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THE INFECTION HAZARD OF CARRIERS OF PROVIRAL BOVINE LEUKEMIA VIRUS AND ITS EVALUATION WITH REGARD TO LEUKOCYTOSIS

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Abstract

The spread of the bovine leukemia virus (BLV) brings significant economic damage to dairy and beef cattle still due to the problems to develop the optimal methods for its prevention. The situation is compounded by the fact that the clinical manifestation of lymphocytic leukemia has been observed in animals during 5 to 10 years after infection in approximately 5-7 % of animals. Subdivision of BLV infected B cells on the producers of mature viral particles and precursors of the formation of lymphomas necessitates of separately consideration of the risk of infecting animals and forecast for development of lymphocytic leukemia. Testing antibodies to viral antigens, or provirus DNA insertion into the host genomes does not allow to reveal the most dangerous animals in view of their role in the infection spread, which is most essential for its control. In this regard, the particular urgency is the method development to assess the infection hazard from carriers of provirus DNA of bovine leukemia virus, associated with a large number of B lymphocytes, producing mature viral particles. In order to evaluate the possibility of using in these purposes not only testing individual animals on the quantity of viral RNA in blood samples, but the proliferative activity of leucocytes, these two characteristics were compared in the present work. The presence or absence of genome integrated provirus DNA in Black-and-White Holstein cows ($n = 57$, commercial herd) was evaluated and the group of infected animals was revealed when using primers designed by us for BLV *gag* and *pol* genes (G.Yu. Kosovskii et al., 2013). The relative quantity expression of provirus in individual infected cows using RT-PCR analysis and primers to the fragment of the BLV *pol* gene were assessed. The counting of leukocytes in blood samples of all cows, included in the analysis, was performed. The number of leucocytes in cows free from infection, with the exception of one cow, was less than 12×10^9 cells/l. This indicator in infected animals divided the investigated cows into two subgroups — in the first subgroup the leucocyte values did not exceed 17×10^9 cells/l, in the second subgroup the number of leukocytes was significantly higher, reaching 28×10^9 cells/l in some animals. It was important to note, that the amount (above 20×10^9 cells/l) of BLV RNA, sufficient to detect by RT-PCR, was revealed only in the second subgroup. The coincidence of the increased leucocyte amount and the BLV RNA in blood of infected animals indicates that particular these cows represent the greatest infection hazard and suggests that the combination of estimates of the number of leukocytes and RNA BLV can be quite a reliable approach for the herd recovery by priority of their liquidation.

Keywords: bovine leukemia virus, BLV, RT-PCR, provirus DNA, *pol* gene, leukocytosis, the infection hazard

Bovine leukemia virus (BLV) belongs to the *Deltaretrovirus* genus of the family *Retroviridae* (*Orthoretrovirinae* subfamily). It is phylogenetically related to human T-lymphotropic virus type 1 (HTLV-1) [1]. HTLV-1 affects CD4+ human T-cells, while BLV infects B cells in cattle, and both viruses integrate their genome copies as a provirus DNA into the host genomes [2, 3]. In most BLV-infected animals no clinical manifestations are observed, however in approximately 5-7 % of animals an increased B cell proliferation occurs during 5 to 10

years after infection resulting in B cell lymphocytic leukemia. The spread of the bovine leukemia virus brings significant economic damage to dairy and beef cattle [4] still due to the problems to develop the optimal methods for animal immunization. Presumably, the problems arising in anti-BLV vaccination are a result of complex influence of the expressed provirus DNA genes on the host immune system [5]. Besides, the strategy for cattle herd sanitation remains disputable since all the animals possessing anti-BLV antibodies or provirus insertion in the genomes are still subjected to elimination without exact identification of the individuals which are dangerous with regard to infection hazard as such.

BLV provirus DNA can be found in less than 1 % peripheral blood cells of BLV-infected cattle [6]. During primary infection, the BLV provirus DNA is massively inserted into transcriptionally active sites of the host genomes, but only some resultant cell clones can further keep the provirus DNA integrated, and only few of them will become malignant [7]. Mass depletion of the cell pull with integrated BLV provirus DNA occurs during infection [6, 7] due to immune response to viral antigens. Besides, BLV provirus location in transcriptionally active sites may gravely affect cell functions, when insertions into regulatory or coding sequences of key structural genes [8].

Thus, natural selection generally suppresses the cells actively expressing BLV provirus. Additionally, there are two subpopulations of the BLV-infected B cells, with and without active expression of IgMs and BLV provirus. Presumably, it is the second population which can form lymphomas as no provirus DNA expression is characteristic of tumor cells [8]. Recent accumulated data attest to the fact that the expression of BLV provirus microRNA found in the lymphocytes not expressing full BLV genome may be key factor to initiate neoplastic transformation of B cells [9-11].

Subdivision of BLV-infected B cells on the producers of mature viral particles and precursors of lymphomas necessitates separate consideration of the risk of infecting animals and development of lymphocytic leukemia [12]. Importantly, current methods of testing antibodies to viral antigens, or provirus DNA in the host genomes do not allow revealing the most dangerous animals in view of their role in the infection spread, which is most essential for its control. Testing total viral RNA in animal blood could be an approach but it is unreliable because of RNA pool lability.

Here, in view to find additional parameters for assessing infection hazard among BLV carriers, we compared expression of provirus DNA, as estimated quantitatively in RT-PCR with primers to the fragment of the BLV *pol* gene, and the counts of blood B lymphocytes. The BLV-infected animals were pre-revealed using primers designed by us for BLV *gag* and *pol* genes to detect integrated provirus DNA [13].

Technique. Blood samples were collected from jugular vein of 2-5-year-old Black-and-White Holstein cows ($n = 57$, commercial herd, Moscow Province).

DNA was isolated from whole blood samples using M-Sorb kit for clinical tests (Syntol ZAO, Russia) according to manufacturer's protocol. Total RNA was extracted from whole blood using ExtractRNA kit (Eurogen, Russia) as to instruction attached. The first cDNA chain was synthesized with MMLV RT kit (Eurogen, Russia) as recommended by the manufacturer. cDNA quality was assessed by amplification of gene encoding ribosomal protein RPLPO. The amplification was performed in 20 μ l of PCR mixture containing 1 $\times$ amplification buffer, 3 mM MgCl₂, dNTPs (0.2 mM each), 1 unit of HS Taq DNA polymerase (Eurogen, Russia), and the pair of primers (0.2 μ M each). Pairs of primers were as follows: for *pol* — 5'-GCAGGCCGATATA-ACCCAT-3' and 5'-TGCTGGCAAAACCTGACAAAG-3', for *RPLPO* — 5'-

CAACCCTGAAGTGCTTGACAT-3' and 5'-CAGATGGATCAGCCAAGA-AG-3'. For amplification, denaturation at 95 °C for 15 sec, annealing at 60 °C for 15 sec, elongation at 72 °C for 15 sec (40 cycles) were performed. PCR fragments were separated by 1.2 % agarose gel electrophoresis.

Comparative expression of *pol* gene was estimated quantitatively in real time qPCR on a LightCycler 480 (Roche, Switzerland) with SYBR Green intercalating dye and qPCRMix-HS SYBR kit (Eurogen, Russia). The presence of genome DNA in cDNA preparation was controlled for more precise characterization of gene expression. A fragment of gene encoding RPLPO ribosomal protein was an internal control.

Lymphocytes were counted for each individual in fresh EDTA-stabilized whole blood aliquot (100 µl) on a Coulter-method based hematological analyzer Abacus junior Vet5 (Diatron, Austria).

Results. Designed primers [13] allowed us to reveal 35 cows free from BLV infection and 22 cows with integrated BLV provirus DNA among 57 individuals tested (Fig. 1, A).

Fig. 1. Bovine leukemia virus (BLV) infection rate among Black-and-White Holstein cows (A) and detection of BLV provirus DNA transcription in infected individuals (B): 1 and 2 — infected and not infected cows, respectively; 3 and 4 — active BLV provirus transcription and no expression, respectively ($n = 57$, commercial herd, Moscow Province).

To evaluate BLV provirus DNA transcription, the cDNA synthesized from total blood cell RNA was normalized against ribosomal protein RPLPO gene. The *pol* gene expression was not detected in cows free from BLV infection but found in 27 % animals with integrated BLV provirus DNA (see Fig. 1, B).

For the cows free from BLV infection, as assessed using specific primers [13], the leucocyte counts varied from 3×10^9 to 18×10^9 cells/l (Fig. 2, A). In this, infected animals were divided into two subgroups. In the first subgroup the leucocyte counts were from 7×10^9 to 17×10^9 cells/l (as the upper limit for most individuals in the subgroup), and in the second subgroup this parameter ranged from $> 20 \times 10^9$ cells/l to 28×10^9 cells/l.

Fig. 2. Blood lymphocyte counts in Black-and-White Holstein cows free from Bovine leukemia virus (BLV) (A, $n = 35$) and BLV-infected individuals (B, $n = 22$) ($n = 57$, commercial herd, Moscow Province).

High expression of the BLV gene *pol* was found in each of six cows from the second subgroup, with no exception, whereas no expression of *pol* could be detected in the first subgroup even in the presence of integrated BLV proviral DNA in animal genomes, which is consistent with a relatively low number of leukocytes in blood samples, compared with the corresponding values in the second subgroup. RT-PCR (Fig. 3) indicated relatively high expression of the pro-

viral DNA in all animals with leukocytosis.

Fig. 3. RT-PCR analysis of Bovine leukemia virus (BLV) *pol* gene expression in BLV-infected subgroup of Black-and-White Holstein cows with the manifestation of leukocytosis ($n = 6$): 1 — 7412, 2 — 7575, 3 — 7652, 4 — 7243, 5 — 7722, 6 — 7345 (the individuals' numbers indicated; commercial herd, Moscow Province). Relative expression of the *pol* gene is indicated as an excess of its amplification product comparing to that produced for reference RPLPO ribosomal protein gene from total RNA in the sample.

Note that the currently accepted stringent regulations for cattle herd sanitation is based mainly on serological tests such as immunodiffusion (RID), or ELISA, i.e. on identification of antibodies produced

in response to contact with a pathogen (usually with BLV *env* gene products). Env gp51 glycoprotein plays an important role in the BLV life cycle, it is necessary for viral entry into the cell and serves the main target for neutralizing antibodies [14].

However BLV diagnosis based on detection of antibodies in virus-infected animals is problematic. Importantly, successful identification of BLV-specific antigens in cattle may be not earlier than in the six-month-old individuals. Besides, antibody response depends on the number of cell clones producing mature viral particles, and, accordingly, on representation of the envelope glycoprotein antigens and their structure. For example, a single mutation affecting N-glycosylation site of surface glycoprotein can result in a sharp increase in BLV pathogenicity. The latter is regarded as evidence for host-pathogen coevolution, which prevents the growth of pathogenicity above a certain limit [15]. No correlation occurs between the kinetics of viral replication and animal immunoreactivity, and BLV can infect not only B-lymphocytes, but other cell populations too [16]. In some cases, no synthesis of the corresponding antibodies is revealed in BLV-infected animals, despite the presence of the integrated proviral DNA [17]. BLV infection is shown to inhibit the regulatory function of T-lymphocytes subpopulation ($CD4^+CD25^{high}Foxp3^+$) resulting in suppression of antiviral activity, particularly in NK-cells [18].

In recent years, PCR is commonly used to identify carriers of retroviral infections by detection of proviral DNA integrated into the host genome. In this, conservative fragments of proviral DNA used as PCR primers, and the lack of their homology to the host genome are the crucial points. However, the mutation rate of retroviruses is rather high reaching 10^{-3} - 10^{-5} per nucleotide in a transcription cycle [19]. Consequently, complications of the existing methods for retrovirus-infected animal identification based on the diagnosis of the of proviral DNA integration into the host genome are due to high volatility of provirus genes and overloading mammalian genomes with endogenous retroviruses and their fragments, since the primers should be chosen to fit proviral DNA portions with inadequate understanding their spontaneous mutability and predisposition to cross-hybridization with dispersed repeats in the host genome. Additionally, the major histocompatibility complex (MHC) class II genotype impacts significantly on the infectious hazard of BLV provirus carriers, as well as on their risk of leukemia. Thus, BLV-infected *BoLA-DRB3*0902* cows incorporated into a BLV-negative dairy herd did not cause infection of the latter for a long time. The infected individuals were positive for BLV antibodies, though in low titers,

and contained BLV proviral DNA in the genome [20].

Thus, current methods for identification of infected animal based on the detection of antibodies to glycoprotein encoded by BLV gene *env*, or integrated BLV proviral DNA, only point out at animal contact with the pathogen, but in general, do not allow to predict an infectious risk or probability of leukemia in the individual.

A persistent leukocytosis associated with BLV-induced increase in proliferation of B-lymphocytes is an additional functional indicator for BLV-infected animal predisposition to leukemia. Blood lymphocyte counts above 10000×10^9 cells/l in adult animals are considered as a preneoplastic state [21]. However, leukocytosis as such is a nonspecific indicator as it is characteristic of a number of noninfectious and infectious diseases associated with underlying disease, particularly in BLV-infected animals [22, 23].

It should be noted that in our study an exceeding blood lymphocyte counts were observed in 19 of 35 cows free from BLV infection according to our data (see Fig. 2, A), whereas in 6 of 22 BLV-infected individuals the number of white blood cells did not exceed the threshold value of 10000×10^9 cells/l (see Fig. 2, B). That is a leukocytosis itself does not specifically indicate BLV infection. It is known that a possession of *BoLA DRB3.2* allelic variants, associated with a relatively high resistance to BLV infection, does not prevent infection by other pathogens, which, in turn, may lead to increased leukocyte count [24].

Consequently, assessment of the BLV proteins antibodies, proviral DNA integration or animal leukocytosis, when used alone, does not allow to reveal the most infectious individuals. Obviously, this complicates herd recovery, but does not exclude elimination of BLV-infected animals from valuable breeding stocks, when no clear threat of spreading infection. Our thought is that the combination of these functional parameters (i.e., relative amount of viral RNA and the level of the peripheral blood leukocytes) seems to be most appropriate in view of identification and founded elimination of infectious animals. BLV RNA levels in peripheral blood, as assayed by qPCR, can be used to estimate the size of the cell population actively producing mature viral particles.

Thus, we found high expression of Bovine leukemia virus (BLV) proviral DNA only in cows with severe leukocytosis: the highest levels of BLV RNA and leukocyte proliferation were characteristic of these animals (unlike the others). It can be expected that the combination of these characteristics will allow to identify animals which are spreading BLV infection and, as the most dangerous, must be removed to recover the herds.

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