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Bovine leukemia viral DNA found on human breast tissue is genetically related to the cattle virus

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ABSTRACT

Bovine leukemia virus (BLV) infection is widespread in cattle and associated with B cell lymphoma. In a previous study we demonstrated that bovine leukemia viral DNA was detected in human breast tissues and significantly associated with breast cancer. Our current study aimed to determine whether BLV DNA found in humans and cattle at the same geographical region were genetically related. DNA was extracted from the breast tissue of healthy ($n = 32$) or cancerous women patients ($n = 27$) and from the blood ($n = 30$) of cattle naturally infected with BLV, followed by PCR-amplification and partial nucleotide sequencing of the BLV *env* gene. We found that the nucleotide sequence identity between BLV *env* gene fragments obtained from human breast tissue and cattle blood ranged from 97.8 to 99.7% and grouped into genotype 1. Thus, our results further support the hypothesis that this virus might cause a zoonotic infection.

1. Introduction

Bovine leukemia virus (BLV) is the causative agent of Enzootic Bovine Leukosis (EBL), a silent lifelong infection of cattle. BLV belongs to the genus *Deltaretrovirus* (order *Ortervirales*, family *Retroviridae*, subfamily *Orthoretrovirinae*) and is genetically related to the primate T-cell leukemia virus type 1 (PTLV-1), PTLV-2 and PTLV-3 [1]. In cattle, BLV infects B-lymphocytes causing polyclonal proliferation of CD5+ cells [2]; however, progression to B cell lymphoma occurs in less than 5% of the infected animals and is characterized by modified B-lymphocyte infiltrates in different organs including lymph nodes, heart, kidney, liver, uterus, eye and udder [3].

As a retrovirus, the BLV genome encodes the *gag, pol* and *env* genes and two identical long terminal repeat chains (LTRs) at the 5′and 3′ ends of the genome [4]. The *gag* and *pol* genes encode for structural proteins and viral enzymes [4]. The *env* gene encodes a highly variable viral envelope glycoprotein (gp51) and a conserved transmembrane protein (gp30). The gp51 is involved in receptor recognition and virus attachment to the host cell [5] and, as such, is a major determinant of viral host range. Because of its heterogeneity, the *env* gene is also used for genotyping studies and recent phylogenetic analysis indicated that BLV could be classified into 10 genotypes [6]. This classification contributes to a better understanding of BLV epidemiology, genetic diversity, and should help to study virus evolution and its role as a possible zoonotic agent.

During natural infection in cattle, BLV is found more abundantly in mammary epithelium than in lymphocytes [7] suggesting that colostrum and milk might be a source of infection to newborn calves and possibly to milk consumer. However, early studies performed in the 1980's using the detection tests available at that time concluded that there was "no epidemiological or serological evidence that BLV could infect humans" [8]. Nonetheless, because anti-BLV antibodies have been detected in humans, that theory is now contested [9]. In addition, several studies indicated that BLV DNA might be found in the breast tissue of women from different regions of the world including Colombia [10], USA [11,12], Australia [13], Argentina [14], Brazil [15] and Iran [16]. Furthermore, BLV DNA was also detected in buffy coat cells of blood specimens from females patients in United States [17] and Iran [16].

Viruses promote changes in the cell cycle and/or cell morphology

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and some viruses are implicated in the development of certain types of cancer, including breast cancer [18]. In humans, breast cancer is a heterogeneous disease characterized by changes in the cellular epithelium and the non-special invasive carcinoma is the most common histological subtype [19]; it originates in the ductal epithelium and accounts for by 65 to 85% of all invasive breast cancer [19]. Worldwide, breast cancer affects approximately 2.1 million women a year and it is considered the most common type of female cancer with the highest number of cancer-related deaths [20]. In 2018, for instance, there were 627 thousand breast cancer related deaths which accounts for approximately 15% of all cancer deaths amongst women [20]. The findings linking BLV DNA to breast cancer in women represents an additional challenge that should be thoroughly investigated. Here, our aim was to determine whether BLV DNA found in women breast tissue and in cattle at the same geographical region and time span period were genetically related.

2. Materials and methods

2.1. Human samples

A total of 59 formalin fixed paraffin-embedded (FFPE) mammary tissue samples were used in this study. All the samples were collected from women in Rio Grande do Sul state, Southern Brazil. Briefly, breast tissues were surgically removed from healthy women submitted to breast reduction surgery ($N = 32$) or women diagnosed with invasive breast cancer submitted to mastectomy or breast segmentectomy ($N = 27$). These samples were subsequently fixed with 10% buffered formalin for 24–36 h and then embedded in paraffin blocks, sectioned (5 μm thick) and dried at −70 °C for 30 min and then mounted on glass microscopic slides for histopathologic and immunohistochemistry diagnosis of breast cancer.

The study was approved by the Committee on Research Ethics (protocol # 2.247.462) of the Universidade de Passo Fundo, and the procedures used conformed to the tenets of the National Commission for Ethic on Research (CONEP) of the Brazilian Ministry of Health.

2.2. Bovine samples

Blood samples were obtained from 30 animals with clinical signs consistent with EBL at different farms in Rio Grande do Sul, southern Brazil. Briefly, 5 mL of blood was collected by veterinarians in EDTA tubes (ethylene diamine tetra acetic acid) and kept refrigerated until reaching the Veterinary Virology Laboratory at the Universidade Federal do Rio Grande do Sul, centrifuged (500 ×*g*, 10 min) to obtain the plasma and buffy coat cells that were then transferred to new tubes and frozen at -20 °C until analysis. The presence of BLV in the animals was confirmed by PCR for the *env* gene and further confirmed by Sanger sequencing.

2.3. DNA isolation

The proviral DNA isolation of paraffin-embedded tissue was performed selecting areas with larger amounts of epithelial tissue cells. In brief, the DNA was isolated from deparaffinized tissue using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion, Austin, TX, USA). For cattle blood, DNA was extracted from the buffy coat layer cells using a standard Phenol–chloroform protocol [21].

Human and cattle DNA were quantified by spectrophotometry using NanoDrop (Thermo Scientific, Waltham, MA, USA) and the quality was evaluated by amplifying the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [11].

2.4. PCR and DNA sequencing

The presence of BLV *env*, *pol*, *tax* and *gag* genes in human mammary tissue and cattle blood was assessed by PCR using 50 ng of DNA from each sample. A 446 bp fragment of the *env* gene was amplified using OBLV1A F and OBLV6A R primer pair [22]; a 279 bp fragment of the *gag* gene was amplified using primers BL2 F and BLRT2 R (present study); for *tax* gene, a nested PCR was conducted using primers that generated a 113 bp fragment [11]; and for the *pol* gene, a nested PCR was carried out using primers that amplified a 157 bp fragment [11]. A partial GAPDH gene (857 bp) was amplified as described previously [11] and used as internal control of DNA quality. Primer sequences and further details are described in Table 1. Precautions were taken to avoid cross contamination between samples and positive control such as use of a DNA free area equipped with a laminar flow chamber with materials and gloves exclusive to the PCR mix and another flow chamber to work with DNA from the samples. Positive and negative samples were used as controls [10] and non-template (DNA-free) water samples were used throughout the assay to assure trustful results. Positive partial *env* [22] and *gag*-PCR products were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen, Carlsbad, CA, USA), according manufacturer's recommendations. Both DNA strands were sequenced with an ABI PRISM 3100 Genetic Analyzer utilizing a BigDye Terminator v.3.1 cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.5. Phylogenetic analysis

The *env* sequences were *de novo* assembled using Geneious Prime 2019 1.3 (https://www.geneious.com). A total of 57 *env* partial nucleotide sequences representative of the 10 previously proposed BLV genotypes [23] were retrieved from GenBank database and aligned using CLUSTAL W (available in MEGA 7). The phylogenetic tree was constructed using maximum-likelihood (ML) inference, K2 + I substitution model in 1000 bootstraps using MEGA 7.

3. Results

3.1. Investigation of the frequency of BLV DNA in human and cattle

Fifty nine formalin-fixed paraffin-embedded (FFPE) women breast tissue were analyzed by PCR to detect BLV *env* [22], *gag*, *pol* and *tax* genes [11]. From these, 86.4% (51/59) of the samples were positive for at least one of the PCR protocols: the most frequently detected sequence was from the *env* gene that was found in 66.1% (39/59) of the samples followed by the *tax* and *gag* genes that were detected in 37.2% (22/59) and 11.9% (7/59), respectively. None of the samples was positive for the *pol* gene. In 18.6% (11/59) of the samples, both the *env* and *tax* genes were amplifies while 10.2% (6/59) were positive for both *gag* and *tax* genes; no positive samples were found for both *env* and *gag* genes (Fig. 1). Only 13.6% (8/59) samples were negative for all BLV genes tested. Amongst bovine samples all were positive for the *env* gene and had a high identity (*E*-value 0.0) to BLV nucleotide sequences deposited in GenBank. The bovine samples were solely tested for partial *env* gene, as it is the region used for BLV genotyping.

3.2. Sequence analysis

The partial *env* amplification products obtained from 39 women and 30 cattle samples were sequenced and analyzed. The nucleotide identity of the sequences obtained from women ranged from 98.8 to 100% amongst them and grouped into genotype 1 (G1), while the sequences obtained from cattle clustered into G1 (98.8–100% nt identity) and G7 (98.4–99.1% nt identity). The nucleotide identity between BLV DNA sequences obtained from women and cattle from G1 ranged from 97.8 to 99.7%.

The ML tree showed a well-supported topology with 10 independent clusters that represent the 10 different BLV genotypes (Fig. 2). From the 39 partial *env* sequences obtained from women breast tissue, only four (Human 9, 14, 25 and 36) were different from each other and, for further

Table 1
BLV and internal control primers and cycling conditions for PCR in human and bovine samples.

BLV gene	→Primer sequences 5′ 3′	Nested PCR	Product lenght	Annealing temperature	Ref.
env	F: CTTTGTGTGCCAAGTCTCCCAGATACA		446 bp	60° C	[22]
	R: CCAACATATAGCACAGTCTGGGAAGGC				
gag	F: TGCTTGGGCACTCCGAT (position 991–1010)		279 bp	60° C	Present study
	R: TTGGGCTGAGCTGATTGTTG (position 1269–1250)				
	F: TAGCCTACGTACATCTAACC	outer	232 bp	52° C	[11]
pol	R: AATCCAATTGTCTAGAGAGG				
	F: GGTCCACCCTGGTACTCTTC	inner	157 bp	57° C	
	R: TATGGGCTTGGCATACGAGC				
	F: CTTCGGGATCCATTACCTGA	outer	373 bp	55° C	
tax	R: GCTCGAAGGGGGAAAGTGAA				
	F: ATGTCACCATCGATGCCTGG	inner	113 bp	55° C	
	R: CATCGGCGGTCCAGTTGATA				
Human GAPDH	F: CCTTCATTGACCTTCACTACATGGTCTA		857 bp	59° C	
	R: GCTGTAGCCAAATTCATTGTCGTACCA				

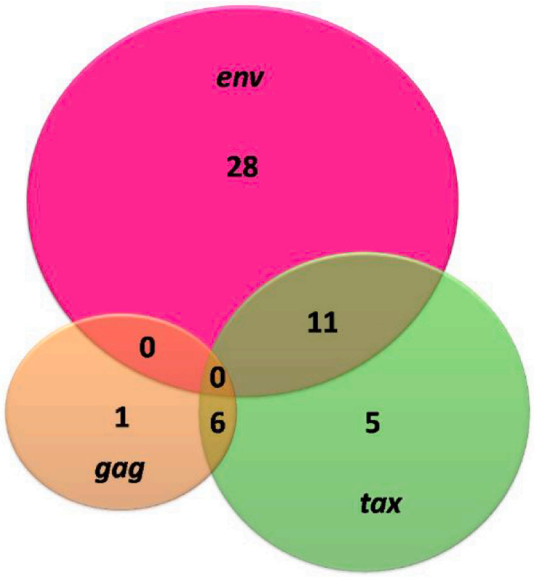

Fig. 1. Frequency of detection of different BLV provirus genes in women breast tissue.

analysis, they were then selected to represent all sequences obtained. Amongst the 30 sequences obtained from cattle, 27 clustered into G1 and from these, the three most diverse (Bov 25, 271 and 2575) were selected to be represented in our sequence analysis. The remaining three samples (Bov29, 32 and 49) clustered into G7.

Non-synonymous mutations were observed in the partial *env* sequences detected in women and cattle (Fig. 3). In addition, silent mutations in BLV DNA sequences obtained from women and cattle were also observed (Supplementary Material 1). Further analysis of amino acid substitutions indicated that they were distributed in different domains of the BLV gp51. Bovine leukemia viral DNA sequenced from human breast tissue had amino acid substitution at the CD4+ (V106L) and E epitope (D190A) when compared with the Brazilian AF399703.3 sequence as reference. Cattle BLV G1-grouped isolates had substitution in the *E*-epitope region (S189R) and G7-grouped isolates had substitution in the neutralizing domain 2 (N134D) and E-epitope (V192F) regions.

4. Discussion

Here, we demonstrate that the nucleotide sequence of BLV *env* gene found on DNA extracted from human breast tissue is genetically similar to that found on cattle blood samples collected at the same time period and geographical region. By nucleotide sequencing we found that all BLV DNA present in human breast tissue grouped into G1 which is the most prevalent genotype within cattle samples.

In addition to bovines, BLV infects capybaras sheep, alpacas and buffalo [3]. Although BLV is a lymphotropic virus, *in situ* techniques demonstrated that in cattle BLV is more abundantly found in mammary epithelium tissue than in lymphocytes [7] and, as such, is transmitted to suckling calves *via* colostrum and milk [24]. The historical intimate contact between humans and cattle and the consumption of cattle-derived products may have given the virus a chance for adapting to a new host. It could be possible that the virus originated in another species or passed through another host before reaching humans but the evidences available so far suggest that the route of transmission was cattle-to-humans.

Out of the 59 human breast tissue samples analyzed, 51 (86.4%) were positive for at least one of the BLV genes. The *env* gene was most consistently found followed by *gag* and *tax*; no sample was positive for *pol* (Fig. 1). Although previous studies reported the presence of BLV DNA in human breast tissue, in at least three different studies BLV DNA was not detected in breast tissue, blood cell lines and human cancer cell lines [25–27] . These conflicting results might be related to the methodology used to detect BLV DNA sequences. DNA amplification by PCR was used in most studies *versus* whole genome sequencing in a single study. Also, it might be possible that the viral DNA sequence targeted by the PCR was not present on the genomes analyzed. Indeed, partial genome deletions following integration into the host cells are common and considered an important mechanism to avoid the host immune response [28]. In a study conducted with the BLV-related PTLV-1, deletions of the host genome-integrated provirus initiated in the *gag* region, followed by deletions of the *pol* and *env* genes; in contrast, the *tax* and *LTR* regions were the less frequently deleted genes [29]. The lack of *tax* expression due to a mechanism of silencing is a strategy to avoid immune detection, however, it results in tumor development [28,30]; this, *per se*, could be a mechanism of BLV-induced tumorigenesis in humans. Depending on the time of integration into the host genome, structural genes are the first to be deleted since their products are targets of the immune system [31]. The high frequency of *env* gene detection in human samples suggests that the species spillover might have been recent and the different rates of detection is and indication that the provirus could be defective in specific genes. In addition, polymorphisms in the primer binding sequence or efficiency of the PCR protocols for detecting different genes could contribute to unequal detection rates. Also, the populations studied were from different regions and with different rates of consumption of cattle-derived products [32] and these could impact on the result reported. And, finally, DNA damaged by the process of preparing samples for FFPE could also contribute to reduce the rate of detection; in our study, to rule out this, we run an internal GAPDH PCR amplification control and all samples resulted positive.

Our comparison of BLV nucleotide sequences showed 13 silent nucleotide substitutions and eight non-synonymous substitutions

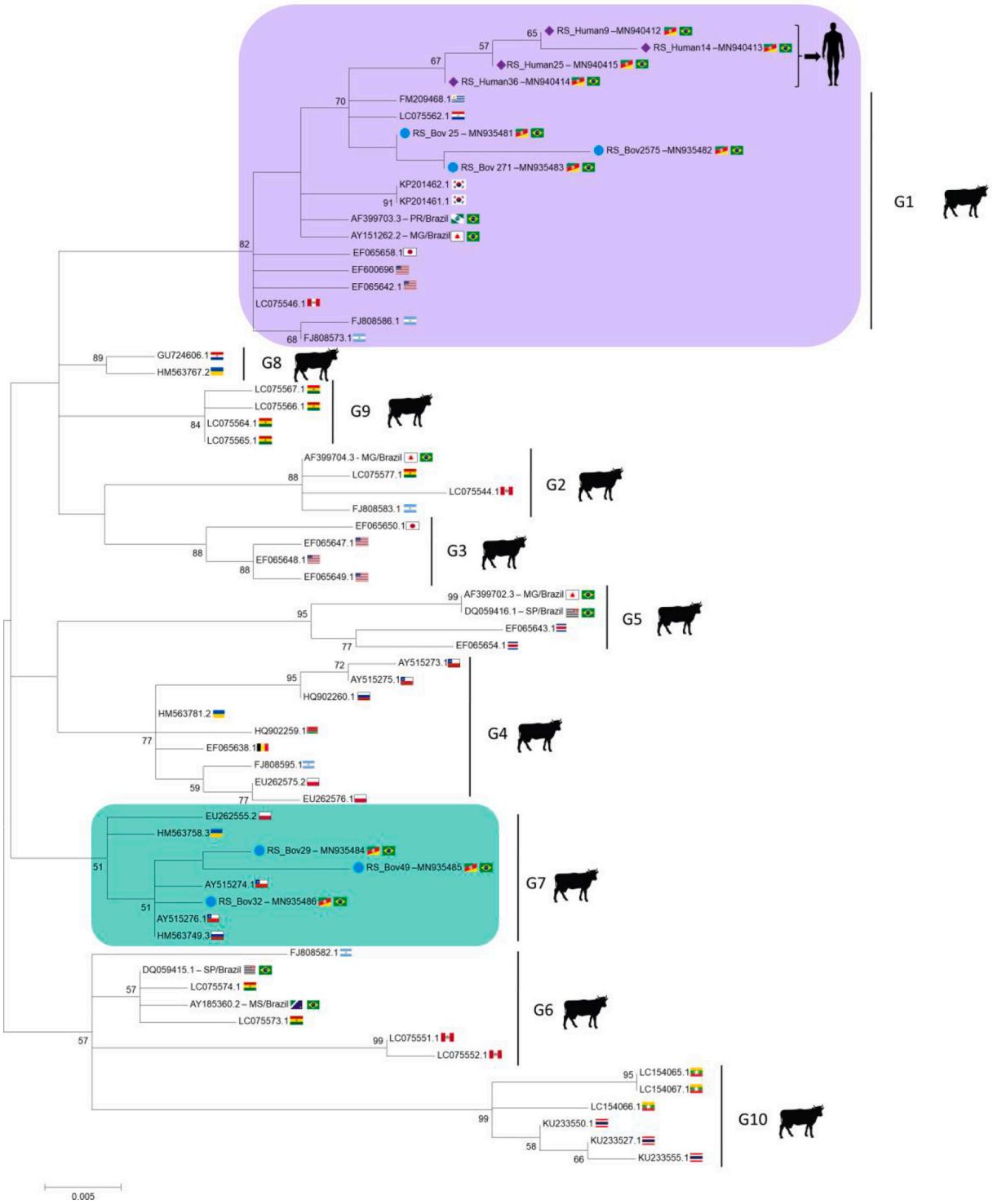

Fig. 2. Phylogenetic analysis of the partial *env* gene nucleotide sequences (446 bp) from BLV DNA obtained in Brazil ( ) and other countries (respective flags). Phylogenetic tree was generated using the Maximum Likelihood method. The ten known major genotypes are labeled G1 to G10. Our human and cattle sequences are indicated with a purple rhombus and blue circles, respectively. The flags represent the countries where the samples were analyzed and reported: Uruguai ( ), Paraguai ( ), South Korea ( ), Japan ( ), United States of America ( ), Peru ( ), Argentina ( ), Croatia ( ), Ukraine ( ), Bolivia ( ), Costa Rica ( ), Chile ( ), Russia ( ), Belarus ( ), Belgium ( ), Poland ( ) and Myanmar ( ).The other flags correspond to Brazilian states: Rio Grande do Sul ( ), Paraná ( ), Minas Gerais ( ), São Paulo ( ) and Mato Grosso do Sul ( ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

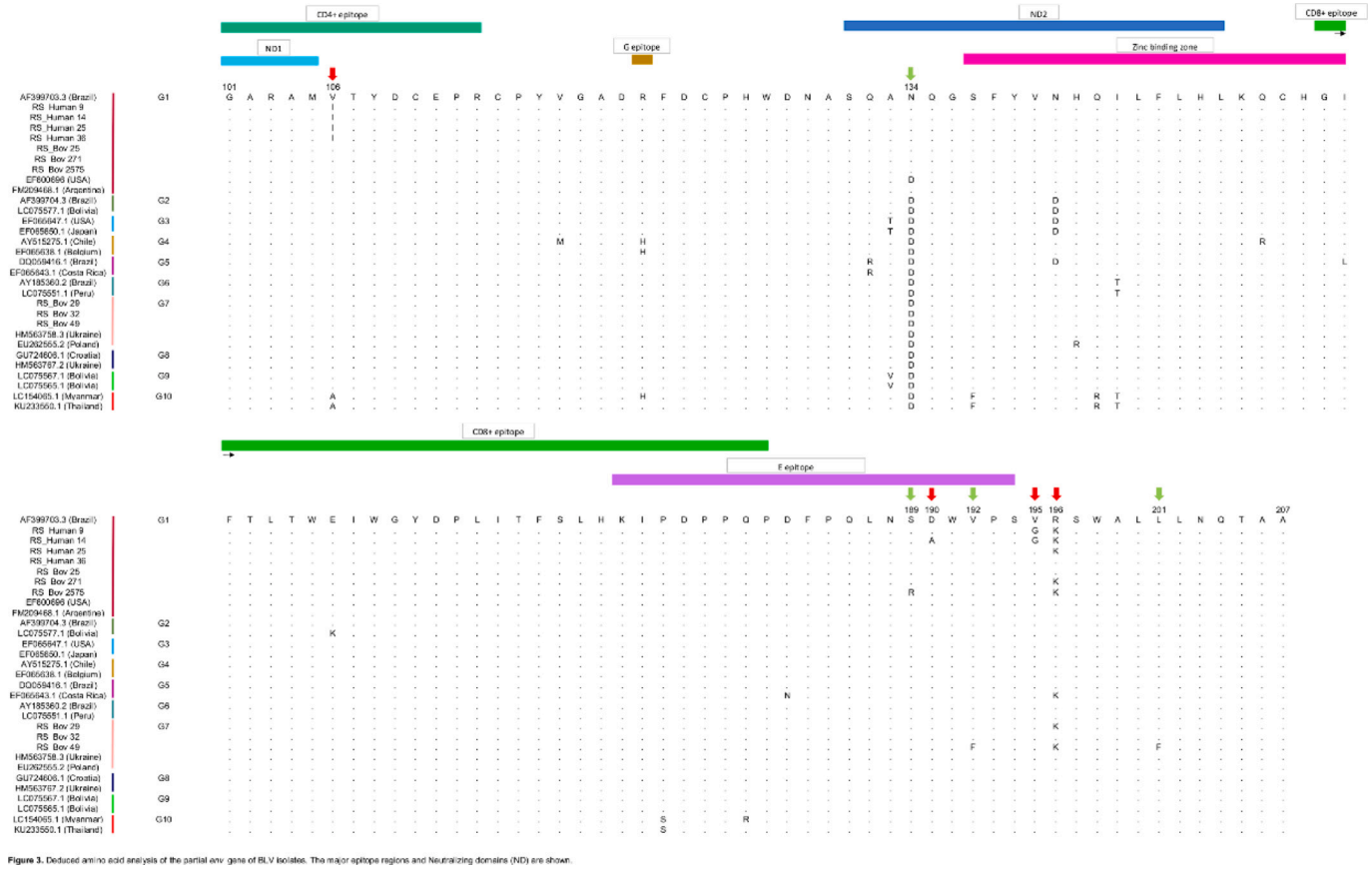

Fig. 3. Deduced amino acid analysis of the partial *env* gene of BLV isolates. The major epitope regions and neutralizing domains (ND) are shown.

(Supplementary Material 1). All human sequences and two bovine sequences from G 10 presented amino acid substitutions in a CD4+ epitope and only sequences represented by Human14 presented substitution in *E*-epitope. The CD4+ epitope is a T-lymphocyte recognition site and mutations on this site suggest that the virus is under pressure from the host immune system [33]. All bovine sequences that grouped into G7 showed amino acid substitutions in the ND2 domain, which is involved in virus neutralization and syncytium inhibition [33]; but, in contrast, only one sequence that grouped into G1 (Bov2575) and one that grouped in G7 (Bov49) had amino acid substitutions in the *E*-epitope which is also involved in virus neutralization [33]. In addition, in our sequences, conserved regions were observed in the ND1, G-epitope, zinc-binding zone and CD8+ epitope. The mutations that occur in viral proteins might allow viruses to infect a wider range of hosts by facilitating interaction with host cell receptors. The cellular receptor for BLV is not yet clearly defined but adaptor protein complex (AP-3) is believed to play an important role in BLV entry into cattle cells [34]. Human cells have five types of AP and these may be receptors analogous to those used by the virus in cattle [35,36]. The AP complexes are involved in the transport of intracellular vesicles and are located in the cytoplasmic membrane and in the Golgi complex [36]. In addition, the mutations observed in the *env* gene might be responsible for changing the viral tropism and may cause the virus to acquire the ability to infect a new host [37].

Emerging diseases are defined by those new infections resulting from the evolution or change of an existing pathogen, resulting in a broader host range, pathogenicity changes and the occurrence of unrecognized diseases [38]. Once a virus has established itself in the human population, the dynamics of virus transmission between people can lead to a public health problem. Like most retrovirus infections, transmission only occurs when an infected cell is transmitted to an uninfected host [39]. Thus, the fact that BLV DNA has already been detected in the blood of humans reinforces this hypothesis [16,17]. Additionally, several lines of evidence accumulated over the past few years indicate that BLV DNA is associated with breast cancer in women from Colombia [10], USA [12,40], Australia [13], Iran [16], Argentina [14] and Brazil [15]. Breast cancer is a multifactorial disease and is the most common cancer amongst women worldwide. In Brazil over 66,000 new cases of breast cancer are estimated for the year 2020 which represents an incidence rate of 43.74 cases per 100,000 women [20]. It is estimated that 16% of the cancers can be attributed to viruses, with higher percentages for developing countries [41].

The way BLV could be transmitted to humans still needs to be elucidated. The finding of BLV gene segments in raw beef and fresh cattle milk destined for human consumption suggests that it could be a foodborne infection [42]. Furthermore, a recent study revealed the presence of BLV RNA in the air and on surfaces at dairy workplaces, which may be a source of occupational infection [43]. It is believed that the virus may have long been integrated into the genome of humans when animal products were not controlled and pasteurization was not obligated in many parts of the world [10]. In this way, after infection, person-to-person transmission could also occur, just as with PTLV [29].

Although we clearly demonstrated the presence of BLV DNA in humans and its genetic relatedness to cattle BVL, we acknowledge that our study might have some limitations. First, *in-situ* hybridization (ISH) and subsequent histologic images would allow us to precisely identify which cell type is harboring BLV DNA and enhance these data considerably; second, we used cattle blood samples rather than mammary tissue; third, sample size was limited because we wanted to test four genes and so we had to limit the number of samples because of budget limitation; and fourth, PCR detection may have had some bias such as sensitivity of the primers and the protocol used. Still, age, genetic factors, eating habits and other factors are potential biases of the study but in no way these would lessen the importance of our findings.

In conclusion, our study indicates that BLV DNA sequences obtained at the same time period and geographical region from cattle and women breast tissue are genetically related. Our data strengthen previous hypothesis that BLV might be a zoonotic agent. Further studies designed to elucidate the transmission between human and cattle, and the biology of BLV infection in human are still required to fully estimate the BLV zoonotic potential.

Author statement

RC conceived the idea of the manuscript, conceptualization, formal analysis, writing and review of the manuscript. MW and RB contributed with methodology, writing and investigation. MS and DS contributed with methodology. CC and LK contributed with conceptualization, funding acquisition, methodology, writing and review of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2021.100252.

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