Bovine Leukemia Virus Presence in Breast Tissue of Argentinian Females and Its Association With Cell Proliferation and Prognostic Markers

Pamela Anahí Lendez[1], Lucía Martínez Cuesta[1], María Victoria Nieto Farias[1], Hua Min Shen[2], Guillermina Laura Dolcini[1], Gertrude Case Buehring[2], Maria Carolina Ceriani[1,*]

[1]*Virology Laboratory, Faculty of Veterinary Sciences, UNCPBA-CONICET (CIVETAN)–CICPBA, Tandil, Argentina*
[2] *School of Public Health, University of California, Berkeley, California, USA*

Corresponding author: Maria Carolina Ceriani, Virology Laboratory, Faculty of Veterinary Sciences, UNCPBA-CONICET (CIVETAN)–CICPBA. University Campus, Paraje Arroyo Seco s/n, 7000 Tandil, Argentina. Tel.: +542494385850; Fax: +542494385850; E-mail: cceriani@vet.unicen.edu.ar

Abstract

Introduction: Bovine leukemia virus (BLV) causes enzootic bovine leukemia, and is closely related to human T-lymphotropic virus type 1. It expresses microRNAs of unknown function and codes Tax, the protein that mediates malignant transformation. BLV is capable of infecting B- and T-lymphocytes, endothelial cells, and mammary epithelial cells of cattle. Several studies demonstrated the presence of BLV DNA in human tissue, and it is significantly associated with breast cancer in case-control studies using in situ Polymerase Chain Reaction (PCR), a highly sensitive and specific technique. The current study was the first report of BLV DNA detection by in situ PCR in tissue from Argentinian females with a diagnosis of breast cancer.

Methods: In situ PCR was done to detect BLV DNA in 85 human FFPE breast cancer tissue samples. The association of BLV DNA and expression of Ki67 and Her-2 was assessed.

Results: BLV DNA presence could be determined in 22.6% of the analyzed samples, and its presence was associated with an increase of the expression and prognostic markers Ki67 (P=0.009) and HER-2 (P=0.044) determined by conventional immunohistochemistry. No statistical significance was observed between the presence or absence of hormonal receptors and the presence of BLV DNA.

Conclusions: The obtained results support the idea that BLV might play a role in malignant tissue transformation.

INTRODUCTION

Breast cancer is the most common cancer in females worldwide, and the second most common cancer overall. It is also a leading cause of cancer death in the developing countries [1], partly since the shift toward lifestyles typical of industrialized countries leads to a rising burden of cancers associated with dietary and hormonal risk factors, and partly clinical advances to combat breast cancer are not always available to females living in such regions. The IARC (International Agency for Research on

Cancer, WHO) estimations for 2012 rank Argentina among the countries with medium-high incidence of cancer with an estimated 17,000 breast cancer cases newly diagnosed each year. Breast cancers comprise 18% of all the cancer cases and 36% of cancers in the females of Argentina [2]. In 2016, all medical associations in Argentina involved in the diagnosis and treatment of breast cancer, including the National Academy of Medicine, followed the model of ASCO/CAP in the US to develop guidelines and a mandate for pathologists to test breast cancer specimens for estrogen receptor (ER), progesterone receptor (PR), growth factor receptor HER-2, and cell division indicator Ki67, for all cases of in situ breast carcinoma, invasive primary breast cancer, and metastatic breast cancer received in their laboratories. These determinations can be done by either immunohistochemistry or fluorescent in situ hybridization (FISH), if laboratories are appropriately certified. Testing for this panel of biomarkers is routine in most of the developed countries as a first step towards individualized patient therapy.

Identifying what contributes to the initiation and early development of breast cancer is important to design adequate strategies to prevent and treat this disease. Some risk factors are identified, e.g. family history, long term exposure to endogenous estrogens, longer time between menarche and menopause, low parity, exogenous hormones, obesity, and sedentary lifestyle [2]. However, these risk factors are not likely to initiate the events that lead to malignant transformation of normal breast epithelial cells. It is estimated that ionizing radiation initiates <5% of all breast cancers. Viruses are estimated to cause 15%-20% of all human cancers. Viruses most frequently investigated in connection with human breast cancer are human papilloma virus (HPV), Epstein Barr virus (EBV), and murine mammary virus (MMTV) [3-5]; recently, bovine leukemia virus (BLV) is added to the virus candidates [6, 7]. The way BLV could enter the cells and infect them remains unknown. Recently a paper was published, where it was shown, in silico and in vitro, the interaction of bovine AP3D1, the putative cell surface receptor, and the viral 51-kDa glycoprotein gp51. This model suggested the receptor-ligand interactions, which could occur in BLV infection, and lead to viral binding and fusion regarding viral entry. The PSI-BLAST analysis of boAP3D1 predicted 88% identity with human

protein for AP3D [8]. BLV is a deltaretrovirus that infects cattle, most of which remain asymptomatic, constituting a continuous source of infection for the rest of the herd. Leukemia/lymphoma, a fatal disease, develops in only about 5% of infected animals, and it always ends with the death of the animal. The prevalence of infected cows in Argentina is more than 40% in dairy cows and 10% to 30% in beef cattle as informed by the National Agriculture Health Service (SENASA) in 2016, and the percentage of infected herds, harboring one or more infected animals, was 84% in 2001, the latest documented record available in Argentina [9]. BLV follows the typical retroviral genomic organization: LTR (long-terminal repeat, promoter region); gag (group-specific antigen, capsid region); pol (polymerase, reverse transcription region); and env (envelope). However, deltaretroviruses have an additional region, tax (trans-activating region of the X gene), which has regulatory functions and is oncogenic to host cells. This protein causes malignant transformation by a pathway different from many other retroviruses. It inhibits DNA repair and trans-activating disruption of cellular growth control mechanisms, instead of inducing tumorigenesis by insertional mutagenesis or integration [10].

In Argentina, the average meat consumption in 2016 was 60 kg/inhabitant/year, as informed by the IPCVA (Instituto de Promoción de la carne vacuna Argentina) a public non-state entity devoted to improving and consolidating Argentine meat products. The milk consumption is around 210 liter/inhabitant/year. This includes milk and all its derivatives, as informed by the FAO (Food and Agriculture Organization of the United Nations), which locates Argentina among the countries with the highest consumption of milk in the world, together with the US, Australia, and Europe. This consumption is distributed in 78% as white milk, 11% derivatives as yoghurts and flavored beverages, and a very little consumption of nursing milks. In the current study, samples were selected from a reduced population, probably not exactly representative of the average inhabitant of the country. According to the location of the city, and that all the biopsies were taken in private clinics, it can be assumed that patients belonged to middle-high income group. It implies that consumption of meat, milk, and its derivatives could be higher than the average of the Argentinian population. These are very important data if the prevalence of BLV

in the herds is considered [9]. There are very few studies on the prevalence of BLV in beef cattle, but it is worth saying, as mentioned before, that milk consumption in Argentina is considered elevated. The prevalence of BLV in dairy cows in Argentina is around 80%. The mechanism by which BLV could enter the human body is still unknown, but there are some hypothesis [6, 7].

Although the zoonotic potential of BLV is not yet proven, there are several lines of evidence that suggest its plausibility. In bovines, there is reported evidence of BLV transmission to calves through milk and blood [11]. In humans, presence of serum antibodies against BLV [12], and retrotranscribed DNA in breast tissue sections [6, 13] and lung tumors [14] are reported. These findings, plus the close relationship of BLV with HTLV-1 and -2, led researchers to hypothesize that BLV could behave as a zoonotic agent, and its association with human breast cancer is postulated [7]. Generally, worldwide trends show that in the developing countries going through rapid societal and economic changes and the shift towards lifestyles typical of industrialized countries lead to a rising burden of cancers associated with reproductive, dietary, and hormonal risk factors. Since there is evidence regarding the presence of BLV proteins and genomic fragments in human breast tissue, a lot of studies should be performed to reassure the issue that BLV can effectively be associated with human breast cancer.

The current study aimed at investigating, for the first time, the presence of BLV DNA in breast cancer tissue of females in Argentina; and, in case of positivity, determining an association of BLV DNA presence in breast malignant tissue with the most common prognostic biomarkers used to predict tumor hormone responsiveness and proliferation aggressiveness; this would help to elucidate if BLV might initiate or help to develop cancer in the human breast.

METHODS

Sample Collection

Archived formalin-fixed paraffin-embedded (FFPE) human breast cancer samples (n=85) were randomly selected disregard of age or diagnosis from the biorepository of a private laboratory, from a city with 140,000 inhabitants, located in the Central area of Argentina. The patient´s age range fluctuated from 29 to 84 years, with a high percentage of the patients over 50 years old (87%), five patients were

29 to 45 (5%), and six 45 to 50 years old (6%). All samples were primary tumors of the breast collected from 2012 to 2015. Tissue sections were cut 5-μ thick, mounted on super adherent glass slides (Fisher Brand, Super frost Plus, Fisher Scientific, USA), and sent to the collaborating research laboratory at the University of California, Berkeley. Extreme care was taken to insure the area of invasive cancer was included in specimens analyzed in the current study. Negative tissue was obtained from patients subjected to reduction mammaplasty (4/4 less than 35 years old).

In Situ Polymerase Chain Reaction (PCR) Amplification

In situ PCR methodology, with slight modifications, was employed to amplify a 114-bp DNA segment from the tax gene region, as indicated by Buehring et al. [7]. Reactions were performed on two adjacent sections cut from each tissue and mounted on the same slide. Samples were permeabilized at room temperature (RT) by digestion with 2 mg/mL pepsin in 0.1 N HCl (40-80 minutes); pepsin inactivation solution (100 mM Tris-HCl, 100 mM NaCl, pH 7.4) was applied for one minute, followed by a rinse with Dulbecco's phosphate buffered saline (DPBS) and 5 minute to 48 hours in absolute ethanol. Each of the two sections from a single patient was surrounded by a separate 15 x 15 mm frame seal chamber (Bio-Rad, Richmond, California, USA); 65 μL of PCR mix was then placed into each chamber, and the accompanying plastic cover was sealed over each frame. The PCR mixture was 4 mM $MgCl_2$, 0.4 mM dNTPs, 0.06% bovine serum albumin (BSA), 0.053 U/μL AmpliTaq Gold DNA Polymerase (Applied Biosystems, California, USA) and 1 μM of each primer. The location of the used tax primers is shown below in base pair numbering according to GenBank accession #EF600696.

Forward (bp 7310–7329):
ATGTCACCATCGATGCCTGG
Reverse (bp 7423–7404):
CATCGGCGGTCCAGTTGATA

Slides were placed into an in situ PCR machine (Hybaid Omnislide Thermocycler, Thermo Scientific, USA) for amplification. The employed cycling parameters were: one cycle of 93°C for 10 minutes, 57°C for one minute, 33 seconds, then 30 cycles of 92°C for 30 seconds, 57°C for one minute, 30 seconds, and 69°C for two minutes, followed by a final extension at 69°C for 10 minutes.

Probe Labelling

Probe labeling for in situ hybridization (ISH) was performed with the PCR Dig Probe Synthesis Kit (Roche) according to the manufacturer's instructions, including recommended adjustments for short probes (<1 kb) with high GC content. Template DNA was from fetal lamb kidney (FLK) cells stably infected with BLV. The reaction mixture was: 5 µL of 10X provided buffer containing $MgCl_2$ and an additional 1 µL of 25 mM $MgCl_2$ (to optimize for the specific primers used in these experiments), 2.5 µL digoxigenin probe synthesis mix, 2.5 µL stock dNTPs, 0.5 µM of each primer, 0.75 µL kit enzyme mix, 200 ng template DNA, and PCR grade water added up to total volume of 50 µL. The cycling parameters were: one cycle of 95°C for two minutes, then 33 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds, followed by a final extension at 72°C for seven minutes. PCR was performed in a conventional thermocycler. The amplified fragments were visualized in 2% agarose gel with ethidium bromide before use.

In Situ Hybridization

Hybridization mixture was prepared at 1:3 ratio of labeled probe: hybridization buffer (Enzo Life Sciences cat# ENZ-33808). Slides were rinsed once in DPBS, once in 2X SSC (saline sodium citrate) at RT for five minutes and once again with 2X SSC at 37°C for five minutes. Sections were fixed 10 minutes with 10% buffered formalin, washed with PBS, and dehydrated with absolute ethanol for five minutes; 20 µL of the hybridization mix was added to one of the two sections on each slide, and the other tissue section on the same slide received hybridization buffer without the probe as a control for false positive reactions due to endogenous peroxidase, melanin inherent in the tissue, or DNA repair by the Taq polymerase. Each section was covered with a separate cover slip (22 mm diameter, Corning® glass cover slip) and placed into the in situ PCR machine. Denaturation of the DNA was performed at 95°C for 10 minutes, and hybridization at 37°C in a moist box overnight.

Detection of the Labeled Probe

Tissue sections were gently rinsed with DPBS. Endogenous tissue peroxidase was quenched for 30 minutes with 3% H2O2, washed with DPBS and soaked with 1% bovine serum albumin (BSA) in 2X SSC for 15 minutes at 37°C. The reaction was developed by adding 1:100 anti-dig-POD antibody (polyclonal anti-digoxigenin antibodies, Roche Diagnostics) to each section, and visualizing the final outcome signal (brown color) with diaminobenzidine (DAB, Sigma Cat # 32750).

Immunohistochemical Determination

Immunohistochemistry was performed according to the manufacturer's recommendation (Vector Lab, Burlingame, Ca. USA) with slight modifications adapted to the working conditions of the current study. Briefly, FFPE sections cut 5-µm thick, mounted on glass super adherent slides, and were dried overnight at 37°C. Prior to antibody staining, the tissue sections were pre-treated to unmask binding epitopes. Briefly, after quenching endogenous peroxide activity (3% hydrogen peroxide in methanol, 30 minutes), slides were immersed in 200 mL of 10 mM citrate buffer (pH 6.0) in a domestic microwave for five minutes on power setting of 1000 W, followed by two cycles, five minutes each, on power of 500 W. Slides were then held for 60 minutes in citrate buffer at RT before washing thoroughly in tap water and rinsed three times in PBS. All reactions were performed at RT. Slides were incubated with a ready-to-use (RTU) mouse anti-ER monoclonal primary antibody (Clone 6F11), RTU mouse anti-PgR monoclonal primary antibody (Clone 312), RTU mouse anti-HER-2 monoclonal primary antibody (clone CB11), and RTU mouse anti–Ki67 monoclonal primary antibody (Clone MM1) for 18 hours. All the monoclonal antibodies were purchased from Leica Biosystems, New Castle, UK. Tissue sections were then washed three times with PBS, and RTU biotinylated goat antibody secondary antibody to mouse/rabbit immunoglobulin (Universal Quick Kit, Vector PK 7200) was applied for one hour. After an additional three washes with PBS, RTU antibody streptavidin–biotin/horse radish peroxidase (HRP) complex (Universal Quick Kit, Vector PK 7800) was added. After rinsing three times with PBS, the final color was visualized by reacting with DAB (SK-4100, Vector Lab, Burlingame, Ca. USA) for 10 minutes in the dark. The slides were washed well in tap water and counterstained with Harris's hematoxylin for 20 seconds to one minute; then dehydrated, cleared, mounted, and observed with a light microscope. Ki67 index was classified as low (+) when less than 30% of the malignant cells were stained and as moderate/high (++ or +++) if more than 30% of the cells were stained.

Statistical Analysis

The association between the presence of BLV, and the hormonal receptors, HER-2, and the proliferative index marker Ki67 in the tissue was estimated with the Chi-square test (P<0.05). A free version of InfoStat (Universidad Nacional de Cordoba, Argentina) was employed.

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┌─────────────────┐   In situ   ┌─────────────────┐
│ Malignant breast│────────────▶│ Positive (n=19) │
│cancer tissue (n=85)│   PCR     │ Negative (n=66) │
└─────────────────┘             └─────────────────┘
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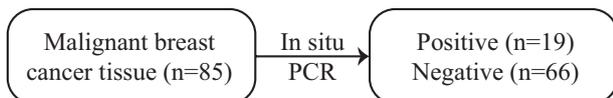
RESULTS

Nineteen of 85 (22.36%) malignant breast cancer samples were positive for the targeted conserved region of BLV tax gene (Figure 1). Four nonmalignant tissue samples were analyzed. Biopsies were obtained from reduction mammaplasties. Only in one case BLV DNA was detected.
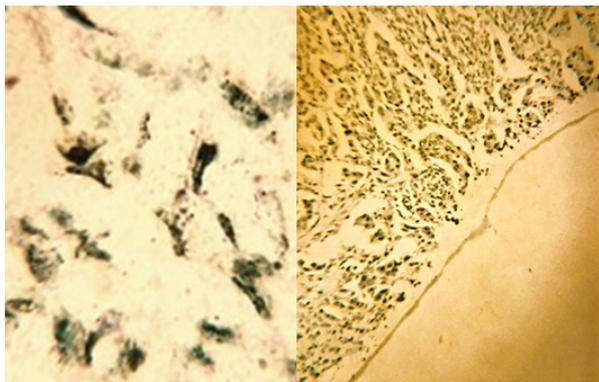
Figure 1: In Situ PCR Hybridization
Left panel: slide (1000X), stained with methylene blue. Dark spots are visualized in cell cytoplasm when reaction was positive for BLV Tax determination. Right panel: 10X magnification.

The presence of hormonal receptors for estrogen (ER) and progesterone (PR) tested by immunohistochemistry are indicated in Table 1. No statistical significance was observed between the presence or absence of hormonal receptors and presence of BLV DNA. The same methodology was applied to determine the expression of the proliferative marker Ki67, which is associated with a poor prognosis, but has a better response to chemotherapy. Samples were classified as moderate to high proliferation index (++, from 15% to 60% positive cells or +++, more than 60% positively stained cells), or low proliferation index (+, less than 15% of positive cells), (Figure 2). A statistically significant association was observed between the proliferative rate and the presence of BLV tax DNA (P=0.009). A high/moderate proliferative index was observed in 13/18 (72.22%)

positive samples and in only 25/63 (39.68%) of negative samples (Table 1). For the oncogen HER-2, there was significant association between the positivity for the marker and the presence of BLV (P=0.044) (Figure 2).

Table 1: Immunohistochemistry Analysis for Hormone Receptors, Ki67 and HER-2 in BLV-Positive Versus BLV-Negative Tissue Samples

	BLV Positive	BLV Negative	P Value
Estrogen Receptors (n=83)[a]			0.178
Positive	12	51	
Negative	7	13	
Progesterone Receptors (n=84)[a]			0.249
Positive	10	42	
Negative	9	23	
Ki 67 (n=81)[a]			0.009
Positive	13	25	
Negative	5	38	
HER- 2 (n=82)[a]			0.044
Positive	5	5	
Negative	15	57	

[a] The difference between the total number of tested samples (n=85) and the data in the table is due to the fact that samples were collected from a repository; information was incomplete.

No significant association was observed between BLV status and either of the hormonal receptors determination. Presence of BLV was significantly associated with both high proliferation index (Ki67) (P=0.014) and the detection of oncogene HER-2 (P=0.042).
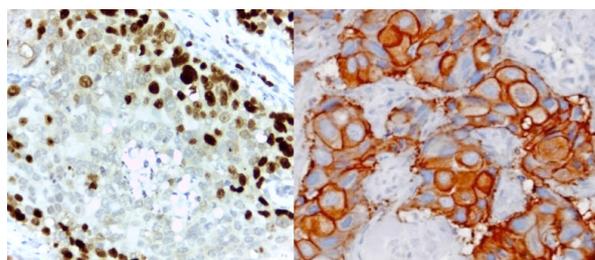

Figure 2: Immunohistochemistry for Prognostic Markers
Left panel: Ki67 determination stained with DAB, positive cells are brown (40X). Right panel: HER-2 determination stained with DAB. Brownish reaction is observed in the cytoplasmic side of plasmatic membrane (400X).

DISCUSSION

BLV is a deltaretrovirus which causes neoplastic

disease of lymphoid tissue in cattle. The main target cells are B-lymphocytes, but it also naturally infects T-cells [15], endothelial cells [11], and mammary epithelial cells [16]. Most infected animals remain asymptomatic, but approximately 30% develop a benign expansion of B-lymphocytes (persistent lymphocytosis) that persists for the entire life of the animal [17]. Less than 5% of infected animals develop malignant B-cell lymphoma, even after a long latency period. These animals are usually culled from the herds and their products are not marketed. Transmission through the herd is due to transfer of infected lymphocytes, rather than extracellular viral particles [17]. In fact, BLV and its close relative human T-cell leukemia virus can be transmitted only by cell-cell contact through cytoplasmic bridges [18]. Infected cells can enter through the injured skin, alimentary, reproductive, or respiratory tract, frequently as a result of iatrogenic procedures [19]. A dam can infect the nursing calf through colostrum or milk in which the whole infected cells are plentiful [11].

Prevalence of BLV infection is much higher in dairy cattle than in beef cattle [20, 21], possibly due to differences in herd management, breed differences in genetic susceptibility to BLV [22], and/or the fact that BLV is hormone responsive and is stimulated to replicate by corticosteroids and progesterone [23, 24] elevated during the constant pregnancy and lactation of dairy cows. High levels of BLV replication in dairy cows would stimulate higher antibody levels, more likely detected by the screening test for BLV.

BLV has the promoter region (3' and 5' long terminal repeat [LTR]) and the coding genes: gag, coding for capsid protein, env, coding for the envelope protein, and pol for the reverse transcriptase (RNA dependent DNA polymerase) enzyme [10]. Unlike other families of oncogenic retroviruses, (alpha, beta, and gamma Retroviridae), it is not mandatory for deltaretroviridae to integrate into host cell DNA in order to initiate malignant transformation [25]. If they do integrate, there is no preferential integration upstream of a cellular oncogene [26]. BLV and the closely related HTLV 1 and 2 viruses synthesize an additional protein called Tax, not observed in other retroviral families. In addition to transactivating regulatory functions, Tax also mediates malignant transformation. While the mechanism of Tax oncogenicity is not fully understood, it disrupts lymphoid cell growth control [27] and inhibits DNA repair [28], which could result in accumulation of cellular genetic defects, possibly leading to leukemia [27]. Other areas of the BLV genome transcribing microRNAs and other RNAs that regulate microRNAs, are recently associated with maintaining the transformed state of BLV-infected cells [29, 30].

Retrotranscribed BLV DNA is detected in human breast tissue by standard solution PCR [13] and in situ PCR [6]. BLV structural proteins gp51 and p24 are also detected in human breast tissue [31]. Robinson et al., [14] detected BLV DNA in human lung carcinomas using a microbial detection microarray with a reference data base including all published sequences of vertebrate viruses [32]. Building upon the evidence of human infection with BLV, Buehring et al., [7] conducted a case-control study on 214 US human subjects using in situ PCR technique to detect sequences in the tax region, which codes for the protein responsible for malignant transformation of cells. They found that 59% of the patients with a cancer diagnosis were positive for BLV DNA, compared with 29% of the subjects with no history of breast cancer. This difference was highly significant with a multiply-adjusted odds ratio of 3.1. Recently, a study conducted in Australia detected the presence of a BLV tax gene before and after breast cancer development. In this opportunity, both standard and in situ PCR techniques were employed to detect BLV DNA in the tissue samples. These results showed that viral DNA could be detected in this tissue many years before the onset of the disease. In 10 cases, it was possible to obtain enough DNA to sequence the viral LTR. The LTR sequences were compared with a reference sequence. After comparison, authors showed that in six samples the LTRs had a 100% homology with the reference sequence and four had at least one substitution [33]. Collectively, these previous studies supported the idea that humans can be infected with BLV and that it might be a risk factor to develop breast cancer.

In the current study, all samples obtained from malignant breast tissue were routinely tested for hormonal receptors (estrogen and progesterone), proliferation marker Ki67, and overexpression of oncogene HER-2. Approximately, 50% of the BLV positive slides were also positive for hormonal receptors, but no association was observed between hormone receptor status and the presence of BLV DNA. In contrast, a significant association was

observed between BLV DNA presence and a high/
moderate proliferative index. Ki67, the marker for
proliferation, recognizes an antigen present on the
nuclear membrane surface of mammalian cells
that are actively dividing. It is undetectable in
stationary cells, not dividing. Ki67 is routinely used
as a prognosis and predictive marker to diagnose
breast cancer, since its expression is an indicator
of the index of growth of the analyzed tissue [34].
The merit of this manuscript, besides being the first
study performed in Argentina, was the relationship
between the typical prognosis factors routinely
quantified in patients with breast cancer and the
presence of BLV DNA. This initial result opens a
new perspective and further supports the idea that
BLV might have a role in the initiation of malignant
transformation of human breast epithelium, which
generally results in more rapid cellular proliferation.
The current study results showed that BLV could
be detected by in situ PCR in 22.36% of the
females that the malignant breast tissue specimens
originated in them. In similar studies performed in
other countries, BLV DNA was present in a higher
percentage of malignant tested tissues [6, 13, 33].
This difference could be due to the fact that samples
were not processed from the beginning by the same
operator, or, instead, since the number of samples
was not large enough. A case-control study could
not be conducted to determine if BLV were more
frequent in malignant tissue than in normal breast
tissue, since the only specimens available were
invasive ductal carcinomas, the most common cancer
in females. It is not usual in Argentina to conduct
breast surgery to remove noncancerous tissue. Also,
the sample size was too small for definitive statistics
and a conclusion that BLV could be associated with
breast malignancy. It is highly important to confirm
that BLV is related to human breast cancer, since
there are several instances in which humans can be
exposed. A study conducted in Colombia reported
that genetic material corresponding to the viral gene
gag was present in fresh milk and raw meat [35].
Seroprevalence of antibodies against BLV in herds
from the most important dairy regions of Argentina
is approximately 90% [36, 37]. Government has no
policies to encourage the elimination of infected
animals or their separation from BLV-negative animals
within a herd. Such measures are implemented in
the European Union, where this infection in cattle is
almost eradicated [38, 39] and in Australia where it

is considered eradicated [40]. Several attempts are
made in order to design an efficient vaccine against
BLV. A novel strategy based on the use of a live-
attenuated BLV provirus is developed. It is being
tested regarding production, storage, and delivery,
as well as safety of the milk produced by vaccinated
cows [41]. In a recent review, the authors suggest
different approaches that could be conducted in order
to end the controversy of whether BLV is certainly
related to human breast cancer development [42].

ACKNOWLEDGMENTS

CONFLICT OF INTERESTS
The authors declared no conflict of interest.

ETHICS APPROVAL AND FINANCIAL SUPPORT

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