Comunicación corta

Report of bovine leukemia virus (BLV) *in situ* detection in primary cultures of bovine mammary epithelial cells

Reporte de la detección in situ de BLV (virus de leucemia bovina) en cultivos primarios de células epiteliales mamarias bovinas

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Abstract
A BLV infected cow, with high proviral load and persistent lymphocytosis, was used for this study. After euthanasia, nipple epithelial material was collected, disaggregated, and primary cultures were established. After 5 passages, viral proteins could be detected by immunocytochemistry, indicating that the virus is capable of expressing proteins in the cytoplasm of the host cell. Proviral load was quantified by qPCR in blood and milk of the infected animal. The presence of viral particles in milk and meat for human consumption has been described previously Therefore this report warns us of the possibility of BLV transmission to humans, with the potential risk of zoonotic disease.

Keywords: bovine leukemia virus, mammary epithelium, viral protein expression, immunocytochemistry

Resumen
Se seleccionó una vaca infectada con BLV, con alta carga proviral. Luego de la eutanasia, se tomó material epitelial de los pezones, se disgregó y se establecieron cultivos primarios. Luego de 5 pasajes, se fijaron las células y se detectó, mediante inmunocitoquímica, la presencia de proteínas virales, indicando que el virus es capaz de sintetizar proteínas en el interior de la célula huésped. La carga viral del animal se cuantificó en sangre y en leche antes de comenzar el estudio. Se ha descripto previamente la presencia de partículas virales en leche y carne aptas para consumo humano, por lo que este reporte nos alerta sobre la posibilidad de que el BLV pudiera transmitirse al humano, con el consiguiente riesgo de provocar una zoonosis.

Palabras clave: virus de leucemia bovina, epitelio mamario, expresión de proteínas virales, inmunocitoquímica

Introduction

Bovine leukosis virus (BLV) is a retrovirus of the *Retroviridae* family, the etiological agent of enzootic bovine leukosis. It is widely spread worldwide, especially in dairy farms, due to handling conditions. Most countries in Europe, New Zealand, and Australia have managed to eradicate it, achieving "leukosis-free" status (Kuczewski *et al.*, 2021). Once the virus infects the animal, it integrates into the host cell's genome and replicates clonally in each replication cycle. After 1 to 8 years post-infection, some animals can develop lymphoma in organs such as the abomasum, heart, lymph nodes, kidneys, uterus, or spleen, causing great economic losses due to reduced milk production, loss of international markets or death of the animal (Erskine *et al.*, 2012).

The host cells of the virus are B lymphocytes, but macrophages and mammary gland epithelial cells have also been described susceptible to infection. Buehring *et al,* (1994), detected the presence of the viral capsid p24 protein in milk cultures and in histological sections of mammary tissue from infected animals by immunohistochemistry, as well as the presence of BLV DNA by PCR (Buehring *et al.*, 1994). On the other hand, Yoshikawa *et al* (1997) detected the presence of the same protein in mammary tissues from infected cattle, but e in infiltrating lymphocytes (Yoshikawa *et al.*, 1997).

As in other exogenous retroviruses, the viral genome is composed of two simple strands of RNA. A reverse transcriptase is responsible for retrotranscribing the genome to be integrated into the host's genome. Once integrated, the provirus is flanked by two LTRs located at 5´ and 3' respectively. In MMTV (murine mammary tumor virus), another retrovirus from the same family, the ability of different factors including MP4, MP5, AP-2, CTF/NF1, MAF, and MGF (Stat5a) to bind to the enhancer region of the LTR, activating tissue-specific viral expression for MMTV, has been described (Pluta *et al.*, 2020), but this issue has not yet been studied for BLV. MMTV plays a defined role in the oncogenesis of mammary tumors in mice, and has been associated with the development of mammary tumors in humans (Stewart *et al.*, 2000).

The incidence of mammary tumors in cattle is very low; only 41 cases of mammary gland tumors have been reported from 1902 to date. A probable explanation for this low incidence could be associated with multiple factors, such as successive pregnancies and lactations, low exposure to estrogens during lactation, and premature elimination of the animals (Piva *et al.*, 2017).

Previous studies from our laboratory have shown that a bovine mammary epithelial cell line (MAC-T) is susceptible to being infected with BLV with peripheral blood mononuclear cells (PBMC) from a stably infected cow. The infection resulted stable, and productive, being capable of reinfecting another cell line with collected supernatant, indicating that viral particles were released to the culture medium (Martinez Cuesta *et al.*, 2018). Also, our previous studies showed the possibility of a mammary human cell line being infected with BLV, but infective particles were not detected in the culture medium (Martinez Cuesta *et al.*, 2020). This finding is of great relevance at this time, when the possibility of a zoonotic role of BLV is being actively studied. Numerous works describe the presence and association of BLV with mammary tumors in humans (Ceriani *et al.*, 2018; Corredor-Figueroa *et al.*, 2021; Martinez Cuesta *et al.*, 2018). This is a topic that deserves to be studied in depth, therefore the aim of this study was to demonstrate that BLV actively and naturally infects mammary gland epithelial cells.

Materials and methods

A BLV infected cow with persistent lymphocytosis and high proviral load was used for this study. The animal, suffering the "downer cow syndrome", belonged to a research herd, and was selected for euthanasia. Blood was extracted from the jugular vein. The detection of BLV was performed by qPCR according to Ladera Gomez *et al*. (2023) after the extraction of DNA from blood and milk.

To establish primary cultures of bovine mammary gland epithelial cells, nipple epithelial material was collected in a sterile container containing Minimal Essential Medium (MEM) supplemented with 100 µg/l of penicillin and 200 µg/l of streptomycin. Samples were refrigerated until transportation and subsequent processing in the laboratory. Euthanasia of the animal was performed through intravenous injection of Procaine HCL following anesthesia with 0.1 mg/kg of acepromazine, in accordance with the Animal Welfare Committee, Facultad de Ciencias Veterinarias, UNCPBA (permit number: Res CA 087/02). Epithelial material from the papillary duct was collected by scraping with a scalpel. The collected material was incubated with a 0.5% trypsin solution at 37 °C for 60 minutes, followed by centrifugation at 3000 *xg* for 10 minutes. The resulting pellet was washed twice with sterile PBS, and resuspended in MEM supplemented with 10% inactivated and gamma-irradiated fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO2 in T-25 flasks. After 5 passages, the cells were trypsinized fixed on slides, and subjected to immunocytochemistry for p24 protein detection. Cells were also lysed to obtain DNA to confirm the presence of BLV, as described previously. Immunocytochemistry was carried out with a monoclonal antibody against an epitope of p24 (1:50), kindly provided by Dr. Buehring, incubated with 0.5µg/ml biotinylated anti-mouse IgG (Vector BA-9200, Vector Labs, USA) for 1h, subsequently incubated with 1µg/ml streptavidin-alkaline phosphatase (KPL 5950-0005, Kirkegaard & Perry Laboratories, Newark, USA) for 30min and washed with PBS. The reaction was developed by the addition of BCIP/ NBT (Color Development Substrate, Moss Inc.) for 10 min, rinsed with water and stained with methyl green.

Results and discussion

The proviral load was estimated as copy numbers present in 30 ng of DNA. The results showed a proviral load of 35.855,58 copies of provirus in PBMC, and 203 copies in somatic cells from milk. Immunocytochemistry detected the presence of the protein in the cytoplasm of infected cells (Figure 1, A), and syncytia formation was visualized, as previously described, as a result of BLV infection (Graves & Jones, 1981). Uninfected MAC-T cells were simultaneously cultured as a negative control (Figure 1, B), and stained in the same way.

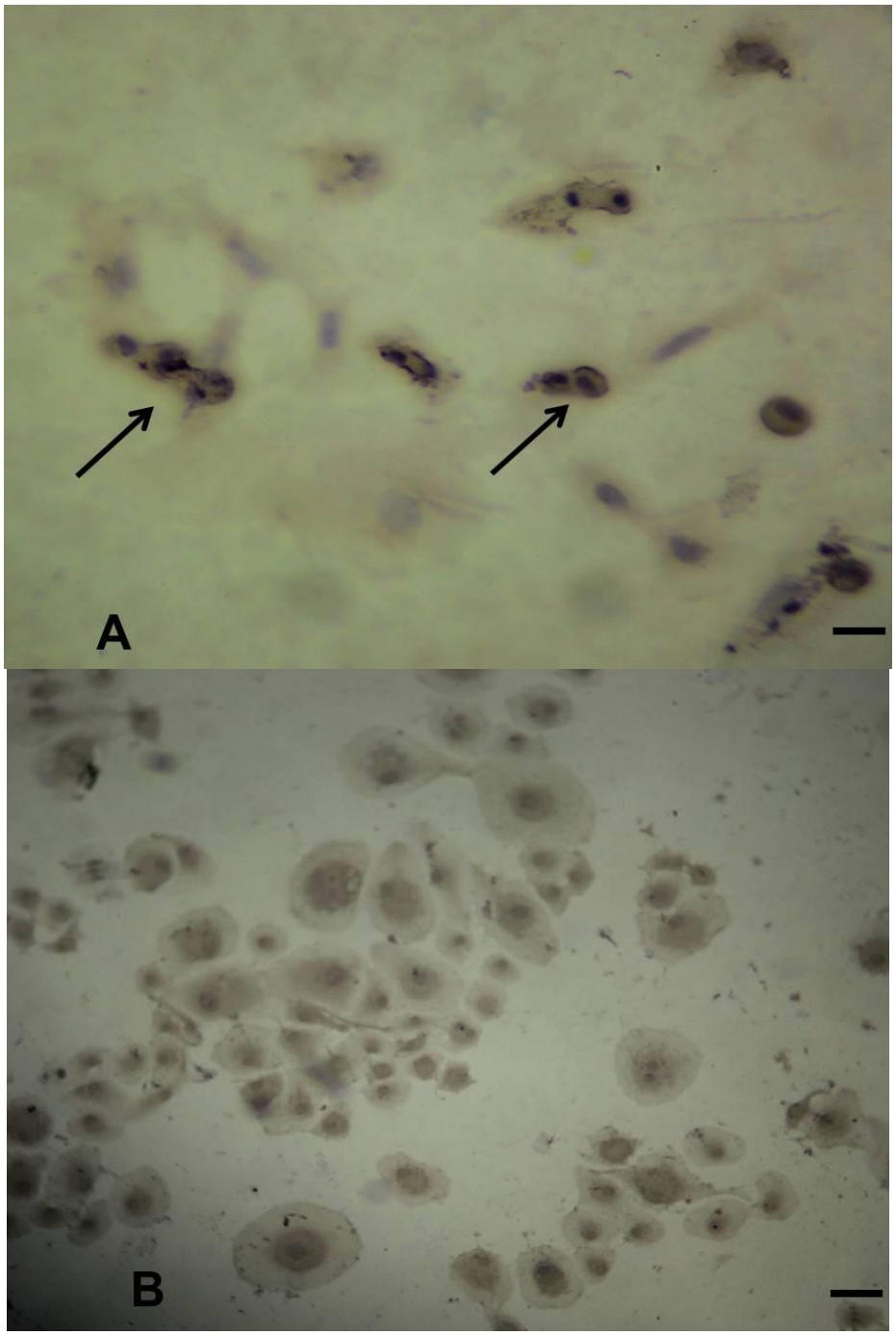

Figure 1. Immunocytochemistry to detect p24 in cell culture. **A**: Primary cultures from mammary epithelial cells, stained with methyl green. Cytoplasms of cells show a strong stain where p24 antibody reacted and small syncytia formation can be visualized. **B**: MAC-T cells (negative control) treated in the same way. No stain in cytoplasm is seen, indicating the absence of p24. Black line in the photograph equals 150 µm.

These results demonstrate that mammary epithelial cells are susceptible to being infected *in vivo*, and that viral proteins can be detected in the cytoplasm, indicating that BLV is being expressed actively. However, we were unable to isolate infectious virions from the primary cultures. One possible explanation is that the proviral load in mammary epithelial cells was significantly lower compared to that of PBMCs. A similar situation has been previously described by our group, as milk somatic cells maintain lower proviral load levels than PBMCs

(Ladera Gomez *et al*., 2023). Although proviral loads in mammary epithelial cells are lower, they should not be disregarded. This finding is particularly important, as it further raises the possibility that products derived from infected cows intended for human consumption could serve as a potential source of viral transmission. During the last few years, the possibility that drinking bovine milk could be associated to human breast cancer has gained momentum. Breast cancer is the most diagnosed cancer in the world, and its incidence rises every year. Approximately 685,000 women died in 2020 from breast cancer, and by the year 2040 the number of newly diagnosed cases is expected as 40%, with 3 million new cases every year (Arnold *et al*., 2022). A study performed in Brazil in 2020 showed that in regions where drinking raw milk was usual, the presence of BLV in tissues from breast cancer patients was much higher than in healthy women (Delarmelina *et al*., 2020). Moreover, BLV was detected in meat and milk for human consumption by different researchers (Corredor-Figueroa *et al*., 2021; De Quadros *et al*., 2023). Even though pasteurization of milk for human consumption is recommended worldwide, in rural areas from many countries this technology is unavailable. Furthermore, the recent increasing trend to consume non-processed food among new generations, is causing a rise in individuals drinking non-pasteurized milk. Pasteurization may not only destroy BLV, but also other pathogenic bacteria responsible of food illnesses.
This research, although preliminary, strengthens the hypothesis of the probable zoonotic role of BLV, and reinforces the requirement of continuing research to determine this fact categorically. The alarm is already raised.

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Conflict of interest
There is no conflict of interest, including financial, personal, or other relationships with individuals or organizations that could inappropriately influence this work

Authors agreement and contributions
PEM and MCC conceived and designed the study and critically reviewed the manuscript. ACH, PEM, and MVNF conducted the experiments, analyzed the data, and drafted the manuscript. PEM, PAL, and MCC collaborated in the execution of the work. GLD and MCC critically reviewed the manuscript. MCC funded the project. All authors approved the final version.

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