



Prevalence of bovine leukosis virus in water buffaloes in West-central Colombia



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Abstract:

Bovine viral leukosis (BVL) is a disease with a high morbidity and low mortality. There are reports of natural infection in some bovine species, but it has been little studied in buffaloes. In Colombia, buffalo production is growing rapidly, and, so far, there are no reports of the disease. The objective of this study was to characterize buffalo production in the coffee-growing region and to determine, by PCR, the prevalence of BVL in buffaloes, as well as its presence in humans, cattle, and sheep near the coffee-growing region. Blood samples from 140 buffaloes and 10 buffalo milk samples were collected, and so were 58 samples from bovines, 35 samples from sheep, and 9 samples from humans that had been in contact with buffaloes. Hematological analyses were performed. Subsequently, DNA was extracted for PCR evaluation. Production information was gathered, and the results were processed and analyzed using the R software. The majority of animals were Mediterranean breed females,

with a birth weight of 33.39 kg, a weaning weight of 202.93 kg, a calving interval of 491.77 d, a time to peak of 67.26 d, and 381.59 L of milk (adjusted to 305 d at two teats). A prevalence of 33.6 % and of 3.4 %, respectively, was detected in buffaloes and in bovines; no milk, sheep or human samples were positive. No risk factors associated with the infection were found; neither were significant alterations of the blood count or factors of production. These results constitute the first molecular report of the bovine leucosis virus (BLV) in the Americas and one of the first in the world.

Key words: BLV, Deltaretrovirus, Bovine Leukemia, Lymphosarcoma, PCR.

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Introduction

Bovine viral leukosis (BVL) is a malignant systemic viral neoplastic disease with high morbidity and low mortality involving a characteristic accumulation of neoplastic lymphocytes in various organs, which leads to immunosuppression and predisposition to secondary pathologies. This has resulted in economic losses, due not only to the increase in the occurrences of diseases but also to the evident negative effect of the infection on milk production. Leukosis is caused by a type "C" RNA virus of the family *Retroviridae*, subfamily *Orthoretrovirinae*, and genus *Deltaretrovirus*, which causes lymphomas, lymphosarcomas, and leukemia. The virus can be transmitted horizontally or vertically, i.e., between bovines or from mother to fetus⁽¹⁻³⁾.

There are reports of leukosis in several species; however, it has been found to occur naturally only in bovines, buffaloes, and capybaras⁽⁴⁾, having been little studied in buffaloes⁽⁵⁾. Sheep have been determined to be highly susceptible to inoculation, showing tumors rapidly; other species of goats, deer, rabbits, cats, monkeys, pigs, and chimpanzees have exhibited persistent antibodies after experimental inoculation⁽⁴⁾, which seems to suggest that the virus can cross the species barrier. However, further research is still needed, especially since one study has shown a link between the presence of the virus and breast cancer in humans⁽⁶⁾.

Water buffaloes have been considered to be resistant to various diseases transmitted by such vectors as ticks, hemoparasites, and certain nematodes⁽⁷⁾. However, a few reports suggest that the bovine leukosis virus naturally infects buffaloes. In the literature, there are reports of

experimental infection⁽¹⁾, but very few reports of natural infection, and although some studies show prevalences, these are close to zero and have been detected using tests that may have allowed that percentage of false positives⁽¹⁾. In Pakistan and Cambodia, there are reports of 0 % prevalence of leukosis in buffaloes evaluated by Western blotting. Other studies have suggested the same percentage, based on serological analyses^(1,8,9). Recently, a study in southeastern Brazil showed a prevalence of 0 % using agar immunodiffusion (AGID), PCR, and ELISA (ELISA-gp51), which reinforces the idea of a possible absence of natural infection in water buffaloes. In the same study, performed with a commercial ELISA kit (ELISA-BLV) on the same samples, 24.4 % of the animals were seropositive; this points to the diagnostic problems associated with this ELISA test, which has a high false positive rate^(1,5). As a result of these studies, PCR has been proposed as a reference test, despite the fact that AGID is considered to be the Gold Standard test⁽⁵⁾.

In a study conducted in the Philippines, a prevalence of 27 % was detected by PCR; however, in a group of unknown origin, the prevalence rate was found to be 0 %⁽⁷⁾. This again opens the question of natural infection of buffaloes, especially in the case of production animals, because although there are multiple case reports of lymphosarcoma in buffaloes in the world^(10,11), many of these cases have been reported as negative for bovine leukosis virus^(5,11), a fact that raises more questions about this disease in buffaloes. Thus, it is necessary to adequately clarify whether natural infection exists in buffaloes, particularly if it is present in the Americas, and specifically in Colombia, where there are no reports of the disease in buffaloes⁽¹²⁾.

It is estimated that there are 3'800,000 buffaloes on the American continent; the American countries with the largest buffalo populations are Brazil with 3'500,000, Venezuela with 350,000 and Colombia with 308,580⁽¹³⁻¹⁵⁾. In Colombia, this is one of the livestock populations that have grown the most in recent years, given the buffaloes' ability to adapt and maintain their performance in difficult conditions where cattle cannot^(14,15). For this reason, buffalo breeding is becoming an increasingly important economic activity, and the study of the diseases that these animals may suffer is an important factor of production. Thus, the objective of this work was to characterize buffalo production in the coffee-growing region, to determine the prevalence of bovine leukosis virus by PCR in buffaloes, and to detect the presence of the virus in humans, cattle and sheep that are in contact or close to the buffaloes.

Materials and methods

Sample size

According to the 2017 national livestock census of the Colombian Agricultural Institute (ICA), the coffee-producing axis includes 34 farms with approximately 3,105 animals⁽¹⁵⁾. Based on this information, the sample size was estimated at 94 animals, taking into account a heterogeneity of 50 %, a margin of error of 10 % and a confidence level of 95%. However, it was possible to perform the evaluation on a larger number of animals; therefore, 140 blood samples were obtained from buffaloes of different breeds including the Murrah, Mediterranean and Bufalypso, most of the animals being in the productive stage. The buffaloes were sampled in eight herds of the coffee-producing axis during the years 2016-2017. Samples were also taken from 58 cattle, 35 African sheep, and 9 humans that were in the same production system or very close to it. In the case of the humans, they were people involved in the handling of the animals. These species were considered because they have been reported to be susceptible to natural BTV infection⁽⁴⁻⁶⁾.

Data collection

In the sampled herds, information was collected on those production and risk factors that may be associated with the presence of leukosis. However, productive information was available only for three herds because there were few or no records for the others. The production information collected included birth weight, weaning weight, weaning weight gain, calving interval, milk production at two teats adjusted to 305 d (considering that two teats are left for the calf), production at peak and days to the peak; while the risk factors considered were breed, herd and age group divided into buffaloes at productive age (3 yr or older) and buffaloes at non-productive age (less than 3 yr old)⁽¹⁶⁾.

The farms where the buffalo samples were taken were located in the central-western region known as the coffee-growing region, mainly comprising the departments of Caldas, Quindío and Risaralda. The farms were located at an average altitude between 917 and 1,575 m asl and an average temperature between 18 and 30 °C. The municipality with the highest number of animals sampled was Marsella, with 44, followed by Chinchiná and Calarcá, with 32 each; Pueblo Rico, with 29; Cerritos, with 18; Pereira, with 8, and Cartago, with 4. The exact origin of 47 of the samples was not determined because they were collected at a slaughterhouse.

The municipality of Cartago was included because, although it is located in the north of the department of Valle, it is considered part of the coffee-growing region.

Sampling and hematological analysis

The project was endorsed by the bioethics committee of the Technological University of Pereira (Universidad Tecnológica de Pereira) in Act 17 of 2015 (code CBE-SYR-172015). Blood samples were taken with BD vacutainer tubes with EDTA as anticoagulant and No. 18 needles, after disinfecting the area with antiseptic alcohol. The buffalo and sheep were sampled from the jugular vein, and the cattle, from the coccygeal vein. In the case of humans, a bacteriologist collected samples after the participants signed an informed consent form. In addition to the blood samples, 10 samples of milk from commercially sold buffaloes were collected. The milk and blood samples were transported in portable coolers at approximately 4 °C to the multiple laboratory of Animal Sciences of the Universidad Tecnológica de Pereira, where the hematological analysis was performed using a URIT 2900Vet plus automated analyzer (URIT®, Guilin, China) in the option for buffaloes, and the following variables were assessed: white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), and platelet (PLT) counts. The remaining blood was used for DNA extraction.

DNA extraction from blood and milk

The blood DNA was extracted with the GE® Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) according to the manufacturer's recommendations. Once the samples were obtained, their purity was evaluated by absorbance ratio analysis using two wavelengths (260/280 nm), with the NanoDrop 2000c (Thermo scientific®, Wilmington, USA). Only DNA samples with an absorbance ratio of 1.8 to 2 and an absorbance concentration above 1.8 were considered 10 ng/mL. The samples were stored in Eppendorf tubes at -20 °C until PCR analysis.

The DNA from milk somatic cells was extracted with the modified salting out method, taking into account two different approaches considering the amount of fat present in buffalo milk. In the first protocol, 14 mL samples of raw milk were collected in Falcon tubes; they were allowed to stand in the refrigerator at 4 °C for 1 h and centrifuged at 3,500 rpm for 8 min; then, the fat layer was broken and removed using a Pasteur pipette, and the supernatant was discarded without disturbing the cell button. Subsequently, 5 mL of saline solution were

added and shaken vigorously until the cell button was dissolved. The solution was centrifuged again (2 to 3 times more until the colorless supernatant could be observed) at 3,500 rpm during 8 min, and the supernatant was discarded without disturbing the cell button. Subsequently, 5 mL of lysis solution (10 mM Tris HCl pH 8.2, 400 mM NaCl, 2 mM Na₂EDTA), 26.5 µL of proteinase K enzyme (2 mg/mL), and 300 µL of SDS solution were added and resuspended by gentle vortexing for 1 min, and the solution was added to the sample. This was then incubated in a water bath at 55 °C for 6 h, after which it was refrigerated at 4 °C for 5 min and allowed to cool, and 1.5 ml of saturated saline solution (6 M) was added. After this, the sample and the solution were mixed in a vortex and centrifuged for 10 min at 3,500 rpm. The supernatant was taken to a 15 mL tube to add 100% ethanol at -20 °C until reaching 14 mL, after which the tube was gently shaken by inversion in order to observe the DNA skein. The sample was washed with 1 mL of 70% ethanol and then centrifuged at 4,000 rpm; the supernatant was discarded and the tube was inverted, allowing the contents to dry. Finally, the button was resuspended in 300 µL of 1X TE buffer pH 8.0 (1 M Tris HCl and 0.5 M EDTA) and stored at 4 °C until analysis. Protocol 2 was the same as protocol 1, except that in this case the cream layer was not broken, nor was the saline solution added; only the centrifugation of the raw milk was followed directly by the addition of the lysis buffer. For both protocols, DNA quantification was performed using a 2000c NanoDrop (Thermo scientific®, Wilmington, USA) using 2 µl of the sample from each extraction protocol. The 260/280 and 260/230 ratios were also taken into account as indicators of DNA purity. The data were stored for further statistical analysis.

Evaluation by PCR

A highly conserved region of the proviral *env* gene was evaluated using the nested PCR technique. We used DNA samples from leukosis positive and negative bovines, which were provided by the Biodiversity and Molecular Genetics Group (BIOGEM) of the National University of Colombia in Medellín, Colombia. The controls provided were obtained from animals that had been previously confirmed by PCR and direct ELISA for the *env* gene of the provirus. The first PCR was performed on a final volume of 25 µL containing approximately 150 ng of DNA, with a final concentration of 0.4 µM of each BLV forward (5'-ATGCCCAAAGAACGACGGACGG-3') and BLV reverse (5'-CGACGGGACTAGGTCTGACCC-3') oligonucleotide, 200 µM of each dNTP, 2.5 µl of 10X Top Taq PCR buffer containing 15mM MgCl₂, and 1U of Top Taq DNA polymerase (Qiagen®, Germantown, Germany). In the second PCR reaction, 5 µl of the PCR product of the first amplification were used as template DNA, with the same conditions as the previous one, but with the Env5032 forward (5'-TCTGTGCCAAGTCTCCCAGATA-3') and Env5608 Reverse (5'-AACACAACCTCTGGGAAGGGT-3') oligonucleotides. The

primers had been reported previously⁽¹⁷⁾, but standardization was carried out on a Labnet TC9610 MultiGene OptiMax Thermal Cycler TC9610 Thermal Cycler (Labnet®, Northlake, IL, USA). The PCR profile included an initial denaturation step at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min, followed by a final extension at 72 °C for 8 min. For the second PCR, a denaturation step was performed at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 45 sec, and finally an extension at 72 °C for 10 min⁽¹⁸⁾.

The products of the second PCR were observed using agarose gel electrophoresis at 2.5% (Amresco, Cochran Road, OH). 5 µL of PCR product mixed in 2 µL of EZ vision stain were poured in (AMRESCO, Sidney, Aus). In each run line, 2 µL of 100 bp molecular weight marker (low range Fermentas, Glen Burnie, MD) were used, along with two 2 µL of EZ-vision. The gels were documented using an ENDURO™ GDS photodocumenter (Labnet, NJ, USA) for photographic evidence. A 598-bp band indicated the presence of provirus in one individual. In all cases, a negative and a positive bovine control were used in the PCR, and so were positive and negative buffalo controls found in this work.

Statistical analyses

Based on the information collected in the herds, descriptive statistics were performed in order to summarize the production and risk factors in the sampled herds. A descriptive analysis of the hematological, DNA quantification, and purity data for milk samples was also performed, and the protocols were compared using a t-student test for DNA concentration, and the 260/280 and 260/230 ratios of the milk samples. Finally, prevalence and their respective 95% confidence intervals were estimated for each of the species sampled. Using the data collected for the buffaloes, a generalized linear model (GLM) was developed to evaluate the effect of infection on the productive characteristics and on the hematological variables white blood cells, red blood cells, hemoglobin, hematocrit and platelets. Subsequently, a logistic regression model was performed to determine whether any risk factors were statistically significant for the presence of the virus in individuals.

Results

Description of herds and productive characteristics

In this study, most of the buffaloes sampled in this study came from the departments of Risaralda (46 %), Quindío (17 %), Caldas (15 %), and others (22 %). Most of the buffaloes were of the Mediterranean breed (29 %), followed by Murrah (19 %), Bufalypso (13 %), and indeterminate crosses (39 %). Taking into account young animals of non-productive age (under three years old) and adult animals of productive age (over three years old), 21.6 % of young animals and 78.4 % of adults were sampled according to their age group.

As for the productive parameters, it was found an average birth weight of 33.39 kg, a weaning weight of 202.93 kg, a weaning weight gain of 136.27 kg, a calving interval of 491.77 d, 381.59 L of milk adjusted to 305 d, and 67.29 d to peak of (Table 1).

Table 1: Productive characterization of water buffaloes in the central-western region of Colombia

Characteristic	Mean	Standard error	Standard deviation
Birth weight, kg	33.89	0.17	4.31
Weaning weight, kg	202.93	2.22	53.43
Kg since weaning, kg	136.27	3.75	96.33
Interval between births	491.77	5.20	63.87
Milk 305 days, L	381.59	28.79	345.49
Days at peak	76.26	6.30	62.38
Production at peak	2.15	0.12	3.07

Hematological description

Regarding the hematological evaluation of the samples, it was found that the white blood cells count (WBC x 103 μ L) was higher in positive animals, but did not differ statistically from uninfected ones; as for red blood cells (RBC x 103 μ L), these were significantly higher in the positive animals, while hemoglobin (HGB%) and hematocrit (HCT%) were similar in positive and negative ones. Platelets (PLT x 103 μ L) were lower in positive samples, but not significantly so (Table 2).

Table 2: Description of hematological variables in water buffaloes positive and negative for bovine enzootic leukosis

Characteristic	Statistic	Total	Negative (n=93)	Positive (n=47)
WBCx10 ³ μL	Mean±SE	32.94±1.85	30.12±3.16	33.45±2.80
	Standard deviation	39.84	20.72	13.14
	Min	126	78	9
	Max	125	77	55
RBCx10 ³ μL*	Mean±SE	46.93±3.11	66.67±6.06	85.64±4.13
	Standard deviation	26.84	39.75	19.37
	Min	80	125	45
	Max	79	124	117
HGB%	Mean±SE	27.74±1.59	20.09±2.47	21.82±3.96
	Standard deviation	22.26	16.2	18.56
	Min	68	66	68
	Max	67	65	65
HCT%	Mean±SE	43.94±2.32	46.07±4.39	46.77±3.57
	Standard deviation	32.71	28.81	16.76
	Min	97	87	17
	Max	96	86	74
PLTx10 ³ μL	Mean±SE	38.78±2.47	45.91±4.89	46.32±6.33
	Standard deviation	32.3	32.09	29.69
	Min	99	98	5
	Max	98	97	98

WBC= white blood cells; RBC= red blood cells; HGB= hemoglobin; HTC= hematocrit; PLT= platelets.

= *Statistically significant difference ($P<0.05$) between negative and positive.

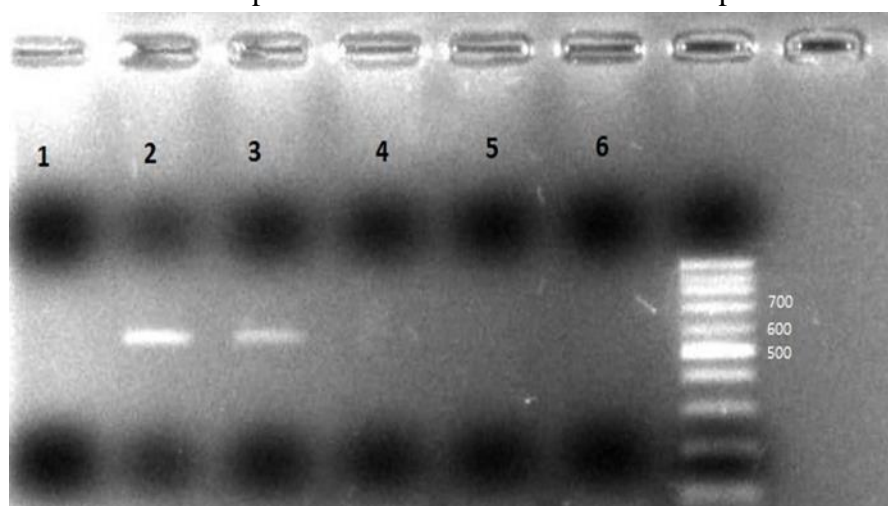
DNA extraction

It was possible to establish a method for DNA extraction from buffalo milk, enabling the obtainment of average amounts above 50 ng/μL, which is the recommended level. Given that the buffalo milk samples have a high concentration of fat; a method was proposed with the extraction of this fat. However, protocol 2 without fat removal showed the best results, with an average concentration of 165.7 ng/μL vs 20.5 ng/μL in protocol 1 ($P<0.05$). The 260/280 ratio of the protocol was 1.74. For DNA extraction from blood using a kit, concentrations above 10 ng/ml were obtained with a 260/280 ratio of 1.8 to 2.

BTv DNA detection

Successful detection of BTv DNA was possible using the leukosis PCR test (Figure 1), and 33.6 % of the 140 tested buffalo samples and 3.4 % of the 58 bovine samples were found to be positive. None of the buffalo milk samples yielded positive results (Table 3); neither the samples from sheep nor those from humans in contact with buffaloes were positive. In all cases, the tests were validated with the respective positive and negative controls mentioned above.

Figure 1: Agarose gel electrophoresis for a fragment of the *Env* gene of bovine enzootic leukosis provirus in buffalo whole blood samples



Lane 1= negative control, lane 2= positive control, lane 3= a positive sample, lanes 4-6 negative samples.

Table 3: Leukosis positivity in buffaloes and other production animals in the central-western region of Colombia

Species	Total No./ Positive	Prevalence (%)	Confidence interval 95%
Buffaloes - Blood	140/47	33.6	26 - 42.1
Buffaloes - Milk	12/0	0.0	0.0 - 60.4
Bovines	58/2	3.4	0.5 - 12.9
Humans	9/0	0.0	0.0 - 37.1
Sheep	35/0	0.0	0.0 - 12.3

Risk factors

The effects of factors such as municipality, herd, age, and breed on the presence of the virus were evaluated by logistic regression, but no significant effect was found in any of the cases ($P>0.05$), so there is no evidence to assume that these are risk factors for leukosis infection in buffaloes. Finally, no relationship was found between BLV positivity and milk production or other productive parameters. Nevertheless, it is important to clarify that the information was not collected homogeneously and completely, since there were not enough records in the buffalo-milk production units.

Discussion

Given the characteristics of water buffaloes for adapting to different climates including tropical conditions, the climate between 18 and 30 °C in which the study animals were found in the central western region, is suitable for dairy, meat or dual purpose production⁽¹⁹⁾. In this study, the Mediterranean breed was the most frequent, as in Argentina⁽²⁰⁾ and Brazil, although the most common dairy breed there is the Murrah^(21,22), and in Costa Rica, the Bufalypto⁽²³⁾. With respect to sex, a larger number of females was found, given the dairy focus of the predominant breed, consistently with a previous study conducted in the coffee-growing region of Colombia⁽²⁴⁾ and in Venezuela⁽²⁵⁾.

Birth weights were similar to those found in other regions of Colombia with weights between 35 to 40 kg and 30 to 37 kg^(12,26). The weaning weight of 202.93 kg was also similar to the weaning weight found in Costa Rica, of 160 to 220 kg⁽²³⁾, and within the range reported in Colombia (204.17 to 356.45 kg) in Murrah females. The calving interval of 491.77 d was higher than that found in Córdoba (Colombia), which was 414 d⁽²⁷⁾. Lactation at 305 d of 381.59 L from two teats was lower than that reported in the same area, which was 1,098 L, but from all four quarters⁽²⁴⁾. It is important to point out that, in the area, it is common practice to milk two teats and leave the other two for the calf, which is why yields tend to be lower than those reported in other studies. Similarly, the production at peak was 2.15 L, which is lower than the 5.0 L reported for the zone, but from the four quarts. It is important to note that most of the production data in this study were obtained from a single herd.

With respect to the extraction of DNA from buffalo milk, in protocol 2 the best method was found to be not to extract the fat layer, since it allowed obtaining higher DNA concentrations with an average of 165.78 ng/ μ L with a 260/280 ratio of 1.74 that allows it to be classified as DNA of acceptable purity. In addition, a 260/230 ratio of 0.41 was found, which suggests

that this method leaves some degree of contamination with phenols. The above results coincide with those found in a study that used six extraction methods in sheep milk⁽²⁸⁾, which included different extraction kits, phenol-chloroform, and lysis with guanidine hydrochlorate; the 260/280 ratio ranged between 1.55 and 1.80, and the 260/230 ratio ranged between 1.43 and 1.80 and had the highest contamination with the lysis solution. The amount of DNA obtained is within the range obtained from goat milk using the salting out method, which was 2.12 ng/ μ L to 610.12 ng/ μ L, although in that study the initial fat layer after centrifugation was discarded⁽²⁸⁾. Finally, the samples were taken and analyzed by PCR to evaluate the possible presence of BLV; upon analysis of these was obtained, with the respective validation of the positive and negative control mentioned previously. This is important, as certain authors suggest a relationship with breast cancer in humans⁽⁶⁾.

The diagnosis of leukosis prior to the use of serological and molecular tests was based on hematological findings, which were supported by the presence of persistent lymphocytosis, in the preclinical phase and in the leukocytic or tumor phase, in up to 30 % of the positive animals⁽²⁹⁾. In this work, although without statistically significant difference, an increase in white blood cells was found in positive animals compared to negative ones, but without discriminating cell lines, so that no specific alterations in lymphocytes can be evidenced. A similar result was found in Brazil in Holstein cows where the white line was $10.3 \times 10^3 \mu$ L in negative and 27.96μ L in positive⁽³⁰⁾. Regarding hemoglobin, it has been reported to be low in positive animals; however, in the present study, there were no differences in this aspect between positive and negative animals. Yet, there were differences in red blood cells, which were significantly higher in positive animals, possibly due to problems of poor regulation in the differentiation and maturation of blood cells after infection with the virus. With respect to the cattle sampled in this study, no statistically significant differences were found in any of the hematological parameters evaluated, although it should be noted that the number of animals was too small to have sufficient statistical power.

Although the buffalo is considered one of the species susceptible to infection by the bovine leukosis virus, there is little concrete evidence of this, since few reports prove the presence of the virus naturally, and those that do base their diagnosis on such tests as ELISA, AGID and Western blot, which detect antibodies, but not proviral DNA. Furthermore, according to some literature reports^(1,5), these tests have a significant percentage of false positives, which, in view of the low prevalence rates, might cast a doubt regarding the true presence of the virus. On the other hand, buffalo have been considered as susceptible in some studies in which the animal is inoculated with the virus in order to monitor its infection and its pathophysiological development.

Some reports of bovine lymphosarcoma in buffaloes have tried to associate the presence of this type of lesion with the presence of bovine leukosis virus. Even in one case report they describe the clinical, hematological, and cellular findings attributing the etiology to BLV, but

without laboratory confirmation⁽¹⁰⁾. Another case of lymphosarcoma was reported in Brazil in a female buffalo that tested negative when diagnosed with a nested PCR test⁽¹¹⁾.

In 2016, a study was conducted in buffaloes from the Amazon and southeastern Brazil, searching for the presence of the virus by agar immunodiffusion (AGID), ELISA and PCR. In this study, 24.6 % of animals were found positive by ELISA, but none positive by AGID which is the test considered as the Gold standard. No positive results were obtained by PCR, so it was concluded that there was no presence of the virus in the animals evaluated; however, in 2000, in the same Amazon region, the presence of antibodies to BTV was found in buffaloes. The tests performed were AGID and two types of ELISA for gp51 antigen and immunoglobulin G detection. The conclusion of this study was the natural presence of the virus in buffaloes⁽³¹⁾ with 87, 81 and 69 positive serum samples for each test, respectively.

All of the above suggests non-specific test results and evidences the few findings of natural infection. One of the few cases reported by PCR is in the Philippines, where a positivity of 27.6 % for BTV was found, which is the only confirmatory report of the virus in buffaloes found in the literature by this method⁽⁷⁾. As in the Philippine study, this work demonstrated the natural occurrence of BTV in water buffaloes in the coffee-producing axis region of Colombia, detected by PCR, being the first report in the Americas, and one of the first worldwide, to use this technique. In the Philippine study, the virus was identified in a total of 272 samples of the Murrah breed (70 positive), Philippine Carabao (5 positive), and undetermined breed (0 positive). In the present study, there were no significant differences between breeds.

In bovines, a positivity of 3.4 % was found; this value is lower than those found in the literature, and even lower than that found in buffaloes in this study, which was 33.6 %. The analysis of bovine samples was also performed with nested PCR. This may explain the difference in regard to studies carried out in Montería, with prevalences of 24 %, and in Mesa de Los Santos, Colombia, with 73 %, as well as in several regions of Chile with 34.7 % where the diagnosis was made with ELISA, with the possible false positives discussed above⁽³²⁻³⁴⁾. In spite of the results found, the transmission of BTV virus between species cannot be ruled out, although it is yet to be confirmed whether the viruses that produce the disease in susceptible species are genetically the same.

The risk factors associated with the presence of the disease may be similar in bovines and buffaloes. These factors are the reuse of needles in tasks such as vaccination, reuse of palpation sleeves in reproductive check-ups, dehorning with saws, and lack of fly control. In the present work, these data were not available; therefore, other factors such as herd, buffalo age, and breed were taken into account. None of these factors was correlated with the presence of BTV. In Montería (Colombia), positive associations have been found in cattle

with respect to the presence of BTV and age, breed and type of production, being higher in females, dual purpose cattle, and crossbreeds of Zebu and European⁽³²⁾.

On the other hand, following the identification of the presence of the virus in humans and its possible association with mammary cancer, it has been suggested that the route of infection is the consumption of raw milk and meat from infected cattle. In this study, 12 samples of buffalo milk purchased in local commerce were analyzed by PCR, of which none tested positive for the presence of the virus. Some studies carried out in Colombia and in Argentina^(35,36) have demonstrated the presence of the leukosis virus in fresh bovine milk with 49 and 59 % positivity respectively; however, there are no reports of similar findings in buffaloes.

Regarding the results of positivity in sheep, these have been considered to be a species susceptible to BTV infection. Although few reports exist, a seropositivity of 0.077 % (2 out of 2,592) was obtained in Brazil. The positive animals were a female of the Santa Inés breed and another female of unknown breed⁽³⁷⁾. In another case, a sheep with lymphoma was tested with AGID, with negative results⁽³⁸⁾. In another study conducted in Iran in 2015, 7.04 % of 95 sheep evaluated by nested PCR tested positive. In this study, samples from 35 African sheep (Camuro) were tested by nested PCR, and none of them proved positive.

In this study, nine blood samples were also collected from personnel who are or have been in contact with cattle and buffalo during their professional practice. None of the samples were positive. Although, as mentioned, it seems that the virus is transmitted to humans through contaminated food, it cannot be ruled out that, as in bovines, the virus is transmitted by needle puncture or in some other way, as in the case of another retrovirus, the HIV virus. In 2003, Buehring and collaborators detected the presence of antibodies to BTV by immunoblot in humans, obtaining positive results in 39 % of 257 volunteers. It should be noted that the volunteers were not agricultural workers⁽³⁹⁾.

Conclusions and implications

The presence of BTV was found in 33.6 % of the buffaloes in the central-western region of Colombia, a value much higher than that found in bovines in the same area (3.4 %). No presence of provirus was found in commercial buffalo milk samples, nor were there hematological or productive alterations in the positive animals. Since this is the first report in Colombia of the presence of BTV in buffaloes, no BTV control programs have been established for this species. When positive cases are found, the programs could be similar to those used in bovines, which are focused on the identification of positive animals, use of

needle per animal, change of palpation sleeves, disinfection of the tattoo machine, and chemical dehorning. These systems have avoided the selective slaughter of positive animals⁽⁴⁰⁾. In addition, disease control reduces economic losses due to lower production and veterinary care, and is fundamental in a potential future export of buffalo meat and dairy products.

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The authors declare that they have no conflict of interest.

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