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Original article

Determination of telomere length and telomerase activity in cattle infected with bovine leukaemia virus (BLV)

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Abstract

Telomeres are repetitive sequence structures at the ends of chromosomes. They consist of the double stranded DNA repeats followed by the short single stranded DNA. In humans and other verterbrates the telomeric sequence is composed of tandem of TTAGGG repeats. With each cells division telomeres shorten by up to 200 base pairs. Telomerase is an enzyme responsible for continuous cell growth and is repressed in most somatic cells, except proliferating progenitor cells, but in more than 85% of cancer cells telomerase expression is observed. Tumour cells with metastatic potential may demonstrate a high telomerase activity, allowing cells to escape from the inhibition of cell proliferation due to shortened telomeres. Determination of telomerase expression was performed with the use of PCR ELISA in samples isolated from bovine leukaemia virus (BLV) infected cows. Telomerase activity was found in almost all investigated samples. The relative telomerase activity (RTA) was higher in infected cows than in healthy animals and the differences were statistically significant (α=0.05). In blood lymphocytes of BLV-infected cows the mean values of telomerase expression determined in real-time PCR were 3534.12 copies, in the healthy group there were 1010.10 copies and these differences were also statistically significant. For telomere length evaluation the Telomere PNA/FITC FISH and Telomere PNA/FITC FISH for flow cytometry were used. The mean fluorescence intensity of telomere sequences calculated on the surface of interphase nuclei of leukaemic blood lymphocytes was lower than that in the control animals and the difference was statistically significant. The mean length of telomeres in BLV- infected and healthy cows was 31.63 ± 12.62 and 38.4 ± 4.03, (p=0.112), respectively.

Key words: bovine leukaemia virus, telomerase, telomeres

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Introduction

Telomerase is a highly conserved enzyme and specialised cellular reverse transcriptase catalysing the synthesis and extension of telomeric DNA with its own template (Allsopp et al. 1992, Counter et al. 1995). It was discovered by Greider and Blackburn in 1985. Telomerase compensates the telomere loss due to the end-replication problems (Blackburn et al. 1997). Normally, in healthy organisms it is expressed in germ cells, proliferative cells, or renewal tissues, as well as in immortal cells (Kim et al. 1994, Herbert et al. 1999). In many tumour cells a high level of telomerase activity was observed, and therefore its activity is considered as specific tumour marker and possible therapeutic target (Rufer et al. 1998, Simickova et al. 2001, Armanios et al. 2012). Reactivation of this enzyme in tumour cells has been observed in humans (Allsopp et al. 1992, Hoos et al. 1998) and in some animal species (pig, dog, and cat), so the telomerase activity has been proposed as a marker of tumour in these mammals (Varon et al. 1997, Biller et al. 1998,Yazawa et al. 1999, Pathak et al. 2000, Biller et al. 2001, Nasir et al. 2001, Argyle et al. 2003). The high level of telomerase activity was determined in cattle infected with bovine leukaemia virus (BLV) in blood lymphocytes, sera, and supernatants of *in vitro* cultured dendritic cell (Szczotka et al. 2009, 2011). Telomerase activity in tumour tissue and blood samples in BLV-infected cows was investigated by Suzuki et al. (2008).

In humans telomerase consists of two major components: human telomerase RNA (hTR), which is composed of 451 base integral RNA providing the template for the synthesis of the human telomeric repeat (TTAGGG)n and human telomerase reverse transcriptase, hTERT, which is a 127-kDA protein with catalytic function to replicate the ends of linear DNA (Feng et al. 1995). The other components such as telomerase associated protein 1 (hTEP1), hsp90 and p53 are present *in vivo* in human telomerase complex, but their role in function of telomerase is not yet clearly determined (Holt et al. 1999). Some authors showed in *in vitro* studies, with the use of rabbit reticulolysate extracts containing transcribed hTR and hTERT proteins, that these two components are sufficient for reconstitution of telomerase activity (Weinrich et al. 1997).

Telomeres, guanine-rich repeats sequences, are present at the end of linear chromosomes. The presence of telomeres protects chromosomes from DNA degradation, fusions end-to-end, rearrangements, and chromosome loss. Blackburn showed that in humans and other vertebrates the telomeric sequence contains TTAGGG tandem array (Blackburn 1991, 1994). These regions serve for protection of the integrity of the coding DNA by shielding it from degradation and recombination events (Ohyashiki et al. 1997, Pardue et al. 1999, Ohyashiki et al 2002). The ends of telomeres shorten with each cell division, up to 200 base pairs because of the end-replication problem, which describes the inability of DNA polymerase to synthesise DNA in a 3' to 5'direction (Counter et al. 1995, Coviello-McLaughlin et al. 1997). After a finite number of cell divisions, the telomeres damage sufficiently to allow potential damage to the coding sequences (Vindelov et al. 1983). The loss of telomeric DNA also leaves telomeric repeat for binding factor 2 (TRF2), which is a telomere-associated protein, and maintains chromatin structure, with a shortage of binding sites, which allows end-to-end chromosomal fusions (Van Steensel et al. 1998). In healthy human somatic cells, telomeres shorten with each cell division (Shay 1995, Covello-McLaughin et al. 1997, Pardue et al. 1999).

The length of telomeres depends both on the age of human/animal and on the number of times the cell has divided and is different in many species of organisms. In yeast the telomere length is about few hundred of bp, but hundreds of bp in mammals. Telomere length in several domestic animal species: sheep, pigs, horses, dogs, and cats range from 10 bp to 30 bp, but in mouse the telomeres are very long up to 100 kb (Nakamura et al. 1997).

Bovine leukaemia virus (BLV) belongs to the genus *Deltaretrovirus*, family *Retroviridae*, and is an infectious agent of enzootic bovine leukaemia (EBL). BLV is very closely related to human T-cell leukaemia viruses (HTLV-1 and HTLV-2) and simian T-cell leukaemia virus (STLV) by genomic organisation and disease progression (Ferrer 1980). Infection of BLV remains subclinical in the majority of cattle, but about one third of infected animals develop persistent lymphocytosis (PL). In about 1%–5% of infected animals lymphosarcoma with or without prior PL is observed after the latency period of 1–8 years (Ferrer 1980, Schwartz and Levy 1994). The B-lymphocyte is the primary cellular target for BLV (Paul et al. 1977). The susceptibility of cells other than B lymphocytes to BLV infection is rather less clear (Schwartz et al. 1994)). Monocytes were first implicated as potential carriers of BLV in sheep on the basis of cell morphology and *in situ* hybridisation (Szczotka 2011). Some authors reported that BLV was detected in 5% to 40% of adherence purified monocytes, but was not found in T cells or granulocytes from BLV-infected cattle with or without PL. T cell susceptibility for BLV infection was evidenced, when immunoaffinity depletion of B cells and monocytes from peripheral blood or when positive selection of T cells with the use of

immunomagnetic beads were performed (Schwartz 1994). The process of progression of HTLV infection in humans and the level of tumour development are relatively similar to those in animals infected with BLV (Tsao et al. 1998, Suzuki et al. 2008). The progression of human acute T lymphoma caused by HTLV infection is correlated with telomerase activity, and according to many author's opinion it is a common reaction in haematopoietic tumours (Ohyashiki 1997, Uchida 1999).

The aim of the present study was to determine telomere length and activity of telomerase in samples taken from BLV infected cows.

Materials and Methods

Animals

Investigations were performed on the group of 17 Polish Black and White Lowland breed cattle at age of 4–6 years, naturally infected with BLV. The viral infection was confirmed by ELISA and PCR tests. The healthy, serologically negative 3 cows, at the same age served as control group.

Samples preparation

The blood and lymphoid organ samples were collected in an abbatoir. Blood samples were taken from the jugular vein to the tubes containing EDTA-K2 as anticoagulant. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by centrifugation in density gradient Histopaque -1077 (Sigma Diagnostic, USA). The mixture 1:1 (v/v) of blood and PBS (without Ca^{++} and Mg^{++}) was layered on the surface of Histopaque (density gradient 1.077 g/ml) in tubes and centrifuged at 200 × g for 30 min. The fraction of interphase was collected and cells were washed twice in RPMI 1640 medium. The telomerase activity and telomere length were determined in these lymphocytes.

Controls

Human cell lines, HeLa, 1301 cell (T cell lymphoblastic leukaemia), avian leukaemia J (ALV-J), and foetal lamb kidney permanently infected with BLV (FLK-BLV) were used as positive controls.

Telomerase activity determination

Investigations were performed in the group of cattle infected with BLV: specific antibodies in sera were detected with ELISA, and proviral DNA in PCR test. Telomerase activity was measured in sera, plasma, and lysates of lymphocytes, spleen, lymph nodes, bone marrow, and in supernatants of these cells, cultured *in vitro*. The samples from healthy cows were used as control.

The expression of telomerase catalytic subunit hTERT was estimated in infected with BLV and control cells by immunofluorescence method (IF) with the use of monoclonal hTERT antibodies. The cell nuclei were counterstained with DAPI. The stained slides were analysed under UV microscope (Olympus).

For determination of telomerase activity, the cells were harvested and counted. For each reaction the cells at concentration of 2×10^5 /ml were used. The cells were centrifuged at 3,000 x g, for 10 min, at 4°C. Then supernatant was removed and the cells were washed with cold phosphate buffer saline, and washing step was repeated. The pellet of cells was resuspended in lysis reagent and incubated for 30 min on ice. Then the samples were centrifuged at 16,000 × g for 20 min at 4°C, supernatant was carefully collected and transferred to new tubes. When telomerase activity was not determined on the same day, the supernatant was frozen and stored at −80°C. The protein concentration was determined and cell extracts were investigated with the use of the TeloTAGGG Telomerase PCR ELISA ^{Plus} kit (Roche) according to producer's procedure. This kit was originally designed for determination of telomerase activity in humans, however, it was shown that bovine telomere RNA sequence was similar to that of human telomerase (Tsao et al. 1998). Telomeric repeats (TTAGGG) were added to the 3'- end of the biotin-labelled synthetic primer by telomerase present in the sample, followed by the amplification of the PCR elongation product. The products were split into two aliquots, denaturated, and hybridised separately to digoxigenin-(DIG)-labelled detection probes. The resulting products were bound to 96-well plate coated with streptavidin- and hybridisation procedure was performed to the DIG-labelled telomeric repeat-specific probe. The antibody to DIG conjugated with peroxidase was bound to the DIG and visualised by virtue of the enzyme ability to convert tetramethyl benzidine (TMB). The absorbance of coloured reaction product was measured at 450 nm using ELISA microtiter plate reader after the stop reagent addition. For determination of reaction specificity, the negative control, where enzyme was inactivated in the sample, was analysed in parallel with the non-inactivated samples. The level of telomerase activity in investigated samples was determined by comparison of the signals from the sample to the signal obtained from a known amount of a control template. Relative telomerase activities (RTA) within different samples were calculated according the formula recommended by the producer.

The human neoplastic haemopoietic 1301 cell line, which is characterised by long telomeres, was used as a telomere length internal control.

Determination of BLV infection

The presence of BLV antibodies in the sera was detected with the use of commercial BLV detection ELISA kit (Pourquier). The presence of proviral DNA was determined by nested PCR. DNA was isolated from blood leukocytes using commercial kit (Qiagen) according to manufacturer's recommendations; 0.5 µg of DNA was added to the mixture containing 10 mM of Tris-HCl (pH 8.8), 50 mM of KCl, 2 mM of MgCl2, 0.1% Triton X-100), 200 µM of dNTPs, 0.5 U of DyNAzyme II polymerase (Finnzymes Oy), and 0.2 µM of each *env* CTGTGATGGCTAAGGGCAGACACGGC ZM2 and *env* ZM3 CTTCCCTCCCTGGGCTCCCGAA primers. The final reaction volume was 50 µl. The thermal cycling conditions were 5 min at 95°C, followed by 35 cycles of 94°C for 30s, and combined annealing and extension step at 70°C for 1 min, followed by 7 min extension step at 72°C. The second nested PCR amplification was performed using 5 µl of the first reaction mixture as template. Mixture composition was similar as in the first PCR reaction. Different pair of *env* internal primers ZM4 CTCGCCCTCCCGGACGCCCA (0.2 µM) and GTCAGGCCTAAGGTCAGGGCCGC ZM5 (0.2 µM) was applied. Thermal cycling conditions were also the same. PCR products were analysed in UV light after the electrophoresis in 2% ethidium bromide-stained agarose gel.

The telomere length in cells was analysed by cytogenetic technique, fluorescence *in situ* hybridisation (FISH) with molecular probe PNA (peptide nucleic acid) conjugated to FITC. Two commercial sets were used: Telomere PNA/FITC FISH kit (Dako) and Telomere FISH kit for flow cytometry (Dako). Both methods are suitable for detection and determination of telomere length in lymphocytes without inclusion of subtelomeric sequences.

Telomere PNA/FITC FISH

Cell culture

Peripheral blood samples with anticoagulant (EDTA/K2; POCH) were centrifuged for 30 min at 1,000 × g at 8°C. The pellet of leukocytes was layered on the density gradient (Histopaque 1,077-Sigma) and centrifuged for 45 min at 1,000 × g at 8°C. Lymphocytes were collected, washed twice with PBS (pH 7.5, Sigma) and placed at a concentration of 1.5 × 10^6 cells/ml in the RPMI 1640 culture medium (Gibco) supplemented with 20% calf serum (Sigma), 0.3 mg/ml of L-glutamine (Sigma), 1:100 antibiotic-antimycotic solution (Sigma), and 50 µg/ml of Concanavalin A (Calbiochem). Cells were cultivated at atmosphere of 5% CO_2 at 37°C for 72 hours. For stopping the cell cycle at interphase, 100 µl of Colcemid (10 µg/ml; Gibco) was added to cell culture and cells were incubated at 37°C for 90 min. Then, to 1 ml of cell suspensions, 8 ml of hypotonic KCl solution (60 mmol/l; POCH) was added and cells were incubated for 30 min at room temperature. After fixation in cold solution of methanol/acetic acid (3:1 v/v), cell suspensions were dropped on cold microscope slides and slides were air dried over night at room temperature. The areas of interphase nuclei were marked.

Fluorescence *in situ* hybridisation (FISH)

During pre-treatment step the slides were rehydrated in TBS (Tris buffered saline, pH-7.5; Dako) and fixed in 3.7% formaldehyde in TBS. After two washes in TBS they were digested with Proteinase K solution (Dako) for 10 min, washed twice, dehydrated in ethanol series (70%, 85%, 95%) and air dried. On the marked area on slide surface the amount of 10 µl of Telomere PNA Probe/FITC ready to use solution (Dako) was added, slides were covered with coverslips and placed for 5 min in incubator at 80°C. Then slides were incubated at room temperature for 3 hours in the dark. Hybridisation was followed by brief rinse at room temperature and post hybridisation wash at 65°C for 5 min in commercial solutions provided by Dako. The slides were immersed in the mixture of antifade (Vector) counterstaining solution containing 0.1 µg/ml DAPI (Sigma) and analysed under UV microscope. The pictures of interphase nuclei and image analysis were performed using Case Data Manager (Applied Spectral Imaging). The level of FITC fluorescence was measured in calculation on nuclei area.

Telomere PNA/FITC FISH for Flow Cytometry

Sample preparation and fluorescence *in situ* hybridisation

The mixture of 1×10^6 cells of lymphocytes and 1×10^6 cells of 1301 cell line (human vector tetraploid cell line, which is characterised by long telomeres) were washed and suspended together in 300 µl of hybridisation buffer with PNA/FITC telomere probe (Dako). Samples were then denatured for 10 min at 82°C and hybridisation was performed overnight in the dark at room temperature. The hybridisation was followed by two post-hybridisation washes at 40°C in wash solu-

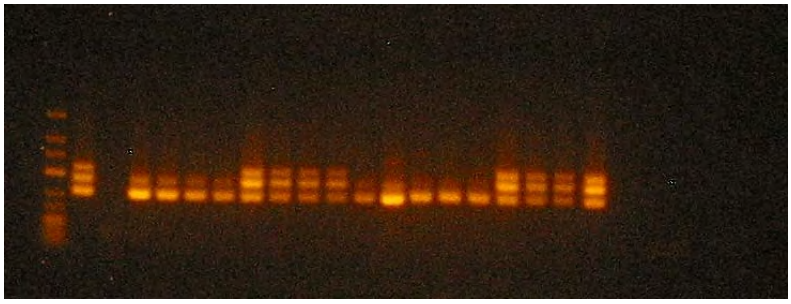

Fig. 1. The infection of cattle with BLV was confirmed by nested PCR. Lanes: M – molecular weight marker 25-700 bp, 1 – positive control, 2 – negative control containing reaction mixture without DNA, 3 − 19 cows infected with BLV, 20 − 22 BLV cows − control group.

Table 1. The telomerase expression (copies number) determined by Real-Time Quantitative Telomerase Detection Kit, Allied Biotech, Inc (QTD Kit).

Organ	Active samples (BLV+)		Active Samples (BLV–)
	Ct (mean values)	Copy number (mean values)	Copy number (mean values)
Lymph node	24.142	4,772.8	900.2
Spleen	22.502	9,068.6	1,408.0
Bone marrow	23.106	5,126.0	2,321.4
Blood	24.63	2,332.0	<500.0

tion provided by Dako. The cells were then resuspended in staining solution containing propidium iodide and RNase A and incubated for 3 hours at 4°C. The control background for every sample was prepared with hybridisation buffer without addition of PNA/FITC telomere probe (Dako).

Flow cytometry analysis

The samples were analysed in a flow cytometer using logarithmic scale FL1-H for fluorescence probe and linear scale FL3-H for DNA staining. During analysis cells which were at S or G2/M phase were identified and removed by gating. The analysis of DNA index was performed in order to obtain an accurate compensation for contents of cellular DNA. DNA indexes of investigated lymphocytes and cell line 1301 cells were measured according to Vindelov (Vindelov et al. 1983). Doublet discrimination also was performed. The cytometric data were analysed by WinMDI software (Windows Multiple Document Interface for Flow Cytometry, Joe Trotter, the Scripps Institute, Flow Cytometry Core Facility; http://www.cyto.purdue.edu/ /flowcyt/software/Winmdi.htm). The values of sample and DNA index of cells were compared to control cell data and relative telomere length (RTL) of each sample was calculated. Percentage of RTL indicates average telomere fluorescence intensity per chromosome/ /genome in the sample cells in comparison to control cells (1301 cell line).

Statistical analysis

Statistical analysis was performed with the use of STATISTICA 10 software (StatSoft Inc, USA). An independent sample t-test was used for comparison of the telomere fluorescence intensity values for peripheral blood mononuclear cells (PBMCs) between animals infected with BLV and healthy controls. The Mann-Whitney U-test was used to compare RTL between both animal groups. To evaluate the relationship between the FISH and RTL results the Spearman's rank correlation coefficient (r) was calculated. The levels of statistical significance was set at $p = 0.05$.

Results

The infection of cattle with BLV was confirmed by nested PCR (Fig. 1). The positive results were obtained in animals infected with BLV and negative in the healthy control cows. Telomerase activity was determined by measurement of enzyme concentration in samples by ELISA (ELISA Kit, Cusabio). The concentrations of telomerase in the serum of BLV- infected cows were from 0.119 ng/ml to 0.354 ng/ml; in the plasma from 0.105 to 0.279 ng/ml. In the supernatants from the *in vitro* cultured lymphoid cells these concentrations amounted from 0.177 ng/ml to 0.482 ng/ml. The telomerase activity was undetectable and the results were negative in samples from the control animals.

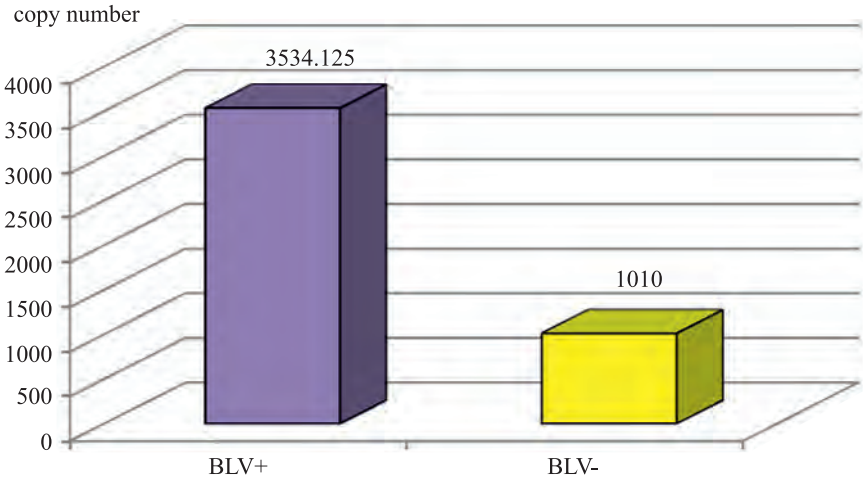

Fig. 2. The telomerase expression (mean values) in cows infected with BLV and control group (real time PCR).

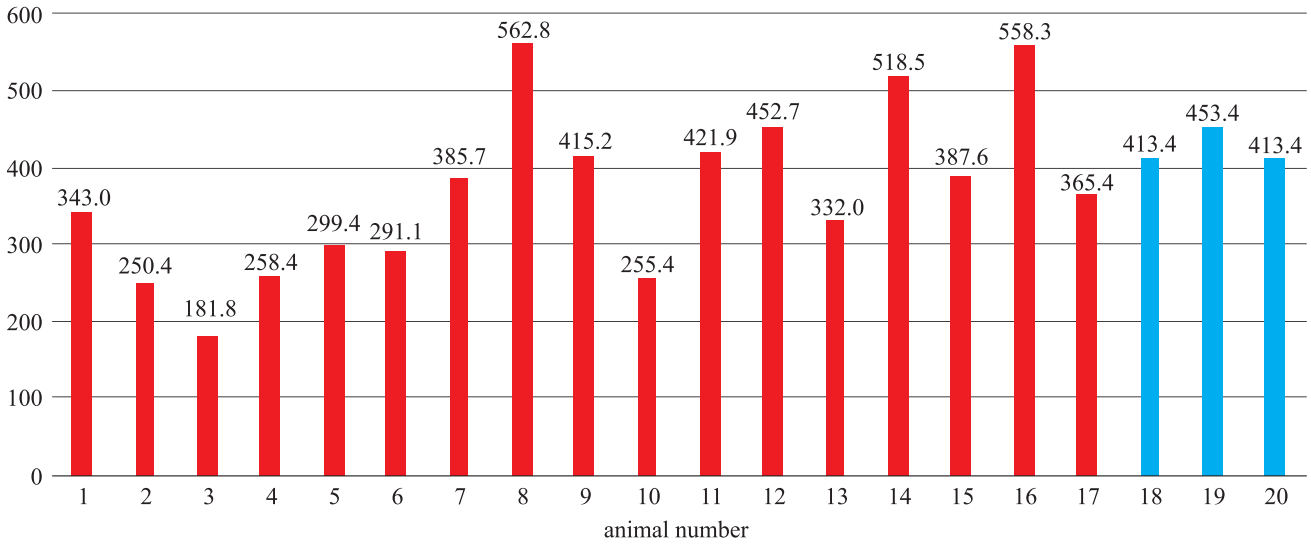

Fig. 3. The values of fluorescence intensity (Telomere PNA/FITC Kit).
Cows: 1–17 BLV-infected; 18–20 control group.

For comparison, the telomerase expression in blood and lymphatic organs was also determined with the use of Real-Time Quantitative Telomerase Detection Kit, Allied Biotech, Inc (QTD Kit). The results are presented in Table 1 and Fig. 2**.** The telomerase expression was very high in BLV-infected cows, and very low or under limit of detection in the control cows. In the infected cows the highest mean values (copy number) of this enzyme expression were found in the spleen (9068.6) and bone marrow cells (5126.0). In lymph node the copy number was 4772.8 and the lowest values were detected in the blood lymphocytes (2332.0). In control samples, the highest mean values of copy number were found in bone marrow (2321.4) and in spleen (1,408), but very low values were in lymph nodes and blood lymphocytes (900.2 and < 500.0, respectively). The telomerase expression - mean of copy number in BLV-infected cows was 3,534.1 but only 1,010.0 copies were determined in the healthy animals (Fig. 2). The Figs. 3 and 4 present the relative telomere length (RTL) determined by two methods: the values of fluorescence intensity (with the use of Telomere PNA/FITC Kit) – Fig. 3 and RTL – (Telomere PNA/FITC Kit for flow cytometry) – Fig. 4. The investigations showed, that this both methods are suitable for telomere length determination in cattle.

It was shown that BLV infection caused changes in DNA content in examined cells. DNA Index (DI value) in leukaemic animals was from DI=0.83 (aneuploid - hypodiploid) up to DI=2.24 (tetraploid). In healthy cows DI was at the level of 1.02 (diploid). In the control cell line 1301, DI=3.15 (tetraploid) was determined (Table 2). The DAPI fluorescence (mean value) in the interphasal nuclei of BLV infected cows was 327.20 and it was higher than in healthy animals, where this value was 300.0. In the control cell line 1301, the DAPI

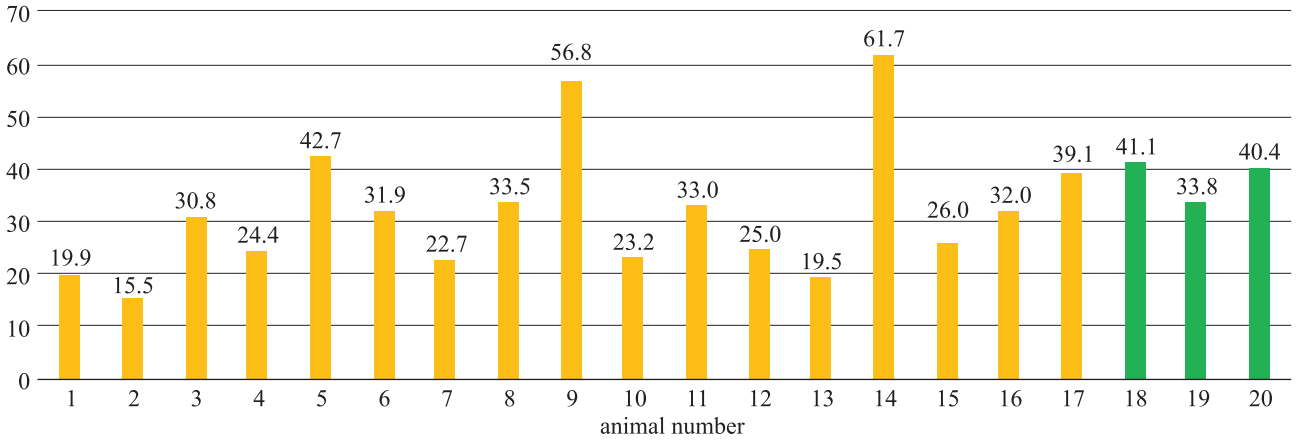

Fig. 4. The values of Relative Telomere Length RTL (Telomere PNA/FITC Kit for flow cytometry). Cows: 1–17 BLV+; 18–20 control, BLV−.

Table 2. The fluorescence intensity, relative telomere length (RTL) and DNA ploidy (DNA index) in BLV-infected and control healthy cows.

Lp	Source of lymphocytes	Mean FITC fluorescence	RTL value (%)	Fluorescence DAPI	DI value
	Positive control (line1301)	502.35	100.00	766.20	3.15
1	BLV+ 1	343.00	19.90	371.90	1.53
2	BLV+ 2	250.35	15.50	259.40	1.07
3	BLV +3	181.82	30.80	265.00	1.09
4	BLV+ 4	258.43	24.40	267.70	1.10
5	BLV +5	299.41	42.70	345.80	1.42
6	BLV +6	291.07	31.90	286.30	1.18
7	BLV +7	385.73	22.70	544.90	2.24
8	BLV +8	562.77	33.50	292.80	1.20
9	BLV +9	415.22	56.80	220.50	0.91
10	BLV 10	255.38	23.20	397.80	1.63
11	BLV 11	452.71	25.00	319.50	1.31
12	BLV 12	331.98	19.50	281.40	1.16
13	BLV 13	518.45	61.70	201.30	0.83
14	BLV 14	387.62	26.00	394.00	1.62
15	BLV 15	421.93	33.00	251.20	1.03
16	BLV 16	558.25	32.00	236.70	0.97
17	BLV 17	365.44	39.10	215.00	0.88
	Mean values for cows BLV+	369.80	31.62	327.20	1.24
18	BLV-1	413.39	41.10	251.30	1.03
19	BLV-2	453.37	33.80	414.30	1.07
20	BLV-3	413.43	40.40	235.60	0.97
	Mean values for cows BLV−	426.73	38.52	300.04	1.02

fluorescence was 766.2. Infection with BLV caused changes in bovine chromosomes and telomere length shortening. The mean values of RTL in BLV infected cows were 31.62% and 38.52%, in the control group (p=0.112). The RTL value measured for positive control cell line 1301 was 100%. These results are presented in Table 2 and Fig. 5. The fluorescence intensity of lymphocyte interphasal nuclei (determined with FISH method) was lower in BLV- infected cows than in the healthy animals (Fig. 6).

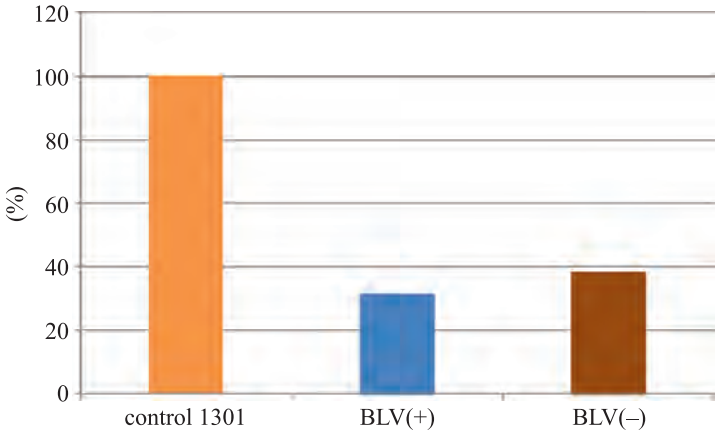

Fig. 5. The relative telomere length (RTL) in internal control 1301 cell line (T – cell lymphoblastoid leukaemia), BLV-infected and control cows (mean values).

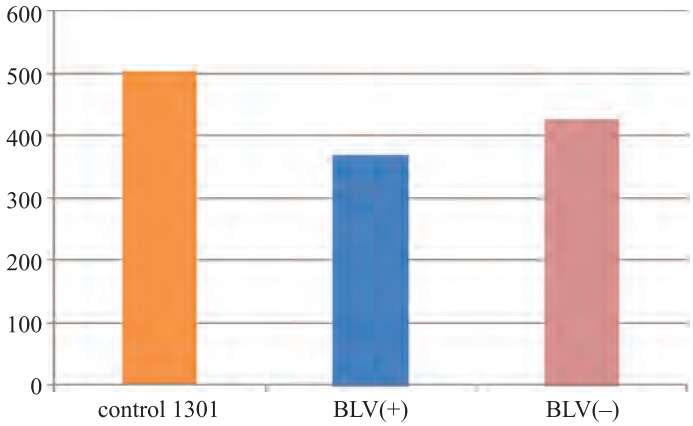

Fig. 6. The fluorescence intensity of lymphocyte interphasal nuclei in internal control 1301 line, BLV-infected and control cows (determination with the use of FISH method and probe of Telomere PNA/FITC FISH for flow cytometry) – mean values.

The results of telomerase activity assay in positive tumour cell lines determined by immunofluorescence method are presented in Fig. 7. The fluorescence was very strong what indicated the high activity of investigated enzyme and the relationship with tumoural proliferation. The FISH results (fluorescence in telomeres of interphasal nuclei) control 1301 cell line, lymphocytes of BLV- cow and BLV+ cow are presented Fig. 8. There were significant differences in the fluorescence intensity; in telomeres in BLV infected cattle the fluorescence intensity was weaker than that in the control cows, where stronger fluorescence intensity was observed.

The number of lymphocyte nuclei from infected cows and uninfected controls were determined by FISH. The results obtained with this method showed significantly lower intensity of telomere fluorescence in BLV-infected cows than that found in the healthy animals which was 369.87 and 420.67, respectively (p=0.000431). The results were shown in Figs. 9A and B.

A significant positive correlation was observed between the results of FISH and flow cytometry, in both studied groups with r=0.527 and p=0.0245 (Fig. 10).

Flow cytometry analysis for fluorescence *in situ* hybridisation was performed for 17 lymphocyte cell cultures from the BLV-positive and three BLV-negative cows. By analysing mean RTL values between infected and non-infected groups, we found that cells of BLV positive cattle showed values of 31.63 ± 12.62 as compared to controls showing 38.43 ± 4.03, (p=0.112) (Fig. 9B).

Additionally, for some samples, relative telomere activity (RTA) was calculated (Table1) and this study indicated a relationship between methods and RTA, so the agreement of the results of two tests used in our experiment was found.

Discussion

Under physiological conditions telomerase is present not only in normal germ and embryo cells, but also in some haematopoietc stem cells. In the majority of the adult somatic cells its activity is repressed (Prowse et al. 1995, Hao et al. 2005). The further expression of telomerase may be the causative factor

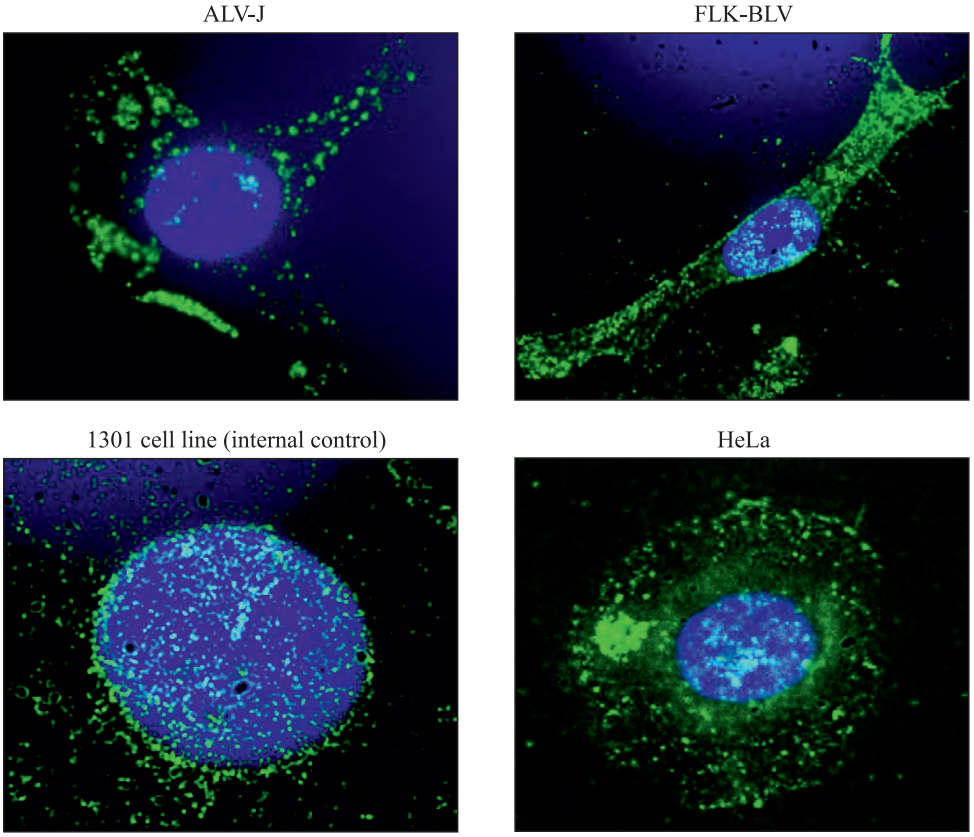

Fig. 7. The expression of telomerase in positive control cell lines.

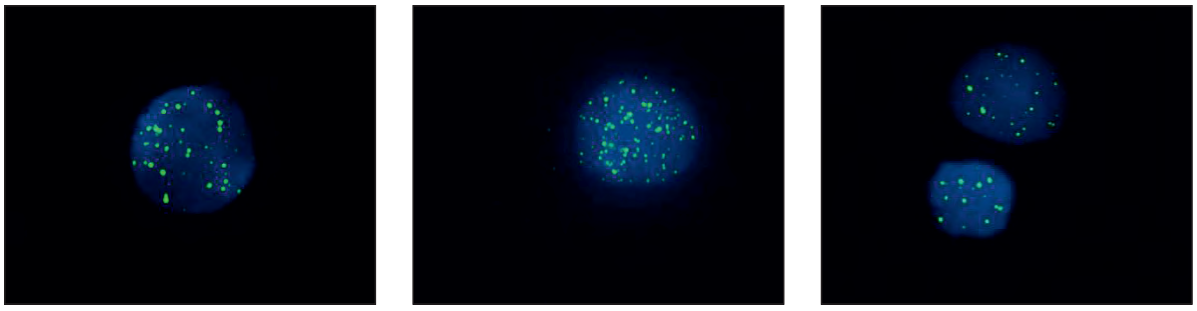

Fig. 8. The FISH results: fluorescence in telomeres of interphasal nuclei; control 1301 cell line, lymphocytes of BLV−cow and BLV+cow.

for uncontrolled proliferation of the cells (Counter et al. 1995). This enzyme is associated with many tumours in humans and has been successfully implemented for distinguishing between malignant and benign cells and to discriminate between more or less aggressive neoplasia (Hoss et al. 1998). The main role of telomeres is to cap eukaryotic chromosome ends and protection of chromosomes from degradation, instability, and fusion (Blackburn 1989, Blasco et al. 1997). Due to the end--replication problem and engagement of telomere ends during the S-phase of the cell cycle, during each round of cell division telomeres shorten (Allsopp et al. 1992, Levy et al. 1992). Telomere shortening cause limitation of the proliferative capacity of primary human somatic cells to 50–80 cell divisions (Allsopp et al. 1992). When telomere reach the a critically length, its dysfunction induces replicative senescence, and at this stage cell cycle is permanently arrested. Telomere shortening is observed in most human organs and tissues during senescence (Djojosubroto et al. 2003). Activity of telomerase in humans is tightly regulated. It is readily detectable during embryogenesis and is suppressed after birth in most tissues. In adult organisms, the activity of telomerase remains only in immature germ cells, certain stem cells, and progenitor cells compartments. In over 80% of human tumours telomerase catalytic activity is reactivated (Satyanarayana et al. 2004). Telomeres are significantly shortened in cancer cells in comparison with non transformed surrounding cells (Plentz et al. 2003, Plentz et al. 2004). Experiments

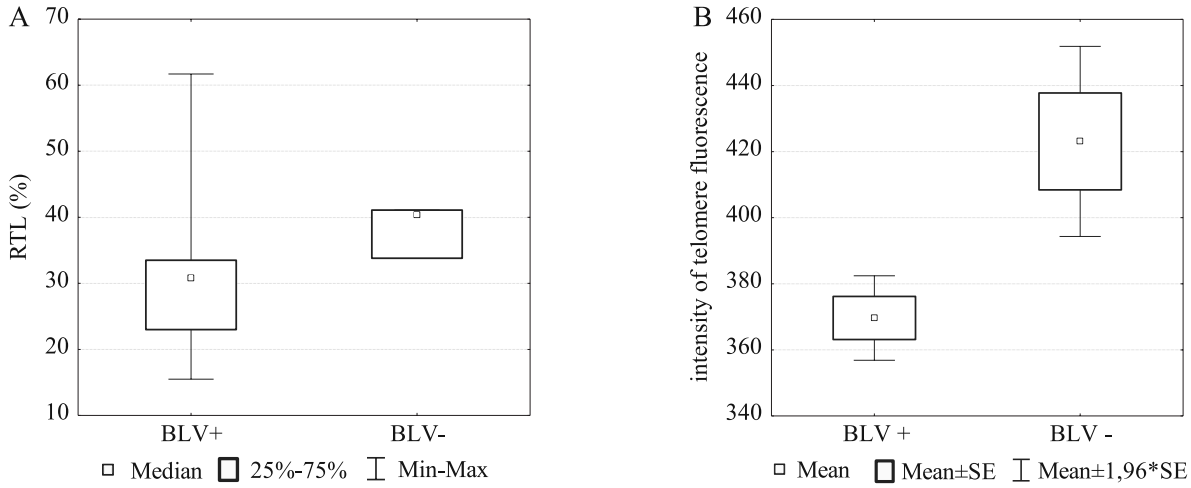

Fig. 9. Box and whiskers plot to visualise the percentage of RTL (A) and intensity of telomere fluorescence (B) between positive and control groups.

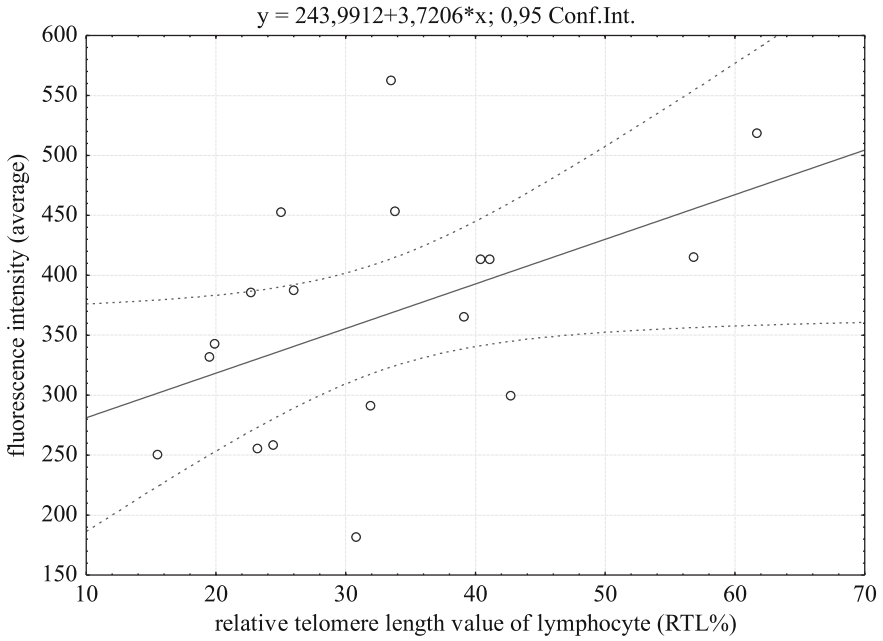

Fig. 10. Correlation (regression line ±95% confidence limits) between Telomere FISH Kit/FITC and Telomere PNA kit/FITC for flow cytometry. Analysis has been performed by Spearman correlation test; the equation is indicated.

in telomerase deficient mice (mTERC) showed that telomere shortening play a dual role in tumourigenesis: enhancing the initiation of early tumours, but at the same time the role of inhibiting tumour progression and development of tumours (Hande et al. 1999, Rudolph et al.1999, Rudolph et al. 2001). The expression of telomerase may cause an uncontrolled cell proliferation (Counter et al. 1995, Hultdin et al. 1998). Suzuki et al. (2008) found in BLV-infected cows that the highest telomerase activity was in the seropositive, non- lymphoproliferative (PBL< 8,000 μl^{-1}) cases (three of seven cases) and was not present (except one case) in the lymphoproliferative cases (PBL<16,000^{-1}). On the basis of these results they suggested that the aleukaemic stage (with the highest telomerase activity) could be defined as "the preneoplastic state" contrary to common statement, that persistent lymphocytosis is considered as a preneoplastic state. The results of our study showed higher activity of telomerase in leukotic cows than in the controls. In lymphoid organs of BLV-positive animals the highest activity of telomerase (the highest copy number) was found in the spleen and bone marrow; in other samples of lymph nodes and blood lymphocytes these values were much lower. The mean values of copy number were three times higher in BLV infected than in healthy animals. The high values of telomerase activity determined in cells of BLV--infected cattle, human tumour cell lines, and foetal lamb kidney cells permanently infected with BLV (FLK-BLV cell line) confirmed, that this enzyme

is responsible for cells proliferation and tumour development (Szczotka et al. 2011). Similar results were obtained in investigations performed on animals with different neoplasia (Varon et al. 1997, Pathak et al. 2000, Argyle et al 2003,). Telomerase activity and telomere length were determined in naturally occurring canine mammary tumours; telomerase activity was detected in almost all investigated samples, but it was undetectable in normal somatic tissues (Yazawa et al. 1999, Yazawa et al. 2001). We found, that BLV changed DNA content in the cells. DI in BLV-infected cells was on the level from aneuploid up to tetraploid, whereas in cells of healthy cows DI was diploid. Tetraploid DI was determined in control cell line 1301. In our earlier investigations (Szczotka et al. 2007) numerous sister chromatid exchanges (SCEs) in metaphasal chromosomes of BLV-infected lymphocytes were found. The obtained results indicated that BLV may cause chromosomal disorders (mutagenesis) in blood lymphocytes of leukaemic cattle. Mutagenesis can be also assayed by sister chromatid exchange (SCE). The SCE is a common and readily quantifiable form of recombination in many mammalian cells. The frequency of the SCE was particularly high within the sub-telomeric regions of chromosomes.

Bechter's investigations indicated, that in patients with B cell chronic lymphocytic leukaemia telomere length and telomerase activity predict survival (Bechter et al. 1998). Telomerase activity was elevated in human blood lymphocytes in chronic lymphatic leukaemia in the advanced stage of the disease (Counter et al. 1995). Our results concerning reactivation of telomerase activity in BLV-infected animals were in agreement with these findings. The telomerase activity and telomere length were investigated in pediatric patients with malignancies undergoing chemotherapy (Engelhardt et al. 1998). In many laboratories the scientists try to find methods which can switch/diminish the telomerase activity and protect telomeres in patients with oncological diseases (Satyarayana et al. 2004, Zhang et al. 2004).

Progressive shortening of telomeres is observed in patients carrying germline mutations inactivating telomerase or telomere maintenance mechanisms, and their cells are unable to counteract telomere damage after each division of cell (Hemann et al. 2001, Hao et al. 2005, Armanios et al. 2012). The germline mutations in several of the telomere maintenance genes are responsible for dyskeratosis congenita (DC), a rare multisystem disorder, where in these humans telomere are drastically shortened and it was estimated that patients with DC have 11-fold increase in cancer incidence as compared with general population (Kannan et al. 2008, Alter et al 2009). DC has been linked to mutations in at least four distinct genes and three of which have now been identified. Dyskerin, the product of these genes, the telomerase RNA (TERC), and the catalytic unit of telomerase (TERT) are part of a ribonucleoprotein complex, the telomerase enzyme, which is essential for the elongation and maintenance of chromosome ends or telomeres. In all patients with DC excessively short telomeres were confirmed, what indicates that the underlying defect in these individuals is an inability to maintain the telomeres (Mason 2005). We found, that in BLV- infected cows telomere were shorter than in healthy control animals, what indicated that bovine leukaemia virus influenced the chromosomes and caused their instability. The results of Vonderheide's (2002) investigations have shown, that telomerase, as an universal tumour associated antigen, can be useful for cancer therapy. Uchida et al. (1999) demonstrated correlation of telomerase activity with the development and progression of adult T-cells leukaemia. Shortened telomeres and high telomerase activity almost always correlate with disease severity in many haematological malignancies, indicating that application of telomere length and telomerase activity measurement might be useful in monitoring disease condition (Shay 1995, Ohyashiki et al.1997).

Conclusions

The obtained results indicated that infection with BLV reactivated telomerase and caused shortening the telomere length in infected lymphocytes in comparison with controls. Additionally, it was shown that BLV influenced DNA ploidy changing the DI from aneuploid - hypodiploid up to DI tetraploid. Telomerase expression (copies number) and values of FITC fluorescence were statistically significant. Telomerase expression and sustaining of telomere length is important for the cancerous process and for the growth of most solid tumours *in vivo* and *in vitro*. High telomerase activity and shortened telomeres almost always correlate with severe or fatal course of the disease in lymphoproliferative disorders in humans and animals. Determination of these parameters might be a prognostic marker and useful therapeutic target in therapy of tumours and haematological diseases in human and animals.

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