Application of *in situ* PCR for the detection of bovine leukaemia virus (BLV) infection in dendritic cell cultures

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Abstract

The aim of the study was to develop an *in situ* PCR (IS-PCR) method for detection of bovine leukemia virus (BLV) in cell cultures. Samples from five BLV positive and five BLV negative cows were collected and dendritic cells (DCs) from blood, bone marrow, spleen, and lymph node were cultured. Cultures prepared from healthy animals were infected with BLV. After two weeks, the cells were tested by nested PCR and IS-PCR for the presence of proviral DNA. As a positive control adherent cell line permanently infected with BLV was used. BLV was successfully detected by IS-PCR in DCs from naturally infected cattle and DCs infected *in vitro*. In control, non-infected DCs, the results of the reaction were negative. The results of provirus detection by IS-PCR were similar with those performed with nested PCR. Additionally, IS-PCR provides many advantages, like specific localisation of infection and smaller number of cells needed as template for PCR.

**Keywords:** bovine leukaemia virus, dendritic cells, *in situ* PCR.

Introduction

Bovine leukaemia virus (BLV) with human and simian T-lymphotropic viruses (HTLV-1, HTLV-2, STLV) belong to the *Retroviridae* family, genus *Deltaretrovirus*. BLV is an aetiological agent of lymphoproliferative disease, enzootic bovine leukosis (EBL). EBL is a chronic infectious disease of the lymphoreticular system. The disease results in impairment of the immune system and tumorous alterations in internal organs (11). Under natural conditions, BLV is infectious for beef and dairy cattle, but also experimental infection of other species including sheep, goat, rat, and rabbit is possible (4). No cases of BLV infection in humans were confirmed. Virus spreads horizontally among animals in a herd, through a direct contact with infected blood. Infection may also occur through milk, semen, insect bites, and veterinary procedures (4).

The target cells for BLV are B-lymphocytes but the virus is also able to infect other cell types, *i.e.* T-lymphocytes, monocytes, macrophages, and dendritic cells (DCs) (6, 21). DCs are professional antigen-presenting cells (APCs). Immature DCs are also called veiled cells because they have a large cytoplasmic “veil” (22). They have unique capacity to take up, process, and present antigen to naïve T-lymphocytes in the immune system. DCs also initiate and modulate primary immune response, stimulate differentiation of B-lymphocytes, and take part in innate immunity against pathogens, as well as in induction of peripheral immunological tolerance (2, 5). Depending on lineages and maturation state, DCs may control a diverse function in immune regulation (15). DCs are constantly produced from haematopoietic stem cells (HSCs) in bone marrow and distributed in small amounts throughout the organism (blood, thymus, tonsils, spleen, skin, and synovial fluid) (22, 24). There are two separate lineages of DCs: myeloid (mDCs) and plasmacytoid (pDCs) (16, 26). For the population of mDCs, surface BDCA-1/3 markers are characteristic and for the population of pDCs, BDCA-2/4 markers are specific (7-9, 18). Precursors of human mDCs and pDCs are mainly present in blood, bone marrow, and lymphoid tissue. The CD14+ monocytes are an accessible source of DC precursors for isolation and *in vitro* culturing. These cells are able to differentiate to myeloid DCs *in vitro* after stimulation with granulocyte-monocyte colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) (19).
DCs are the most efficient primary activators of naïve T cells from all antigen presenting cells. They can also be administered in situ to prime T cells without additional adjuvants (24). Because of these unique immunostimulatory properties, there is a considerable interest in the development of DC-based vaccines (10). DCs also constitute very attractive potential therapeutic target for immunotherapy of virus-associated diseases and cancer treatments (10). Application of in vitro cultures provides an interesting model of leukaemogenesis and allows for detailed studies of changes within cells in the course of BLV infection.

In situ PCR is a molecular technique that combines sensitivity of traditional PCR and anatomical localisation of in situ hybridisation (16). During this process, cells or tissues are fixed and permeabilised, then specific nucleic sequences are amplified, and PCR products are visualised through immunohistochemical reaction. In situ PCR could be an excellent technique for the detection of BLV infections in cultures of adhesive DCs. This technique allows for direct signal localisation, without necessity of DNA extraction (17) and is more sensitive than other in situ techniques because of addition of PCR reaction.

The aim of the study was to develop in situ PCR for the detection of proviral DNA of BLV in DCs from naturally infected cattle and DCs infected in vitro.

Material and Methods

Animals. Experiments were performed on the group of cows of Polish Black-and-White Lowland breed aged four-seven years. Blood, bone marrow, spleen, and lymph node samples were collected from five BLV infected cows and from five healthy animals. Blood samples were collected from jugular vein to the tubes containing EDTA/K$_2$ (POCH) as anticoagulant. The presence of BLV antibodies was detected with the use of a commercial ELISA kit (Pourquier). The presence of proviral DNA was determined in blood samples by nested PCR. In nested PCR, DNA from FLK-BLV cell line was used as positive control (20).

Cell preparation and culture. Peripheral blood leukocytes and cell suspensions from bone marrow, spleen, and lymph nodes were layered on the density gradient (Histopaque 1.077, Sigma) and centrifuged for 45 min at 1000 x g at 8°C. Cells from interphase were collected, washed with PBS, and counted. For magnetic isolation, 10$^7$ cells were used. The cells were suspended in isolation buffer (PBS, 0.5% calf serum, pH 7.2; Sigma) and incubated for 15 min at 8°C with 20 µL of human CD14 MicroBeads (Miltenyi Biotec). After incubation, the cells were washed and centrifuged at 300 x g for 10 min, and again resuspended. Magnetic separations were performed on LS Columns (Miltenyi Biotec), according to the manufacturer’s instruction.

CD14+ monocytes were collected and cultured in RPMI 1640 (Gibco) medium with 20% calf serum (Sigma), 0.3 mg/mL of L-glutamine (Sigma), antibiotic-antimycotic solution (Sigma) in dilution 1:100, GM-CSF (Bio-Rad/Serotec) in dilution 1:500, and 10 pg/mL of IL-4 (Endogen). The cells were cultured in atmosphere of 5% CO$_2$ at 37°C in 8-well Lab-Tek chamber slides (Becton Dickinson) until the optimal covering of growth surface was achieved. DC cultures from BLV-free animals were infected with BLV using cell-free (culture medium) supernatant from FLK-BLV cell culture. After two weeks of incubation DCs were harvested.

Nested PCR. The presence of BLV proviral DNA in DC cultures was confirmed by nested PCR. DNA was isolated from 10$^6$ cultured DCs using DNeasy Blood and Tissue kit (Qiagen) according to manufacturer’s recommendations. Amplification reaction was performed in a volume of 50 µL in the UNO II Termoblock (Biometra, Germany). The 0.5 µg of genomic DNA was added to the mixture containing: 10 mM Tris-HCl (pH 8.8; at 25°C); 50 mM KCl; 2 mM MgCl2; 0.1% Triton X-100; 200 µM of dNTPs; 0.5 U DyNAzyme II polymerase (Finnzymes Oy); 0.2 µM of primers: ZM2; CTGTGATGGCTAAGGGCAGACA CGGC and ZM3; CTTCCCCTCCTGGGCTCCCGAAGA. The conditions of reaction were as follows: 5 min at 95°C, 30 cycles at 94°C for 30 s, combined annealing and extension step at 70°C for 1 min, and 7 min extension step at 72°C. The second amplification was performed using 5 µL of the first reaction mixture as template and the pair of internal primers ZM4 CTGCCTCTCCGGAGCGCCA (0.2 µM) and GTCAGGCTAAAGGTAGGCGCC ZM5 (0.2 µM). Other reaction conditions including thermal cycling were as described above. PCR products were analysed under UV light after the electrophoresis in 1.5% agarose gel stained with ethidium bromide. As positive control DNA from FLK-BLV cell line was used.

In situ PCR. Adhesive cultures of DCs were fixed in 40% acetone in PBS at -20°C and air dried. The cells were then treated with proteinase K solution (10 µg/mL, Sigma) for 10 min at 37°C, incubated for 15 min in 0.4% formalin solution, briefly rinsed in distilled water, and air dried. In situ PCR was performed in chamber slides sealed with heat-resistant adhesive tape (Bionovo) in volume of 150 µL. Reaction mixture contained: 10 mM Tris-HCl pH 8.8 at 25°C; 50 mM KCl; 2 mM MgCl2; 0.1% Triton X-100; 200 µM of dATP; 200 µM of dCTP; 200 µM of dGTP; 190 µM of dTTP; 10 µM of digoxigenin-11-dUTP (DIG-11-dUTP; Roche); 2 U of DyNAzyme II polymerase (Finnzymes Oy), and 0.2 µM of ZM2 and ZM3 env primers. Thermal conditions of reaction were as follows: 95°C for 5 min, 35 cycles of 94°C for 1 min, 68°C for 1 min, 72°C for 1 min, and final elongation step for 7 min at 72°C. The amplification products were detected by colorimetric-based assay. Specimens were
washed twice with TBST (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20). To each chamber, 150 µL of 1:100 diluted conjugate anti-DIG-AP (antidigoxigenin with alkaline phosphatase; Roche) in TBS (100 mM Tris-HCl, pH 7.5; 150 mM NaCl) with 1% blocking reagent (Roche) were added. After 1 h incubation at 37°C, the specimens were washed twice in TBST and once in AP buffer (100 mM Tris-HCl, pH 9.5; 150 mM NaCl, 50 mM MgCl₂; 1 mM levamisole; 0.1% Tween 20). Then 150 µL of substrate solution, containing 0.38% X-phosphate (Roche) and 0.5% NBT (Roche) in AP buffer was added and 1 h incubation in the dark was performed. Substrate solutions were discarded and slides were removed from chambers. The slides were briefly rinsed in redistilled water, air dried, and analysed under microscope (Olympus). FLK-BLV cells were used as positive control. Adhesive cultures of DCs of BLV-free cows were used as negative control.

Results

Nested PCR was used to confirm the presence of BLV proviral DNA in DC cultures from naturally infected cows and in vitro infected DC cultures. Expected length of the product was 218 bp. Positive results were observed in all DC samples from naturally infected animals. Proviral DNA of BLV was also present in all in vitro infected DC cultures from blood, bone marrow, spleen, and lymph node. In DC cultures of BLV-free animals, results of nested PCR were negative. The results are shown in Fig. 1.

![Fig. 1. Agar gel electrophoresis of 218 bp BLV env gen fragment. L – 50 bp DNA Ladder (Fermentas), 1 – negative control, 2 – positive control (FLK-BLV); DC cultures from naturally infected cows: 3 - lymph node, 4 - spleen, 5 - bone marrow, 6 - blood; DC cultures from BLV-free animals: 7 - lymph node, 8 - spleen, 9 - bone marrow, 10 - blood, DC cultures from BLV-free cows two weeks after in vitro BLV infection: 11 - lymph node, 12 - spleen, 13 - bone marrow, 14 – blood](image)

To confirm the data obtained by nested PCR, in situ PCR, as the most appropriate technique, was used in all DC cultures. Positive result of in situ amplification is seen as very characteristic purple-violet precipitate localised in the nuclei of infected DC's. FLK-BLV adhesive cultures were used as positive control (Fig. 2).

![Fig. 2. 1 - positive control of adhesive FLK-BLV culture, 2 - control reaction for FLK-BLV (with unlabelled dNTP's)](image)

Positive results of in situ PCR were observed in all DC cultures obtained from blood, bone marrow, spleen, and lymph node of naturally infected animals. Fig. 3 presents results similar as in the case of nested PCR. Control reactions contained unlabelled dNTP's instead of DIG-labelled dNTP's.

The positive results of in vitro infection were observed in DC cultures originating from blood, bone marrow, lymph node, and spleen. However, intensity of signals was weaker in spleen DC cultures infected in vitro than in DCs isolated from spleen of naturally infected cows. To avoid false negative results in the detection of BLV proviral DNA in DCs infected in vitro, two types of control reactions were performed: in situ amplification using non-labelled dNTP's and in vitro infected cells, and in situ amplification using DIG-labelled dNTP's and DC cultures before BLV infection. The results are shown in Fig. 4.

The results of nested PCR and in situ PCR used for the detection of BLV proviral DNA in DC cultures are presented in Table 1. BLV provirus was detected successfully by nested PCR and in situ PCR in all examined samples of DCs from all tissue types of naturally infected animals. The same results were obtained from group of DCs infected in vitro. Moreover, proviral DNA of BLV was detectable in those cells two weeks post infection. In control group of non-infected DCs, results of nested PCR and in situ PCR were negative in all samples. The results are identical regardless of the detection method and type of DC cultures.
**Fig. 3.** Results of BLV detection by *in situ* PCR in adhesive DC cultures from naturally infected animals: 1 - lymph node, 2 - spleen, 3 - bone marrow, 4 - blood; control reactions with unlabelled dNTP's: 5 - lymph node, 6 - spleen, 7 - bone marrow, 8 - blood

**Fig. 4.** Results of BLV detection by *in situ* PCR in adhesive DC cultures infected *in vitro*: 1 - lymph node, 2 - spleen, 3 - bone marrow, 4 - blood; control reaction with unlabelled dNTP's: 5 - lymph node, 6 - spleen, 7 - bone marrow, 8 - blood. Negative control with DC cultures before viral infection: 9 - lymph node, 10 - spleen, 11 - bone marrow, 12 - blood

**Table 1.** Comparison of BLV detection results in DCs by *in situ* PCR and nested PCR

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Type of DCs</th>
<th>Nested PCR</th>
<th>PCR in situ</th>
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<tr>
<td>Blood</td>
<td>Naturally infected</td>
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<td></td>
<td><em>In vitro</em> infected</td>
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<td></td>
<td>Non-infected</td>
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<tr>
<td></td>
<td>Naturally infected</td>
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<tr>
<td></td>
<td><em>In vitro</em> infected</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
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<tr>
<td></td>
<td>Non-infected</td>
<td>- - - - - - - - - -</td>
<td>- - - - - - - - - -</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Naturally infected</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
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<tr>
<td></td>
<td><em>In vitro</em> infected</td>
<td>+ + + + + + + + + +</td>
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<tr>
<td></td>
<td>Non-infected</td>
<td>- - - - - - - - - -</td>
<td>- - - - - - - - - -</td>
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<tr>
<td>Spleen</td>
<td>Naturally infected</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
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<tr>
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<tr>
<td></td>
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<td></td>
<td>Non-infected</td>
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As it can be seen in Table 1, BLV was detected in all examined samples of DCs from naturally infected animals and DCs infected in vitro by both methods, regardless of tissue type. In control group, the results of both methods were negative in all samples from blood, bone marrow, spleen and lymph node.

**Discussion**

Dendritic cells play an important role in the immunity and infection. Their main function is to process antigens and present them on the surface to other cells of the immune system. They act as messengers between the innate and adaptive immunity. Because the role of DCs in BLV pathogenesis is poorly understood, it is interesting to study their biology in vitro and in vivo. Due to the fact that some viruses infect cells of the immune system latently their detection in the newly infected hosts or cell cultures by traditional methods may be problematic (14). These problems may be overcome by PCR (6, 12). Solution phase PCR (SP PCR) has been successfully applied for the detection of BLV infections in tissues and blood of infected animals, as well as in cell cultures (12). It is a very useful technique for the detection of replicating viruses in cells infected in vitro. To perform SP PCR, a large number of cells is required for DNA extraction. Considering the fact that DCs and their precursors are available only in limited number in the body, it is necessary to maintain them in cell culture. To detect viral infection of DCs, the efficient methods must be used. SP-PCR and its potential detection of low copy number viruses are enhanced significantly by reamplification of the product with internal primers.

*In situ* hybridisation (ISH) allows the detection of specific sequences directly in cells but it is not enough sensitive for the detection of low replicating viruses (6, 16). *In situ* PCR permits histological localisation of low copy of DNA and RNA targets because of their amplification before the detection (16, 17). *In situ* PCR was successfully used by Duncan et al. (6) for BLV detection in tissues. In this study, *in situ* PCR based on previously published protocols was adapted for detection of BLV sequences in DC cultures (6, 13). This method was chosen because of the limited number of DCs available and possibility of direct detection of BLV without DNA isolation.

Nuovo (16) showed that protease digestion is a critical step during *in situ* PCR to achieve reliable results of immunohistochemical detection. Different types of specimens and tissues have various resistance to proteolytic digestion. Every type of sample requires different conditions of permeabilisation to ensure successful experiment (16, 17). In this study, proteolytical treatment of DC cultures was optimised. The best results were obtained using 10 μg/mL of proteinase K for 10 min at 37°C. The use of longer incubation times caused a damage of the cells making them unsuitable for BLV proviral DNA detection.

Another factor that should be taken under consideration during *in situ* PCR is the appearance of background due to the non-target nucleic acid, which is defined as the presence of a signal in cells that do not contain the target of interest (17). The most important factors (17) leading to the appearance of the background are: probe concentration, hybridisation time, and the post-hybridisation wash conditions. In the present study, the optimal concentration of DIG-11-dUTP for DC specimen labelling during amplification step was 10 μM. Primers for *in situ* PCR and for the first reaction of nested PCR were used according to Kubis et al. (13). Reaction conditions described above were based on published reports on BLV detection in DCs, with some modifications (6, 13). To minimise the background occurring during signal detection, levamisole was added to inhibit alkaline phosphatase activity. The use of non-infected DC cultures as additional negative control confirmed the specificity of the reaction. In conclusion, the results of this study revealed that *in situ* PCR is an efficient method for the detection of BLV provirus in DC cultures infected *in vitro* and isolated from BLV positive animals.

**References**


