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Short communication: Relationship between the level of bovine leukemia virus antibody and provirus in blood and milk of cows from a naturally infected herd

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ABSTRACT

We explored the relationship between the level of bovine leukemia virus antibodies and provirus load during natural infection. For that purpose, a set of 50 blood and milk paired samples were analyzed for the presence of bovine leukemia virus provirus and antibodies. Additionally, provirus load and antibody titers were measured and the relationship between these variables was investigated. Bovine leukemia provirus was detected in 59% of milk samples and a negative correlation was observed between the level of milk provirus load and milk antibody titers. By the consumption of raw milk, calves might be exposed to bovine leukemia virus favoring the perinatal transmission of this disease.
Key words: bovine leukemia virus, mammary gland secretions, antibodies, dairy cattle, perinatal transmission

Short Communication

Bovine leukemia virus (**BLV**) is distributed worldwide, with the exception of western Europe. The virus was first described in Argentina in 1973 (Ciprian, 1973). At the beginning of the 21st century, the individual cow prevalence was 33% (Trono et al., 2001); however, more than 80% of farms were infected with BLV, denoting a rapid spread of the virus in the territory (Trono et al., 2001). In a recent study, an individual prevalence of 80% has been observed in dairy farms of the main productive areas of Argentina (Gutiérrez et al., 2012).

Several routes of transmission of BLV have been described (Hopkins and DiGiacomo, 1997). In addition to horizontal and intrauterine transmission, the ingestion of colostrum and milk with provirus or free virus particles might be important for BLV perinatal transmission in calves. It has been demonstrated that the mother's provirus load (**PVL**) and the duration of lactation are associated with the risk of perinatal transmission of human T-lymphotropic virus and human immunodeficiency virus (**HIV**), 2 related viruses (Kinoshita et al., 1984; Miotti et al., 1999; Li et al., 2004; Martin-Latil et al., 2012; Milligan and Overbaugh, 2014). On the other hand, the administration of BLV-specific antibodies (**Ab**) has been effective blocking the oral infection with BLV (Van Der Maaten et al., 1981). However, information is scarce about the factors involved in transmission and progression of BLV in Argentinean dairy herds (Monti et al., 2005; Gutiérrez et al., 2011).

To explore the relationship between PVL and Ab in blood and milk of lactating cows under natural settings, a set of 50 paired samples was analyzed. The samples were obtained from a dairy farm located in Rafaela, Santa Fe, Argentina (31°16′S, 61°29′W). This region belongs to the main dairy producing area of the country. The herd was composed of 332 Holstein cows (>1 lactation) and was naturally infected with BLV. The procedures used for animal handling and sampling were approved by the Institutional Animal Care and Use Committee of the Instituto Nacional de Tecnología Agropecuaria. The guidelines described in the Institutional Manual were followed at all times.

Plasma-specific Ab against the whole BLV viral particle were measured by indirect ELISA as described previously (Trono et al., 2001). Briefly, ELISA plates were coated with antigen purified from fetal lamb kidney cells persistently infected with BLV by centrifugation on a sucrose gradient. The samples to be tested were added to the plate in duplicates. Based on preliminary data plasma samples were prediluted 1:40. The Ab titers were assayed by the end-point dilution method using 2-fold dilutions of sera. After incubation and washing, anti-bovine IgG peroxidase conjugated was added to each well. The presence of secondary antibody was revealed with 3,3′,5,5′-tetramethylbenzidine and H_2O_2.

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Reaction was stopped using H_2SO_4 and the absorbance was read at 450 nm. Normalized results were obtained as a sample-to-positive ratio. A weak positive control serum was used to calculate the ratio; its reactivity was set to 100% and all tested samples were referred to it. A cut-off level of 25% was established in the original work (Trono et al., 2001) over 339 serum samples, using PCR and Southern blot as confirmatory tests. The sensitivity and specificity of the assay were 97.2 and 97.5%, respectively; those samples with reactivity above the cut-off level were considered positive. Titers were expressed as the reciprocal of the last dilution with reactivity above the cut-off level. The presence of BLV-specific Ab was detected in 94 and 90% of plasma and milk samples, respectively (n = 50; Figure 1A). Although the proportions of BLV Ab in plasma and milk samples were similar, the mean Ab titer in the plasma was 10-fold higher than in milk (493 vs. 36, respectively; Mann-Whitney U-test, $P < 0.0001$; Figure 1A).

Whole blood and milk DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. As not enough material was obtained from 1 blood and 4 milk samples (<5 ng/μL), these were excluded from the following analysis. The BLV DNA was detected in 82 and 59% of blood and milk samples, respectively (n = 49 and 46, respectively; Figure 1B). Furthermore, PVL was quantified by a real-time quantitative PCR (**qPCR**). Briefly, each qPCR reaction contained Fast Start Universal SYBR Green Master Mix (Roche), 800 n*M* forward and reverse primers (BLVpol5f: 5′-CCTCAATTCCCTTTAAACTA-3′; BLVpol3r: 5′-GTACCGGGAAGACTGGATTA-3′) and 500 ng of DNA template. The reaction was performed on an ABI 7500 machine (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) with the following cycling conditions: 2 min at 50°C, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 15 s, and at 60°C for 1 min. The specificity of each reaction was confirmed by dissociation curve analysis. As standard, a plasmid pBLV1 (provided by Jacek Kuzmak, National Veterinary Research Institute, Pulawy, Poland) containing BLV pol fragment was used. Ten-fold dilutions of this standard were made from 1×10^6 to 1 copy/μL. A strong and a weak positive control and 2 negative controls were included in each plate. The limit of detection of the assay was 5 BLV copies per reaction (10 BLV copies/μg of DNA). The PVL in blood was significantly higher than PVL in milk (Mann Whitney U-test, $P = 0.0006$; Figure 1B) and no correlation was observed between PVL measured in these body compartments (Figure 2A). Furthermore, plasma and milk BLV Ab titers positively correlated with blood PVL (Figure 2B and C, respectively) and a positive correlation was noted between the level of Ab in plasma and milk (Figure 2D). In contrast, a weak negative correlation was observed between milk Ab titers and milk PVL (Figure 2E). To further explore the relationship between milk Ab titers and milk PVL, milk samples were divided into 2 groups based on their Ab titer, (1)

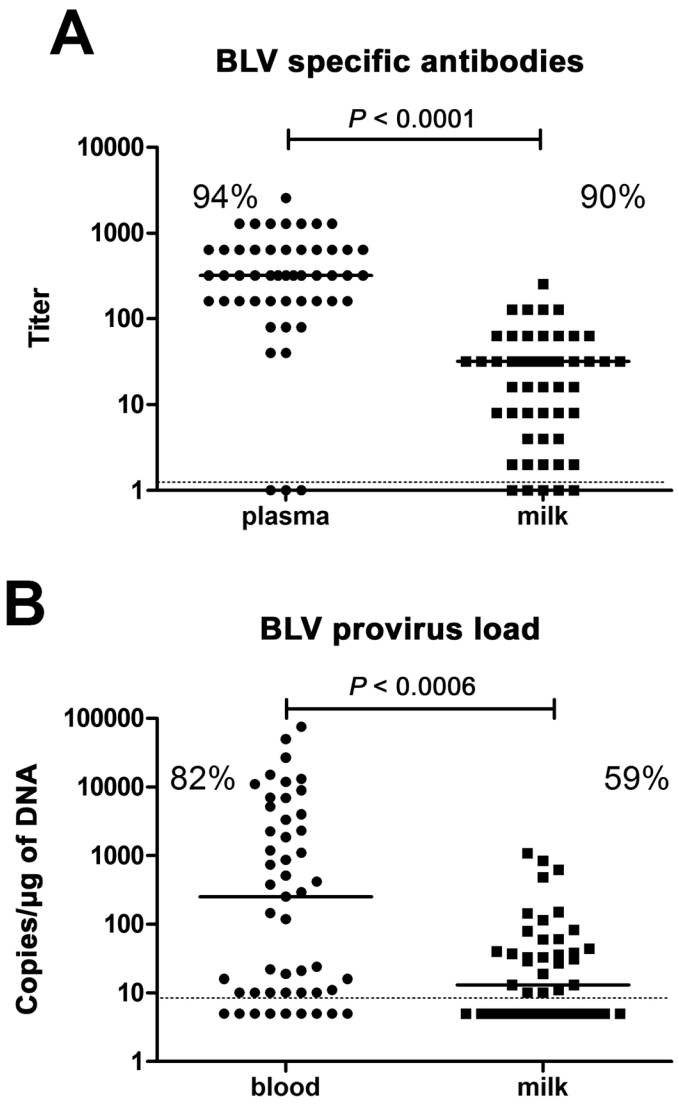

Figure 1. Comparison of bovine leukemia virus (BLV)-specific antibody titers and provirus load (PVL) in blood and milk. (A) BLV antibody titers and (B) PVL in cow's blood (or plasma) and milk samples were plotted. Horizontal solid bars represent median values. The *P*-value is indicated (Mann-Whitney U test). The proportion of positive samples is stated as a percentage in each graph. The BLV antibody titers were assayed by ELISA and PVL by real-time PCR; the horizontal dotted line shows the limit of detection for each assay. Fifty plasma and milk samples were used for the BLV antibody analysis. Forty-nine blood samples and 46 milk samples were used for the BLV and PVL analyses.

low (Ab titer <32; n = 20) and (2) high (Ab titer ≥32; n = 26), and the PVL was compared between them (Figure 2F). This arbitrary categorization was made based on the median value of milk Ab titers distribution (Figure 1A; median Ab titer = 32). In agreement with the previous result, this complementary analysis showed that milk PVL was significantly higher in milk samples containing lower titers of Ab (Mann Whitney U-test, $P = 0.03$).

The role of mammary gland secretions—colostrum and milk—in natural transmission of BLV to the offspring remains undefined. Different studies that have been published to date leave unclear whether these secretions primarily play a protective or an infective role (Ferrer and Piper, 1981; Van Der Maaten et al., 1981; Lassauzet et al., 1989; Lairmore, 2014). Some studies linked the presence of BLV virus or provirus in colostrum to a higher risk of transmission (Sprecher et al., 1991; Meas et al., 2002). In contrast, another group of studies observed a protective role of specific Ab present in these secretions (Van Der Maaten et al., 1981; Nagy et al., 2007; Kobayashi et al., 2010). Studies in HIV and simian-human immunodeficiency virus, 2 related retroviruses, have shown that a delicate balance between amount and quality of Ab and virus might affect both protection and transmission (Richardson et al., 2003; Barin et al., 2006; Dickover et al., 2006; Lynch et al., 2011). Although the mechanisms that favor or block transmission remain to be elucidated, it has been demonstrated that extending the duration of lactation was associated with a higher risk of perinatal transmission of HIV (Miotti et al., 1999).

In a previous study, we showed that colostrum with different levels of BLV Ab shed BLV provirus and highlighted the potential risk in colostrum containing high PVL and low levels of Ab (Gutiérrez et al., 2015). Continuing with the study of BLV natural transmission, the goal of the present study was to analyze the relationship between the levels of Ab and PVL in blood and milk of cows from a naturally infected herd.

The samples for the present study were collected from a dairy herd with a serologic prevalence of 94%. This prevalence was in agreement with recent studies that showed an overall seroprevalence of 86.5 and 90%, respectively, considering cows with more than one lactation and from different herds (Gutiérrez et al., 2011, 2012).

As expected, a positive correlation was observed between blood PVL and the level of Ab in plasma and milk. The reason for this might be the higher antigenic stimulation that occurred in those animals with higher PVL. Although the proportion of BLV Ab in milk and plasma samples was similar (90 vs. 94%, respectively), the level of Ab in milk was more than 10 fold lower compared with plasma. It is known that the active secretion of IgG to the lumen of the mammary gland that takes place during colostrum production is reduced during milk production (Tizard, 2013).

Milk PVL was significantly lower than blood PVL, and no correlation to PVL was noted between these body compartments. In addition, milk Ab titers negatively correlated with milk PVL. When milk samples were divided into low and high Ab titer groups, a significant increase was observed in milk PVL in the low group compared with the high group. The variation of the level of Ab and PVL and the different relationship between both variables in milk compared with blood could be due to different factors. First, It might be possible for some components present in the milk (i.e., proteases, complex polysaccharides, lipids, Ca^{2+} ions) to interfere with the DNA extraction and qPCR reaction, decreasing the sensitivity of the assay compared with blood. The difficulty associated with the measurement of BLV provirus in milk samples has been previously reported (Kuckleburg et al., 2003; Logar et al., 2012). If this was true, milk PVL data might be biased; however, we did not observe any interference when the limit of detection of the BLV qPCR was assessed using a milk matrix. Second, another explanation for these differences could be the presence of fewer B-lymphocytes in milk compared with blood (Reber et al., 2005). Therefore, fewer target cells containing integrated BLV provirus would found, making it more difficult to detect B-lymphocytes in milk. The migration of B-cells and the secretion of Ab from blood to peripheral tissues are 2 independent mechanisms. Hence, the levels of these immune components in the milk do not necessarily mirror what is observed in the blood. In addition, any change in the expression of adhesion molecules and cytokines in persistently infected B-cells could affect the migration of B-cells carrying provirus to peripheral tissues. Alternatively, BLV Ab in milk could accelerate the clearance of infected B-cells in the mammary gland compared with blood (i.e., favoring the phagocytosis by macrophages through opsonization). Further studies are granted to explore the dissemination of BLV and specific Abs among different body compartments during the natural infection.

Although the different hypotheses for the negative correlation between milk antibody titers and milk PVL remain to be explored, it is important to highlight that the ingestion of raw milk containing provirus or free virus particles and low levels of Ab could favor BLV transmission to calves. A similar situation has been reported in other infections caused by retroviruses (Kinoshita et al., 1984; Miotti et al., 1999; Li et al.,

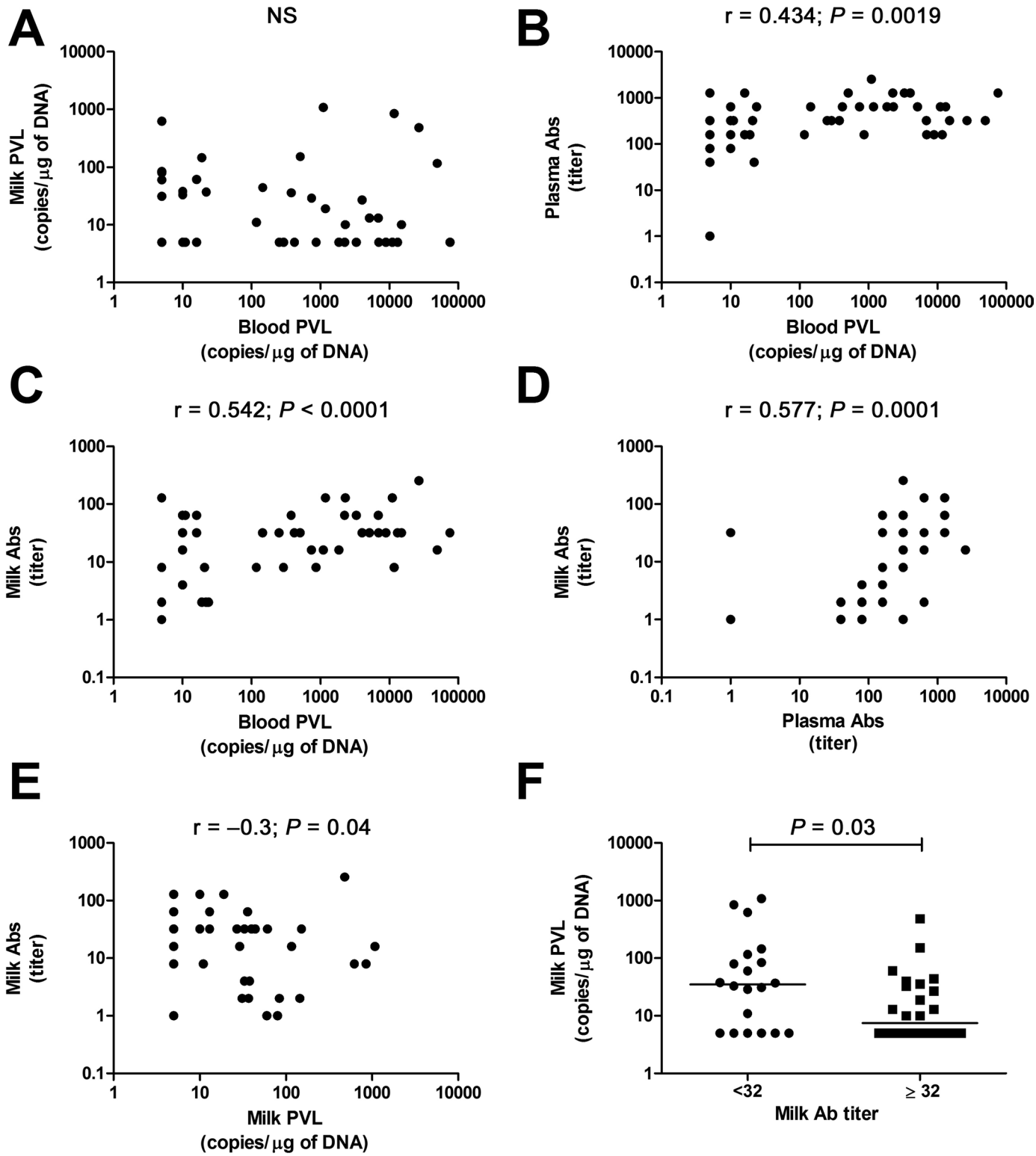

Figure 2. Relationship between bovine leukemia virus (BLV) provirus load (PVL) and antibody titers in blood, plasma, and milk. Correlation between (A) milk PVL and blood PVL (X,Y pairs = 45); (B) plasma antibody titers and blood PVL (X,Y pairs = 49); (C) milk antibody titers and blood PVL (X,Y pairs = 49); (D) milk and plasma antibody titers (X,Y pairs = 50); and (E) milk antibody titers and milk PVL (X,Y pairs = 46). Spearman correlation was used, and correlation coefficient and *P*-values are given. (F) Milk samples were grouped as low (titer <32; n = 20) and high (titer ≥32; n = 26) according to the level of antibodies and PVL in these milk samples were plotted. Horizontal solid bars represent median values. The *P*-value is indicated (Mann-Whitney U test).

2004; Martin-Latil et al., 2012; Milligan and Overbaugh, 2014). Moreover, this could explain the high prevalence observed in heifers in Argentina before they were in contact with older animals (Gutiérrez et al., 2014) and when risk factors associated with horizontal transmission were controlled.

Performing pasteurization or freeze and thaw cycles to bulk milk before it is administered to calves might help to the prevention of early BLV infections. In the long term, this could limit the spread of the disease under natural settings. An interesting alternative would be the passive immunization of calves using BLV-specific Ab, as they might be able to block infection. Even if protection was not achieved, passively administered Ab could boost calves' endogenous immune responses, as reported for other retroviruses (Jaworski et al., 2013). Further studies are needed to explore if the levels of Ab and PVL in individual and bulk milk are associated with the risk of perinatal transmission of BLV.

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