virus se amplificó una región del gen *env* viral, mediante PCR anidada. La presencia del VLB fue mayor en la raza HV seguido por ChS (83.3% y 60% respectivamente), VEL y LUC tuvieron el mismo porcentaje (50%), en CAS, CCC y CQT la presencia del virus fue de 26.7%, 23.3% y 16.7% respectivamente; no se encontró el virus en BON, SM y RS. En las razas foráneas la presencia fue de 83.3% para H y 6.7% para B. Se encontró dependencia altamente significativa entre la presencia del VLB y la raza, el sexo y región de origen de la muestra. El promedio de presencia en las razas criollas fue menor que en las foráneas, menor en los machos que en las hembras y en la región norte que en el suroccidente y el centro del país.

Palabras clave: Diagnóstico molecular, ganado criollo, leucosis bovina enzoótica.

Introduction

Enzootic bovine leukosis (EBL) is an infectious disease caused by a retrovirus –bovine leukosis virus (BLV)- which affects B lymphocytes from the lymphocyte lineage (Beyer *et al.*, 2002; Dequiedt *et al.,* 1999). It is characterized for inducing tumors (lymphosarcoma, LS; malignant lymphoma, ML) and/or a steady increase in the absolute number of lymphocytes in the bloodstream (persistent lymphocytes, PL) (Dees *et al.*, 1996).

BLV belongs to the Retroviridae family, it has a retrotranscriptase which synthetize a DNA copy from viral RNA. The synthetized DNA (provirus) integrates into the host genome and stays in the nuclei of diverse cells (Evermann, 1992).

The medical manifestation of EBL starts after cattle are two years old with anemia, emaciation and infertility. The most evident sign is the bilateral symmetrical growth of the lymphatic ganglia. Exophthalmia is considered as a more specific sign of the disease, as well as different subcutaneous tumor masses at different places (lymphoadenopathy) (Chamizo, 2005; Malatestinic, 2003; Shell *et al.*, 2004).

Infection is transmitted horizontally or iatrogenically. From six months old cattle, the prevalence increases, the highest incidence is between two and three years, and it is higher on meat cattle than on dairy cattle farms (Chamizo, 2005).

The most used serology tests to diagnose the disease are: radioimmunoassay (RIA), agar gel immunodiffusion (AGID) and immunoenzymatic assay (ELISA). AGID is a reliable indicator of BLV infection and has a high specificity degree due to, somehow, the high stability of the viral genome. This diagnosis test cannot recognize between passively acquired antibodies (antibodies from colostrum) and the ones acquired by natural infection. RIA and ELISA tests are more sensitive, the last one has the advantages of being less expensive, easier to perform and it can be used in milk instead of blood samples.

(González *et al.*, 2001). A disadvantage of these diagnosis tests is that they cannot detect the infection in young animals or animals with an early disease stage. (Chamizo, 2005).

The acid nucleid hybridization test "dot blot" is highly repeatable and it correlates with the AGID assay. Dot blot analysis results take shorter time than AGID´s (Chamizo, 2005).

PCR has been used for early detection of BLV in animals younger than six months old to avoid false positive reactions caused by passive transference of immunoglobulin from the colostrum. Another advantage is the ability to detect the virus in immune-tolerant animals. It is also 96% sensitive and 45% specific in comparison with the AGID test (Agresti *et al.*, 1993; Fechner *et al.,* 1996).

One of the main disadvantages of PCR is the variation in the nucleotide sequence of some virus isolates, which can induce oligonucleotide hybridization errors and, consequently, reduce sensitivity (Fechner *et al.*, 1996).

According to the World Organization for Animal Health (OIE, 2009) between 1996 and 2004 there were 198 disease outbreaks, 1083 cases and 39 deaths due to bovine leukosis in Colombia. Studied about BLV presence in Colombia are variable because they depend on the sampling region and the serology test used. At the Northeast of the country presence percentage is between 3.9 and 14.64% using the AGID test (Aguilar *et al.*, 1989; Trujillo, 1989; Ruiz, 1995). Ramírez *et al.* (2002) report a presence of 37.5% in young cows and 71.9% in cows. 21.5% positives were found in Cordoba using ELISA test (Betancur and Rodas, 2008), while in the Sabana de Bogotá –main dairy area of Colombia- presence of 45.28% was reported (Alfonso *et al.*, 1998). Griffiths *et al.* (1982) found prevalence of 24.9% in dairy cattle in the Andean region, 14.4% in the Caribbean and 15.3% in the foothills of the Eastern plains. Using molecular techniques (nested-PCR), Muñoz *et al.* (2008) found 25% of virus presence.

Colombia is considered as one of the countries with the highest biodiversity on zoogenetic resources since, in each of the hydrographic areas of the Orinoco and Amazonas river there is a creole bovine (*Bos taurus*) adapted breed. These breeds correspond to Romosinuano and Costeño con Cuernos for the Atlantic coast (north zone), Chino Santandereano and Blanco Orejinegro in the temperate mountain zone, Hartón del Valle in the Cauca river valley; Sanmartinero and Casanareño in the eastern flat zone; Caqueteño in the Department of Caquetá; and synthetic Colombian breeds such as Lucerna del Valle and Velásquez in Caldas (Martínez-Correal, 1992). Creole breeds and synthetic breeds are denoted as creole Colombian breeds (CCB) which has 18,231 animals belonging to the 0.08% of the total cattle population in the country (Martínez-Correal, 2010).

The objective of this work was to determine the presence of the bovine leukosis virus in creole and synthetic Colombian breeds by using nested-PCR.

Materials and methods

Blood samples of 30 individuals of each creole bovine breed (Blanco orejinegro (BON), Chino Santandereano (ChS), Costeño con cuernos (CCC), Caqueteño (CQT), Casanareño (CAS), San Martinero (SM), Romosinuano (RS), Hartón del Valle (HV)), from each synthetic breed (Velásquez (VEL) and Lucerna (LUC)) and from foreign breeds (Holstein (H) y Brahmán (B)) were used. Animals were sampled in the Departments of Atlántico, Arauca, Bolívar, Caldas, Caquetá, Cauca, Meta, Santander and Valle del Cauca in 2009, for a total of 240 creole breeds samples, 60 synthetic breeds samples and 60 foreign breeds samples. Sample size was determined based on the data related to BLV presence reported by Muñoz *et al.* (2008) using 90% of confidence and 0.01 absolute maximum error. In Valle del Cauca were sampled HV (six farms), B (five farms) and H (two farms); ChS, CQT and SM were sampled in in two farms each located in Santander, Caquetá and Meta, respectively. The other breeds were sampled in only one farm. BON, originated in Antioquía and Caldas, was sampled in Cauca. 90% of sampled males were active breeders.

DNA was extracted from blood using the Salting Out protocol (Miller *et al.*, 1988). DNA concentration was determined with DNA of the Lambda bacteriophage using known concentrations and visualizing it in 0.8% agarose gels dyed with ethidium bromide (Hernández *et al.*, 2007). DNA was diluted to 10 ng/µl. Each PCR reaction had positive and negative controls coming from the Genetics Institute of the Universidad Nacional de Colombia. Control DNA was isolated from serum using the methodology described by Klein *et al.* (1997).

A highly conserved region of the *env* viral gene was amplified from the isolated DNA using nested-PCR (Beier *et al.*, 2001). The first reaction was done in 30 µl composed of DNA (100 ng), primers [1.25 mM] (Forward-TCTGTG-CCAAGTCTCCCAGATA and Reverse-AACAACA-ACCTCTGGGGAGGGT), each dNTP [0.2 mM], PCR buffer 1x, $MgCl_2$ [2.5 mM] and Taq DNA polymerase (1U). Second reaction used as template 3 µl of the PCR product from the first reaction and the same concentrations of the other reactives described using the primers Forward-CCCACAAGGGCGGCGCCGGTTT and Reverse-GCGAGGCCGGGTCCAGAGCTGG. PCR reaction included an initial denaturation at 94 °C for 5 minutes, followed by 40 cycles of 94 °C for 30 seconds, 57 °C for 30 seconds and 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes. The second reaction had the same conditions, except for the hybridization temperatura which was 68 °C (Beier *et al.*, 2001). Amplifications were done on a thermocycler PTC-100® Teltier Thermal Cycler, BIO-RAD. Amplified products were visualized in 1.2% agarose gels dyed with ethidium bromide on a SUB-CELL® GT, BIO-RAD camera. An amplicon of 444bp indicated the protovirus presence on an individual (Beier *et al.*, 2001).

Statistical analyses were done using the following groups: creole breeds (BON, CAS, CCC, ChS, CQT, HV, RS and SM), synthetic breeds (LUC and VEL) and, foreign breeds (H and B). Percentage of virus presence in each

breed and gender was determined. Animals were group according to region of origin of the sample, i.e. North (CCC, ChS and RS), Center (VEL), East (CAS y SM) and Southwest (BON, CQT, HV y LUC). Chi-square (X^2) test were done using SAS v.9.1 software (SAS, 2003) to determine the dependence between virus presence and breed, gender and origin of the sample.

Results and discussion

This is the first study reporting the use of molecular techniques to detect BLV in creole Colombian breeds. The results refer to BLV detection in bovine blood samples.

Table 1 shows BLV presence (%) according to breed and gender for each one of the groups. Percentage of BLV presence was higher in HV and ChS breeds (83.3 and 60%, respectively), VEL and LUC had the same percentage (50%) and, percentage of BLV presence in CAS, CCC and CQT was 26.7%, 23.3% and 16.7% respectively.

BLV was not found in the breeds BON, SM y RS. Given the high prevalence of this disease in Colombia, this finding suggests a possible resistance to BLV in those breeds. This requires studies in a larger number of farms to correlate virus presence with EBL symptoms and with genetic markers associated to disease resistance.

The presence average in creole breeds (26.7%) and synthetic breeds (50%) was lower than the average for H (83.3%) but higher than B (6.7%). These results are in agreement with Chamizo (2005) who declares that there is a higher BLV presence in *Bos taurus* than in *Bos indicus*, and with Orjuela *et al.* (2000) who reported that 9.4% of *Bos Taurus* and 1.4% of *Bos indicus* were positive for the disease, while the crossed animals were not.

Virus presence in foreign breeds was 6.7% in B and 83.3% in H. According to Chamizo (2005) virus presence is higher in dairy cattle than in beef cattle since they are more exposed to infectious sources like syringes for vaccination, surgical material, palpation gloves, among others.

Independence or association test by chi-square (X^2) suggested that virus presence depends on breed ($X^2c = 29.62$; $P < 0.001$), which is opposite to the reports of Betancur and Rodas (2008) whom did not find relation between zebu, european and crossed breeds with the disease. It is important to highlight that virus presence was not detected in BON, SM and RS breeds, which could be a good indicator of good management and sanitary control in the farms sampled or, it could be related to virus resistance. The last one could be proven in future studies involving a larger number of farms, in which, besides studying virus presence, the disease symptoms are studied as well.

The association test also showed significant differences ($P < 0.05$) for virus presence and gender ($X^2c = 0.0842$; $P < 0.0771$). It was higher in females than in males in both creole breeds (21.7% and 4.6%, respectively) and synthetic breeds (40% and 10%, respectively) (Table 1). It is important to highlight that around 90% of the sampled males were active breeders. The above information is consistent with Betancur and Rodas (2008), which reported as disease positive 68.6% of the females and 31.4% of the males. Chamizo (2005) found that management practices as palpation routines, artificial insemination and milking, among others, make females more prone to the infection than males. Dependence relation between virus and region of origin of the samples was highly significant ($X^2c = 63.88$; $P < 0.001$). Sampled breeds from the North zone of Colombia (CCC, CHS and RS) had 25% of virus presence, 13% was found in the eastern region (CAS and SM), 50% in the Central region (VEL) and 33% in the Southeast (BON, CQT, HV and LUC). Griffiths *et al.* (1982) found on dairy cattle a prevalence of 24.9% in the Andean region. VEL, originated in the central zone of Colombia, showed 50% of disease presence in this study. The differences between this value and the one reported by Griffiths *et al.* (1982) are due to the scope of the research since they diagnosed the disease presence not the virus presence.

Table 1. Bovine leukosis virus (BLV) presence (%) in creole Colombian, creole synthetic and foreign breeds..

Breed group[a]	Breed	Presence (%)	By sex			
			Male		Female	
			No.	(%)	No.	(%)
Creole	BON	0.0	8	0.0	22	0.0
	CAS	26.7	6	3.3	24	23.3
	CCC	23.3	3	3.3	27	20.0
	ChS	60.0	5	16.6	25	43.3
	CQT	16.7	5	0.0	25	16.7
	HV	83.3	4	13.3	26	70.0
	RS	0.0	7	0.0	23	0.0
	SM	0.0	3	0.0	27	0.0
	Promedio	26.7		4.6		21.7
Synthetic	LUC	50	0	0.0	30	50.0
	VEL	50	11	20.0	19	30.0
	Promedio	50		10.0		40.0
Foreign	B	6.7	1	3.3	29	3.3
	H	83.3	0	0.0	30	83.3
	Promedio	45.0		1.65		43.3

a. Creole: Blanco Orejinegro (BON), Casanareño (CAS), Costeño con Cuernos (CCC), Chino Santandereano (ChS), Caqueteño (CQT), Hartón del Valle (HV), Romosinuano (RS) and San Martinero (SM). Colombian synthetic: Lucerna (LUC) and Velásquez (VEL). Foreigns: Brahman (B) and Holstein (H).

In the samples collected in the Eastern of Colombia, SM did not showed virus and CAS had a presence percentage of 26.7%, a higher value than the one reported by Griffiths *et al.* (1982) for dairy cattle (15.3%) in the same zone of the country.

In bovines from the North region of Colombia virus presence was 0% for RS, 23.3% for CCC and 60% for ChS. Orjuela *et al.* (2000) work in this region using the immunodetection technique and detected BLV presence in 1.5% of the tested animals. In the Department of Córdoba 21.5% of animals were positive for BLV using ELISA (Betancur y Rodas, 2008) while Griffiths *et al.* (1982) reported a prevalence of 14.4% in dairy cattle. With the exception of Romosinuano animals, the values for virus presence reported in literature are lower than the ones found in this study.

In HV and LUC, the percentage of virus presence was 83.3% and 50%, respectively. Using nested-PCR, Muñoz *et al.* (2008) found 25% of virus presence by evaluating samples of different breeds from a DNA bank in Valle del Cauca, which is a value that is lower than the one found in the present study.

Conclusions

BLV presence was not detected in BON, SM and RS breeds. Since EBL has a high prevalence in Colombia, these findings suggest a possible resistance of these breeds to BLV. High

presence of BLV in breeds such as HV an ChS should be investigated more closely. It is required to follow up this work with studies that sample a higher number of farms, evaluating management practices of the animals and that correlate virus presence with EBL symptoms and with genetic markers for disease resistance.

Acknowledgments

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