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**Stable infection of a bovine mammary epithelial cell line (MAC-T) with bovine leukemia virus (BLV)**

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Highlights:

- Bovine mammary epithelial cell line is susceptible to infection with BLV.
- BLV infection increases TNF-α expression on infected cells.
- Infected mammary epithelial cell lines expresses BLV actively.

**Abstract**

Bovine leukemia virus (BLV) is a retrovirus that affects cattle causing a lymphoproliferative disease. BLV infection has been associated with misbalance of the immune response causing a higher incidence of other infections. Mastitis is one of the most important conditions that affect milk production in cattle. The aim of this study was to stably infect a bovine mammary epithelial cell line (MAC-T). MAC-T cell line was successfully infected with BLV and the infection was confirmed by nested PCR, qPCR, immunocytochemistry, western blot and transmission electron microscopy. This is the first report of a bovine mammary epithelial cell line stably infected with BLV. This new cell line could be used as an *in vitro* model to study the effect of BLV on the immune response in the mammary gland and the relationship with other agents causing mastitis.

**Abbreviations:**

BLV Bovine leukemia virus; MAC-T Bovine mammary epithelial cell line; EBL Enzootic bovine leukosis; BHV-1 Bovine Herpesvirus 1; BHV-4 Bovine Herpesvirus 4; BVDV Bovine viral diarrhea virus; FDM Foot and Mouth Disease; PBMCs peripheral blood mononuclear cells; FLK fetal lamb kidney; HPL high proviral load ; p.p.i passage post-infection

**Keywords:** Bovine Leukemia Virus; MAC-T; Infection; Mastitis; TNF-α**1. Introduction**

Bovine leukemia virus (BLV) is a δ-retrovirus responsible for Enzootic Bovine Leukosis (EBL), a lymphoproliferative disease that affects cattle. With a size of 60 to 125 nm, this enveloped virus naturally infects B-lymphocytes causing a chronic infection. Its genomic organization is typical of

the retroviral family: it has two identical long terminal repeats (LTRs) flanking structural genes *gag*, *pro*, *pol,* and *env*. These genes encode viral structural proteins, integrase, protease and reverse transcriptase. Structural proteins gp52 (Env) and p24 (Gag) are indicative of active viral replication and since they are the major antigenic proteins, they are commonly used for serological diagnosis. Especially, p24 is the major core protein of BLV and is highly immunogenic. Furthermore, BLV encodes regulatory proteins such as Tax, Rex, G4 and G3 in a region called pX (Gillet et al., 2007) and it also expresses regulatory microRNAs (Durkin et al., 2016).

Due to the slow progress of the disease, clinical signs and symptoms appear many years after infection (Gillet et al., 2007). Thus, this silent infection is spread worldwide with the exception of Europe, where it has been eradicated (Bartlett et al., 2014). Transmission occurs naturally and iatrogenically through the transfer of infected cells, mainly by blood or milk (Florins et al., 2007). Based on this assumption, the mammary epithelium could play an important role in viral transmission. Previous studies report the presence of BLV in the bovine mammary epihtelium (Buehring et al., 1994; Yoshikawa et al., 1997). However, most recent works are focused in the detection of BLV infected mononuclear cells in milk (Jaworski et al., 2016; Kuckleburg et al., 2003), and its potential risk to human (Olaya-Galán et al., 2017; Sellers et al., 2008).

BLV infection negatively impacts on dairy production by diminishing the milk yield and increasing deaths and veterinary costs. Moreover, it has been described that BLV infection might lead to a higher susceptibility to other infections due to its effects on immune cells (Della Libera et al., 2015; Emanuelson et al., 1992; Erskine et al., 2011; Frie and Coussens, 2015). In this way, BLV could be related to an increased incidence of mastitis, one of the most common diseases in dairy cattle.

Mastitis is an inflammation of the mammary gland that is characterized by an increase in TNF-α expression (Alluwaimi, 2004). TNF-α is a pleiotropic cytokine that plays an important role in local and systemic inflammatory response (Fu et al., 2013). It stimulates the activation and proliferation of many immune cells and the release of other cytokines (Wojdak-Maksymiec et al., 2013). The relationship between infection of the bovine mammary epithelium by BLV and TNF-α production has not been explored yet.

Many viruses have been associated with bovine mastitis, such as bovine herpesvirus 1 and 4 (BoHV 1 and 4), bovine viral diarrhea virus (BVDV) and foot and mouth disease (FMD) (Wellenberg et al., 2002). Furthermore, it has been demonstrated a greater incidence of clinical and subclinical mastitis on herds affected with FMD (Lyons et al., 2015).

The aim of this study was to stably infect with BLV a bovine mammary epithelial cell line and to analyze the effect of this infection on TNF-α expression. For this purpose, we isolated peripheral blood mononuclear cells (PBMCs) from a heavily BLV infected cow and co-cultured them with a bovine mammary epithelial cell line (MAC-T). Our results show that MAC-T cells can be stably infected with BLV, releasing viral particles that can be detected in cell culture supernatants.

## 2. Materials and Methods

### *2.1 Cell cultures:*

The bovine mammary alveolar cells-T (MAC-T) is an adherent cell line which was produced after the stable transfection of alveolar mammary cells with SV-40 large T-antigen (Huynh et al., 1991).

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Natocor, Cordoba, Argentina) and 1 µg/ml hydrocortisone (Sigma-Aldrich) at 37°C with 5% CO2.

FLK is an adherent cell line derived from fetal lamb kidney persistently infected with BLV. This cell line was maintained in DMEM supplemented with 10% FBS at 37°C with 5% CO2.

*2.2 Sample collection*

Blood samples were obtained from an adult Holstein (Holando-Argentino) dairy cow belonging to a herd from Tandil region (Provincia de Buenos Aires, Argentina). Previous studies (Farias et al., 2016) demonstrated that this animal was BLV infected and carried a high proviral load (HPL). For peripheral blood mononuclear cells (PBMCs) separation, 10 ml of heparinized blood (5U/ml) were obtained by jugular venipuncture. Blood was transferred to 15 ml tubes and centrifuged for 15 min at 3000 rpm at 4 ºC. Buffy coat was mixed with 11 ml of cold ammonium chloride buffer (150 mM NH4Cl, 8 mM Na2CO3, and 6 mM EDTA) for 1 min to completely lyse red blood cells. PBMCs were obtained by centrifugation at 3000 rpm for 10 min at 4 ºC. Cells were washed with PBS and centrifuged at 2500 rpm for 7 min at 4 ºC. PBMCs pellets were resuspended in RPMI supplemented with 10 % FBS and incubated at 37°C with 5% CO2 until use.

*2.3 Infection of a bovine mammary epithelial cell line (MAC-T) with BLV*

Once MAC-T cells reached 90% confluency, they were co-cultured for 24 hours with 3 x $10^6$ PBMCs from a cow with high BLV proviral load. Cells were then washed with PBS and then trypsinized to remove any PBMC that could remain attached. The infected cell line was named MAC-T BLV. Analysis of this new cell line begun after the sixth passage post infection (p.p.i).

*2.3.1 DNA extraction*

DNA from MAC-T and MAC-TBLV was extracted using Qiagen columns (QIAamp DNA Mini Kit, Germantown, MD, USA) according to the manufacturer´s protocol. DNA was eluted in 50 µL of water. DNA concentration and purity were determined by absorbance at 260 nm and 260/280 coefficient, respectively, in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Purified DNAs were stored at -20 ºC until use.

*2.3.2 Detection of BLV genes by nested PCR.*

Obtained DNA was used as template to amplify the viral gene *env* by a nested PCR optimized in our laboratory and previously described (Juliarena et al., 2013).

*2.3.3 Proviral load determination*

An absolute quantification method by real-time PCR (qPCR) was used for proviral load determination as described previously(Farias et al., 2016). A plasmid carrying the complete BLV genome was used for the construction of the standard curve. Standards and samples were analyzed in duplicates. The amplified sequence was a fragment of the viral pol gene and results were expressed as BLV copies/ 30 ng DNA.

*2.3.4 Western Blot Analysis*

MAC-T, MAC-T BLV, and FLK cell culture supernatants were run in 12% polyacrylamide gels and transferred onto a Hybond $^{+}$ nylon membrane (Amersham Pharmacia, Sweden). After blocking with MTBS, the membrane was incubated 2 h with an anti-BLV-p24 monoclonal antibody (1:500) (a kind gift from Dr. Buehring, UCLA, Berkeley, USA)(Buehring et al., 1994), washed 3 times for 15 min each with PBS-Tween and incubated with 0.5 µg/ml biotinylated anti-mouse IgG (Vector BA-9200) for 1 h. The membrane was washed with PBS-Tween and incubated with 1 µg/ml streptavidin-alkaline phosphatase (Vector SA-5100) for 30 min. After washing with PBS, BCIP/NBT (Color Development Substrate, Moss Inc.) was added for 10 min to develop the reaction and stopped by rinsing with tap water.

*2.3.5 Immunocytochemistry to detect viral protein p24*

One thousand cells were seeded on a glass coverslip in a 6 wells plate and incubated for 24 h at 37°C. Cells were washed twice with PBS and fixed with buffered formalin for 10 min, washed with PBS and treated with 0.5% Triton X-100 for 10 min at room temperature. After 30 min incubation with equine fetal serum as a blocking agent, cells were incubated for 2 h with an anti-BLV-p24 monoclonal antibody (1:500). Coverslips were washed three times with PBS and incubated with 0.5 µg/ml biotinylated anti-mouse IgG (Vector BA-9200) for 1 h. After three washes with PBS, coverslips were incubated with 1 µg/ml streptavidin-alkaline phosphatase (Vector SA-5100) for 30 min 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.

*2.3.6 Transmission electron microscopy (TEM):*

Cells were harvested with a cell scraper, centrifuged and fixed with 2% glutaraldehyde in PBS for 2 h at 4°C, washed three times with PBS pH 7.2 and subsequently treated with 1% osmium tetroxide (Sigma-Aldrich) for 2 h at 4°C. In the next step, cells were washed again with PBS and sequentially dehydrated in solutions with increasing concentrations of acetone. Finally, samples were included in epoxy resin (Spurr) and ultrathin sections were obtained with an ultramicrotome Ultracut Reichert-Jung E. Sections were contrasted with uranyl acetate/acetone for 3 min, washed with distilled water and colored with lead citrate for 2 min before observation with the Zeiss EM 109T equipped with a Gatan ES1000W digital camera.

*2.3.7 Measure of TNF-α expression at the mRNA and protein level*

*2.3.7.1 mRNA gene expression*

*2.3.7.1.1 RNA extraction and DNase I treatment*

Total RNA extraction was performed from MAC-T and MAC-T BLV using TRIzol Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RNA was eluted in 50 µl of RNase-free water. RNA concentration and purity were determined by the OD value at 260 nm and 260/280 coefficient, respectively, in a NanoDrop 2000 Spectrophotometer. RNAs were kept at -80 ºC until use. To remove any possible contaminating genomic DNA, 1 µg of each RNA sample was treated with RNase-free DNase I (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

*2.3.7.1.2 Reverse transcription*

Reverse transcription (RT) reaction was carried out on 0.5 µg of total DNase-treated RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany), according to the manufacturer's instructions. The copied DNA (cDNA) was used immediately or stored at -20 ºC until use.

*2.3.7.1.3 qPCR conditions for mRNA gene expression*

PCR reactions mix contained 0.5 µM specific forward and reverse primers, 1 x PCR Master Mix with SYBR Green (FastStart Universal SYBR Green Master Rox, Roche) and 2 µl of cDNA, in a final volume of 20 µl. Amplification and detection of specific products were carried out using an Applied Biosystems 7500 cycler, under the following conditions: 2 min at 50 ºC, 10 min at 95 ºC, 40 cycles of 15 s at 95 ºC and 60 s at 60 ºC. For each sample, three biological replicates and two technical replicates were processed. Negative controls for cDNA synthesis and non-template control were included in all cases.

*2.3.7.1.4 Primers*

Primers used for amplification are listed in **Table 1**.

*2.3.7.1.5 Determination of amplification efficiency and statistical analysis*

The amplification efficiency for all genes evaluated was determined by standard curves. Briefly, 5 serial dilutions (4-fold) of a reference cDNA calibrator were amplified in triplicate and the amplification efficiency was determined by a linear regression model according to the equation: E = 10[-1/slope] (Pfaffl, 2001).

Target gene expression was normalized to that of the endogenous gene, GAPDH. Relative Expression Software Tool (REST®, Qiagen Inc., Germantown, MD, USA) was used to obtain the relative expression ratios and for the statistical analysis (Pfaffl et al., 2002). Statistically significant differences were considered when $p < 0.05$.

*2.3.7.2 TNF-α protein analysis by ELISA*

TNF-α protein was measured by commercial ELISA (NordicBiosite LS-F13473-1, Täby, Sweden) following the manufacturer´s instructions. A standard curve of TNF-α was used to determine the concentration of the sample. The experiment was conducted using biological replicates and the results were analyzed by ANOVA using Infostat®.

**3. Results:**

*3.1 Infection of target cells with BLV:*

Co-cultivation of MAC-T cells with PBMCs obtained from a high proviral load BLV infected cow resulted in a new cell line, called MAC-T BLV. Infection was demonstrated performing a nested PCR (nPCR) to amplify the viral gene *env*. Figure 1 shows a fragment of 444 pb amplified from DNA obtained from FLK and MAC-T BLV. Amplicons were not detected when DNA from the uninfected counterpart, MAC-T, was used as a template (Figure 1 line B).

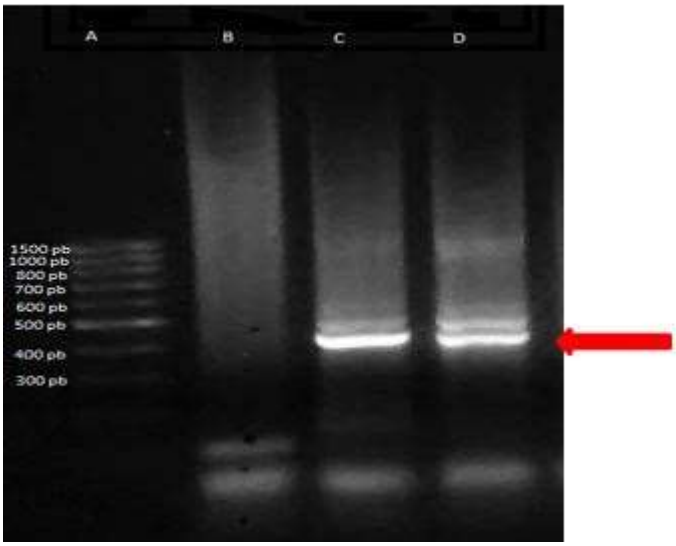


**Figure 1. Fragment of BLV *env* gene amplified by nPCR.** (A) MW marker (B) non infected MAC-T (negative control) DNA, (C) MAC-T BLV DNA; (D) FLK DNA (positive control). The red arrow indicates the 444pb *env* fragment.

Moreover, an absolute quantification of the viral gene *pol* was performed by qPCR. MAC-T BLV had 3486 BLV copies/ 30 ng DNA while amplification of this gene in MAC-T cells was not detected.

To further confirm PCR results, western blot and immunocytochemistry were performed. Western blot analysis revealed a specific band of approximately 24 kDa in MAC-T BLV supernatant, which was also visible in the positive control, FLK. This protein band was not detected in MAC-T cell culture supernatant (Figure 2).

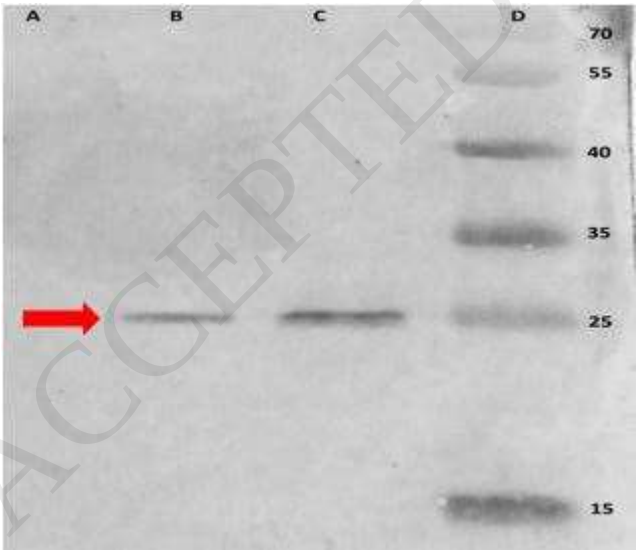


**Figure 2. Western Blot analysis of viral protein p24 in cell culture supernatants.** (A) MAC-T (negative control); (B) MAC-T BLV; (C) FLK (positive control); (D) MW Marker. The red arrow indicates the BLV 24 kDa protein.

Immunocytochemistry for BLV p24 detection shows strong positive staining in MAC-T BLV and FLK cells (Figure 3). On the other hand, the cytoplasm of MAC-T cells exhibited only green staining indicating that viral p24 was not present.

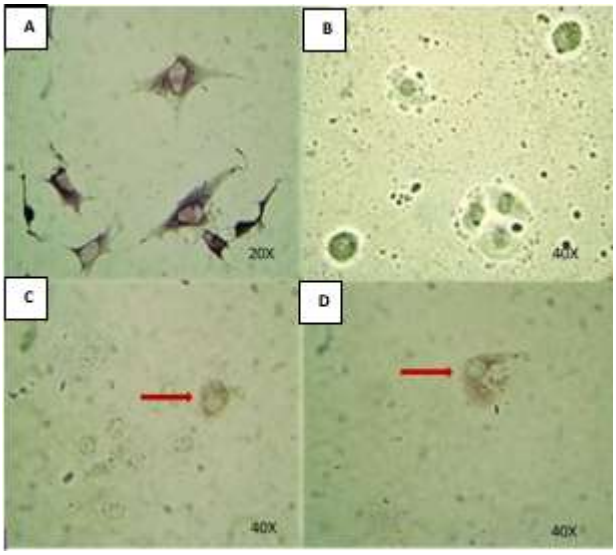


**Figure 3. Immunocytochemistry for viral p24 detection**. (A) FLK (positive control): note cytoplasmic violet staining (20X); (B) MAC-T (negative control), only the counterstain is visible in the cytoplasm (absence of p24) (40x); (C & D) MAC-T BLV, red arrows indicate infected cells that express p24. Non-infected cells stained with methyl green surround infected cells (40X).

*3.2 Visualization of BLV particles in the infected cell line, MAC-T BLV*

Electron microscopy images confirm BLV particles production. In the cytoplasm of MAC-T BLV cells inclusions of viral particles can be observed (Figures 4 A and B).

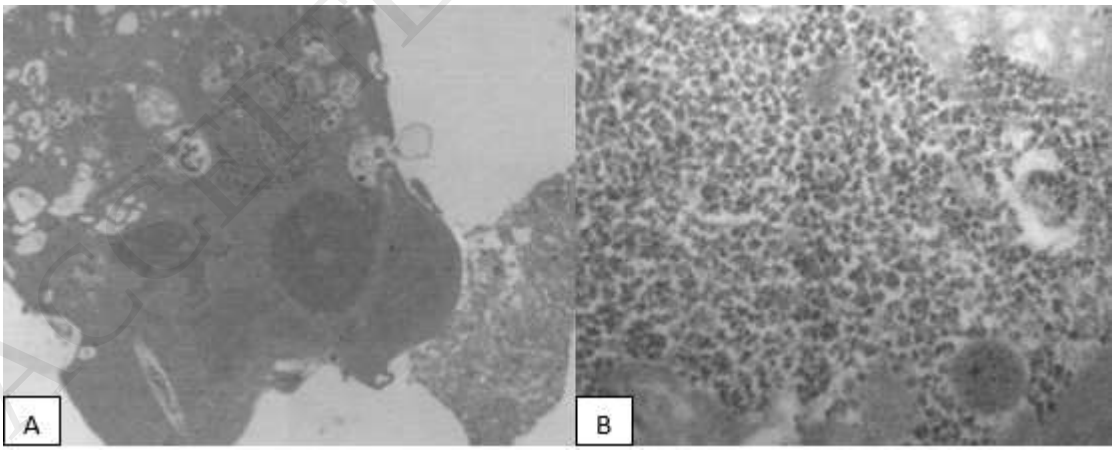


**Figure 4: BLV particles visualized by TEM in infected MAC-T BLV cells cytoplasm**. A Scale bar: 0.5 µm. B Scale bar: 100 nm.

*3.3 Analysis of TNF-α protein and mRNA expression*

Relative expression of TNF-α mRNA was measured using qPCR. Expression of this target gene was normalized against bovine GAPDH as a reference gene. Mean TNF-α mRNA expression level was higher in MAC-T BLV cells ($p < 0.001$) compared with uninfected MAC-T cells(Figure 5). This cytokine is up-regulated 24.4 times (range 5.4-129.5). Moreover, when analyzed by ELISA, the supernatant of infected MAC-T cells (average 14563.3 pg $ml^{-1}$) showed a higher protein expression ($p = 0.014$) of TNF-α than the non-infected counterpart (average 2222.5 pg $ml^{-1}$) (Figure 6).

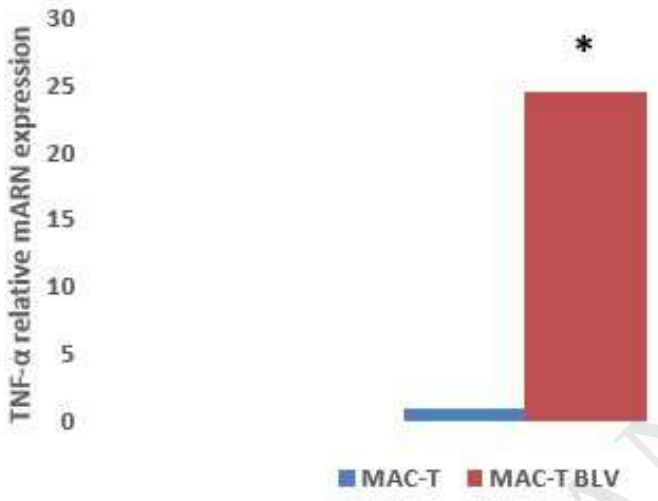


**Figure 5. TNF- α relative expression levels in MAC-T and MAC-T BLV cells by qPCR.** An asterisk (*) indicates statistically significant differences ($p < 0.05$) in relation to uninfected control (MAC-T).

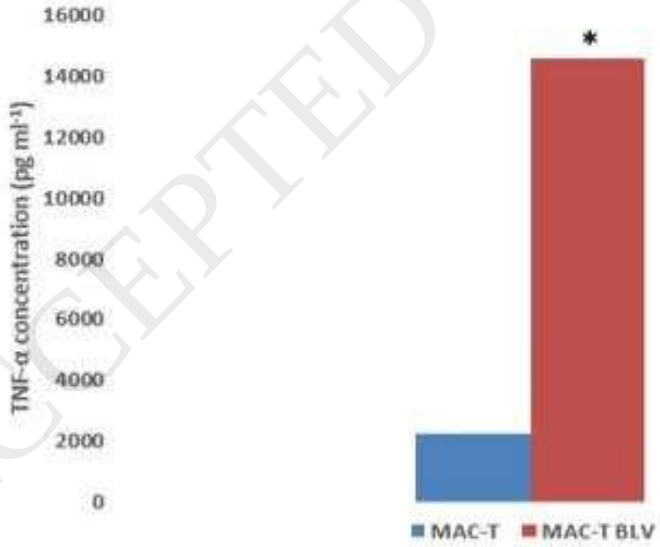


**Figure 6. TNF-α protein concentration measured by ELISA**. An asterisk (*) indicates statistically significant differences ($p < 0.05$)

**4. Discussion**

In this study, a bovine mammary epithelial cell line was infected with BLV. This is the first report of an *in vitro* infection of bovine mammary epithelium with this virus. Although previous studies have demonstrated the presence of BLV in mammary epithelium and tissues of infected cows (Buehring et al., 1994; Yoshikawa et al., 1997), this is the first report of a stably BLV-infected cell line.

Our results demonstrate that MAC-T is susceptible to infection with BLV present in its natural target cell, the B lymphocytes. The new cell line, MAC-T BLV, synthesizes and expresses BLV particles that can be detected in the cell cytoplasm and in cell culture supernatants. Indeed, the presence of viral protein p24 indicates active viral production (Gillet et al., 2007). Moreover, detection of viral genes in MAC-T BLV DNA and visualization of viral particles by electron microscopy confirm the infection of this bovine mammary epithelial cell line with BLV. The absolute quantification of the proviral load shows that there are 3486 proviral copies integrated in 30 ng of MAC-T BLV cellular DNA.

Although it is well known that BLV is characterized by its latency *in vivo*(Gillet et al., 2007), viral transcription is activated after infected PBMCs are placed in cell culture(Lagarias and Radke, 1989). This i*n vivo* viral repression may be caused by a plasma factor related to fibronectin (Gupta et al., 1984.; van den Heuvel et al., 2007), which is not present in cell lines infected *in vitro*. For this reason, FLK, bovine leukocytes and other BLV stably infected cell lines express large amounts of viral particles (Harms and Splitter, 1992; Mamoun et al., 1981; Rössler et al., 1985; Van Der Maaten and Miller, 1975). The capacity of MAC-T BLV to produce virus is consistent with the results observed in previous BLV producing cell lines. Unfortunately, this does not reflect the number of viral particles that could be produced *in vivo* by an infected bovine mammary epithelial cell. Nevertheless, this cell line could be used to study *in vitro* the effect of the virus on this epithelium and is a useful and simpler tool than the use of primary cell culture form mammary epithelium.

These results have two major implications. The fact that BLV can infect the bovine epithelial mammary gland supports the possibility of viral transmission through breastfeeding, an issue that nowadays is still controversial (Gutiérrez et al., 2015; Mekata et al., 2015; Nagy et al., 2007; Van Der Maaten et al., 1981). Some studies demonstrate that the virus is present in the milk of infected cattle and others authors ensure that milk maternal antibodies protect against calf infection. It is required to conduct more research in this area, analyzing whether there is a threshold of proviral load in milk above which antibodies lose their capacity to neutralize the infection. In this case, continuous production of virus in the infected mammary gland could favor the infection of calves. Transmission of BLV needs a cell to cell contact due to the instability of isolated viral particles (Lairmore, 2014). Until now, BLV transmission through breastfeeding was considered to be caused only by infected lymphocytes recruited to the mammary gland which are finally secreted in milk. Therefore, these results are a starting point to further investigate the role of bovine mammary epithelial cells in BLV transmission.

On the other hand, it is now well known that BLV infected cattle have alterations in the immune response that cause immune exhaustion, disease progression and more susceptibility to opportunistic infection (Frie and Coussens, 2015; Konnai et al., 2017). In this context, BLV infection of the mammary epithelium could affect the immune response in the mammary gland favoring the development of bacterial mastitis and, consequently, decreasing milk quality and quantity.

Furthermore, during mastitis, there is an increase in epithelial permeability allowing a bidirectional passage from blood to milk (Rainard et al., 2016). This permeability could favor the passage of BLV infected cells to milk and finally, to calves.

Furthermore, BLV could be itself a cause of mastitis since it has been suggested that the virus might play a role in this disease (Wellenberg et al., 2002). In fact, viral particles compatible with BLV have been found in mammary tissues of BLV-infected cows with subclinical mastitis (Yoshikawa et al., 1997). However, studies about the incidence of mastitis in BLV infected cattle are not conclusive (Emanuelson et al., 1992; Sandev et al., 2004).

In this study, we demonstrate that MAC-T BLV cell line expresses more TNF-α mRNA and protein than its uninfected counterpart. The increase of pro-inflammatory mediators such as TNF-α is one of the mechanisms responsible for the alteration of mammary epithelial cell tight junctions (Madara and Stafford, 1989). Epithelial cells are one of the first barriers in the defense against pathogens. Secretion of pro-inflammatory cytokines contributes to the recruitment of immune cells (Medzhitov and Janeway, 2000). TNF-α infusion into the normal mammary gland reduces milk proteins expression and increases the influx of neutrophils as a result of the weakening of the milk-blood barrier; effects that resemble those of mastitis (Watanabe et al., 2000).

TNF-α is up-regulated in bovine infectious mastitis caused by different types of bacteria and besides its beneficial role in immune response, it is also associated with tissue damage due to the inflammation process (Bannerman, 2009; Burvenich et al., 2003; Gilbert et al., 2013; Günther et al., 2011; Lahouassa et al., 2007). This mammary gland damage causes an increase in somatic cell counts (that reduce milk quality), diminishes milk production through fibrosis of the cow udders, and alters the immune response favoring the growth of other pathogens (Sharma and Jeong, 2013; Zhang et al., 2016; Zhao and Lacasse, 2008). In this scenario, the increase of TNF-α caused by BLV infection could favor the development of other pathogens in the mammary gland; thus, leading to a higher incidence of mastitis in BLV infected cattle.

**5. Conclusion**

This is the first description of an experimental infection of a bovine epithelial cell line with BLV. This new stably infected cell line (MAC-T BLV) could be used as a model to investigate *in vitro* the effect of the virus on the immune response in the mammary epithelium. MAC-T BLV cell line could be a useful tool to evaluate the relationship between BLV infection and the incidence of mastitis in dairy cattle.

Furthermore, this study shows that infection of MAC-T with BLV causes an increase in TNF-α expression. Considering this cytokine effects, it could be possible that BLV infection increases inflammatory cytokine production and alters the epithelium permeability favoring bacterial infections that cause mastitis. In addition, BLV infection could affect the normal immune response thus allowing bacterial growth in the affected tissue. However, more research needs to be done including the study of the effect of BLV on the epithelium response to different bacterial infections.

**Conflict of interest**

The authors declared no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial and personal relationships that could inappropriately influence this work.

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| **Gene** | **Primer sense** | **5'-3' sequences** | **Reference** |
|---|---|---|---|
| GADPH | Forward | CACCCTCAAGATTGTCAGCA | |

| | | | |
|---|---|---|---|
| | Reverse | GGTCATAAGTCCCTCCACGA | (Okuda et al., 2010) |
| TNF-α | Forward | AGCCTCAAGTAACAAGCC | (Konnai et al., 2006) |
| | Reverse | TGAAGAGGACCTGTGAGT | |

**Table 1:** Primers used for the amplification of mRNA.