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Differential expression of complement subcomponent C1qA in blood samples of healthy and BLV-infected Polish Holstein-Friesian cows*

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The aim of the study was to compare mRNA gene expression and serum protein levels of complement subcomponent C1qA in blood samples of Polish Holstein-Friesian cows reared in a bovine leukemia virus (BLV) seropositive herd. Using qRT-PCR and the $2^{-\Delta\Delta Ct}$ method of relative quantification, we studied C1qA transcript expression in vivo in blood cells of 23 naturally BLV-infected cows, including 8 animals in subclinical persistent lymphocytosis (PL), 15 in clinically silent aleukemic state (AL) and 13 BLV non-infected controls. In comparison to the BLV uninfected animals, the C1qA transcript level was found to decrease approximately 4.7-fold and 1.75-fold in the PL and AL stages, respectively. Furthermore, the C1qA protein level in serum was examined with the competitive ELISA technique and it was found to decrease in BLV-infected animals. The average content of C1qA in serum was recorded at 31.6±3.78 µg/ml and 54.5±5.94 µg/ml in PL and BLV non-infected groups, respectively, with the difference found to be significant ($p \leq 0.05$). In search of a causative DNA polymorphism within the bovine C1qA regulatory sequence, a 576bp PCR-amplified DNA fragment, containing the transcription start site and 472 bp of the proximal C1qA promoter, were sequenced. Three SNPs were identified; however, due to the low minor allele frequency their contribution to the observed C1qA gene differential expression needs to be verified in future research. We concluded that the differential expression of the complement subcomponent C1qA suggests that the bovine C1qA gene should be considered as a potential candidate gene associated with BLV resistance or pathogenesis.

KEYWORDS: BLV / C1qA complement subcomponent / dairy cattle / gene expression

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Relatively little is known concerning the bovine complement subcomponent C1q when compared with what is known on its human and murine counterparts. C1q is most acknowledged as a recognition unit of pentameric complex C1 ($C1q\text{-}C1r_2\text{-}C1s_2$) in the classical pathway of complement activation, involved in pathogen or pathogen-infected cell lysis or phagocytosis and in the processing of immune complexes [Stoermer and Morrison 2011]. However, in recent years numerous studies have shown that C1q binds directly not only IgMs and IgGs, but also a multiplicity of other biological structures including diverse pathogen-associated molecular patterns (PAMPs) and host-associated ligands and receptors. This has resulted in emerging novel functions for C1q, which have been extensively reviewed recently [Kouser *et al.* 2017, Ghebrehiwet *et al.* 2017]. The complement subcomponent C1q molecule is composed of 18 polypeptide chains, arranged to a batch of a tulip-like structure with 6 triple helical strands, each strand consisting of 3 distinct polypeptide chains A, B and C, encoded by 3 distinct genes C1qA, C1qB and C1qC, respectively [Lu *et al.* 2008]. In cattle, these genes are highly clustered within a 25kb DNA segment on chromosome 2 [Zimin *et al.* 2009].

The complement system was identified as a mediator of protection or pathology during infection by retroviruses. It was shown that human serum efficiently lysed a variety of animal retroviruses by triggering the classical pathway of the complement, as a consequence of direct binding of C1q to viral envelope proteins, which did not require anti-virion antibodies [reviewed by Thielens *et al.* 2002]. In contrast, human retroviruses HIV-1 (human immunodeficiency virus type 1) and HTLV-1 (human T-cell lymphotropic virus type 1) are not lysed by the human serum or complement, due to the evolutionarily developed mechanism to resist complement-mediated destruction by incorporating host cell regulators of complement activation, such as the decay-accelerating factor and protectin into the viral envelope lipid bilayer [Spear *et al.* 1995, Saifuddin *et al.* 1997]. However, in the case of the HTLV-1 system, it was reported that the interaction and binding of human complement subcomponent C1q with the gp-21 protein of HTLV-1 inhibits viral infectivity [Ikeda *et al.* 1998].

Bovine leukemia virus (BLV) is the primary retroviral threat in cattle industry, being an etiological agent of enzootic bovine leukosis (EBL) and causing considerable economic losses worldwide. EBL is a chronic lymphoproliferative, fatal neoplastic disease, marked by 3 illness stages: clinically silent aleukemic (AL), subclinical persistent lymphocytosis (PL) and clinical leukemia/lymphoma [Gillet *et al.* 2007]. BLV is closely related to HTLV-1, it is characterised by a similar genomic organization, gene expression strategies and pathologies and used as a model system in HTLV-1 research [Aida *et al.* 2013]. Previously, using the microarray approach, we performed global gene expression profiling of leukocytes from BLV non-infected and BLV-infected cows in order to identify genes that may play a role in molecular events leading to PL and involved in host response to BLV infection. The results showed the occurrence of numerous regulatory processes that are targeted by BLV-infection and lead to disease progression; however, BLV associated immune suppression emerged

as one of the main themes. The gene set enrichment meta-analysis demonstrated that bovine complement system activation could be potentially disturbed, with a 2.37-fold decrease in the level of mRNA for C1qA found in the BLV-infected group with PL [Brym and Kamiński 2017]. Although currently it is not known whether bovine C1q is able to bind to any of the BLV proteins, the differential expression of the bovine C1qA gene could be of interest, taking into account that human C1q inhibits infectivity in the related HTLV-1 viral system [Ikeda *et al.* 1998]. Therefore, the main aim of the study was to compare mRNA gene expression and serum protein levels of complement subcomponent C1qA in blood samples of Polish Holstein-Friesian cows reared in a BLV-seropositive herd. Furthermore, the minor aim was to identify DNA polymorphism within the bovine C1qA proximal promoter sequence which could affect gene expression.

Material and methods

Animals, sample collection and BLV diagnostics

The local ethics committee of the University of Warmia and Mazury in Olsztyn, Poland (permission no. 13/2008/N/T) approved all the applied procedures. All sampled animals were lactating cows of Polish Holstein-Friesian Black-and-White variety and were reared in a BLV-seropositive large dairy herd from the West Pomerania region of Poland. The analysed cows were reared under identical feeding and welfare conditions. No other diseases except for EBL were recorded by the veterinary service. Milking performance traits of cows were officially registered and evaluated by the Polish Federation of Cattle Breeders and Dairy Farmers. Blood sampling was performed within two days and soon after the herd was culled by the owner's decision, due to the high BLV-seroprevalence and the strict BLV eradication policy requiring compulsory slaughter. Blood was collected from the jugular vein using a VACUETTE® Blood Collection Set and evacuated using blood collection tubes: VACUETTE® Serum, VACUETTE® EDTA (Greiner Bio-One) and VACUETTE® Tempus™ Blood RNA Tubes (Applied Biosystems, Greiner Bio-One). Serological diagnosis of BLV infection was performed by ELISA using POURQUIER® ELISA Bovine Leukosis Screening (Institute Pourquier) according to the manufacturer's protocol. Furthermore, the diagnosis was corroborated by BLV proviral DNA detection employing the nested PCR method [Markiewicz *et al.* 2003]. Automated blood cell counting was performed using a Sysmex SF3000 analyzer according to the manufacturer's instruction. Total leukocyte and lymphocyte counts were used to classify BLV-positive cows into those with aleukemic (AL) ($<12x10^3$/μl leukocytes and $<8x10^3$/μl lymphocytes) and persistent lymphocytosis (PL) ($>12x10^3$/μl leukocytes and $>8x10^3$/μl lymphocytes) stages of the disease, respectively [Stachura *et al.* 2016]. Based on serological (ELISA), molecular (nested-PCR proviral detection) and hematological assays as well as the quality of isolated RNA, 36 animals were analysed in the study, including 23 naturally BLV-infected cows (8 in PL and 15 in AL) and 13 BLV non-infected controls.

Relative quantification of bovine C1qA gene expression by real-time qRT-PCR

Total RNA extraction was performed using the VACUETTE® Tempus™ Blood RNA Tube (Applied Biosystems, Greiner Bio-One) RNA stabilization system and the protocol described in detail [Brym and Kamiński 2017]. Purified RNA concentration, its purity and integrity were determined by A_{260}, A_{280} and A_{230} measurements using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies) and an Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit (Agilent Technologies). As the quality of isolated RNA is the critical component of gene expression analysis, only samples of RIN ≥9.0, 28S/18S rRNA ≥1.7, A_{260}/A_{280} ≥2.0 and A_{260}/A_{230} ≥2.0 were used. There were no significant differences in averaged RNA quality parameters between any groups in comparison. RNA samples were aliquoted and stored at -80°C until use. One µg of anchored - oligo$(dT)_{18}$ primed total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Roche). The final cDNA products were diluted 100-fold prior to use in qRT-PCR and then aliquoted and stored at -20°C. Bovine C1qA primers were designed using PRIMER-BLAST and sequences deposited in the NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). As a reference gene for qRT-PCR data normalisation the UCHL5 (ubiquitin carboxyl-terminal hydrolase L5) gene was used, as the most stable reference gene for this experiment, based on a previous determination from a panel of 10 putative reference gene candidates [Brym *et al.* 2013]. The primers, the target and reference amplicons details are presented in Table 1. Real-time qRT-PCR reactions were performed in a 96-well plate format on a LightCycler® LC 480 II (Roche) using LightCycler® 480 SYBR Green I Master (Roche) reagents according to the manufacturer's instructions. Each qRT-PCR assay was conducted in two replicates for each sample and in three replicates for the

Table 1. Quantitative reverse transcription-PCR (qRT-PCR) primer sequences applied in the study and specification of amplicons.

Gene symbol	Primer sequences (forward/reverse) (5'-3')	Anneal-ing temp. (°C)	T_m (°C)	Amplicon size	E	*Error*	Reference / AN
C1Qa	F – GGCAGCCCCGGGAACATCAAG R – AGAGCAGCGGAACCTGCCTGT	60	87.8	153	1.999	0.002	in house design / NM_001014945
UCHL5 reference gene	F – ACAAAGACAACTTGCTGAGGAACCC R - GGCAACCTCTGACTGAATAGCACTT	60	78.5	79	2.029	0.013	Brym *et al.* [2013] / NM_174481

E – PCR efficiency calculated using formula $E = 10^{-1/slope}$, *Error* value – mean squared error of the single data point fit to the regression line, T_m – melting temperature curve peak analysis, AN – accession number, C1qA – complement C1q chain A, UCHL5 – ubiquitin carboxyl-terminal hydrolase L5 (reference gene).

negative (no template) control. The cycling conditions, melting curve analysis, PCR efficiency and quantification cycle (Cq) determination were carried out as described previously [Brym *et al.* 2013]. Furthermore, the specificity of C1qA and UCHL5 amplicons was confirmed by DNA sequencing. The relative quantification of C1qA gene expression in the BLV-infected groups in comparison to the BLV non-infected group was calculated according to the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen [2001], since PCR efficiencies for both C1qA and UCHL5 amplicons were estimated as close to 2.

Quantification of bovine C1qA protein in serum

Bovine C1qA concentration in serum was determined employing the competitive enzyme immunoassay technique. The blood samples collected into tubes with a clot activator (VACUETTE® Serum, Greiner Bio-One) were centrifuged for 5 min. at 1000 x g, and serum samples were stored at -20°C until use. A commercial kit, Bovine complement C1q subcomponent subunit A (C1QA) ELISA (Cusabio), was used according to the manufacturer's protocol. Absorbance at 450 nm was measured with a MultiScan FC plate reader (Thermo Scientific). The CurveExpert 1.3 software (Hyams Development) was used for standard curve fitting, with the Morgan-Mercer-Flodin (MMF) found to be the best regression model and applied to calculate serum C1qA concentration in µg/ml.

Identification of DNA polymorphism within 5'flanking sequence of bovine C1qA gene

The position of the promoter sequence was assigned based on comparative genomics using the Genomatix Software Suite (promoter record GPX_3883484, transcript NM_001014945) starting at 130795643 nt and ending at 130796398 nt on the minus strand. Putative regulatory DNA of the 5'flanking region of the bovine C1qA gene was amplified with the standard PCR method, using the PRIMER-BLAST designed forward 5'ACATGCAGCGAGGAAGTGGCAGTCT3' and reverse 5'CCCAGTCCAGTCTCCTGGGCCACCT3' primers and annealing temperature of 60°C. Afterwards, the obtained amplicons (576 bp) from animals under study were sequenced using the Sanger method. The resulting sequences were compared with one another and with the NCBI reference sequence AC_000159 [Zimin *et al.* 2009]. The identified SNPs were analysed in terms of potential changes in transcription factor binding sites, using the MatInspector tool from the Genomatix Software Suite ver. 3.1.

Results and discussion

Analysis of C1qA gene expression at mRNA level in blood cells

In comparison to the BLV uninfected animals, C1qA gene expression at the transcript level was found to decrease 4.76-fold and 1.76-fold in the PL and AL stages, respectively ($p \leq 0.01$), (Tab. 2). The currently estimated 4.76-fold C1qA down-regulation, in the BLV-infected PL animals in comparison to the BLV non-infected group, indicated

down-regulation to be 2 times greater than the 2.37-fold down-regulation identified by the microarray approach [Brym and Kamiński 2017]. This difference is probably due to the technical aspects of mRNA quantification with the qRT-PCR technique thought to be a gold standard in gene expression analysis and much more precise [Derveaux *et al*. 2010]. The measurement results were not affected by the starting RNA quality, as we included in our analysis only those samples, which were marked by high RNA quality indices and there were no significant differences in the averaged RNA quality parameters between any groups in comparison (data not shown). As BLV-associated pathology is intrinsically linked with polyclonal proliferation, affecting mainly the B-cell lineage and as it could change the hematological profile and alter the gene expression assessment, we examined leukocyte cell counts to test whether this effect occurred. Differential cell counting was performed using an automated hematology analyser and the results of averaged counts for lymphocytes, neutrophils, monocytes and eosinophiles were shown with regard to the BLV-infection status (Fig. 1). BLV-infected cows in the PL stage were characterised by higher numbers of lymphocytes ($p \leq 0.001$) and eosinophiles ($p \leq 0.05$) in comparison to the BLV-infected, but aleukemic group and the BLV non-infected cows. Additionally, the number of neutrophiles was higher in the BLV-infected PL group when compared to the BLV-non-infected group ($p \leq 0.05$). In contrast, in each case no differences were found either in the number of monocytes among the analysed groups or aleukemic BLV-infected and BLV non-infected animals. The latter is of particular interest, because monocytes and dendritic cells are considered as the main sources of the C1qA transcript in blood [Lu *et al.* 2008, Ghebrehiwet *et al*. 2017]. Assuming that circulating monocytes and DCs should be the only source of C1qA mRNA in blood, the observed differences in C1qA mRNA contents could be attributed to a BLV infection or BLV-associated processes rather than to any changes in

Table 2. Peripheral blood leukocytes fold change expression of C1qA in BLV-infected groups relative to BLV uninfected animals calculated according to the $2^{-\Delta\Delta Cq}$ method [Livak and Schmittgen 2001]

BLV status	Average Cq for C1qA	Average Cq for UCHL5	ΔCq	ΔΔCq	$2^{-\Delta\Delta Cq}$	Down-regulation fold change relative to BLV non-infected animals
BLV- (non-infected)	20.34 ±0.53	25.93±0.33	-5.59±0.62	0.00±0.62	1.00	1.00^{AB}
BLV+ (AL)	21.73±0.67	26.5±0.41	-4.77±0.79	0.82±0.79	0.57 (0.98-0.33)	1.76 (1.02-3.05)^{A}
BLV+ (PL)	23.06±0.39	26.41±0.18	-3.34±0.44	2.25±0.44	0.21 (0.28-0.15)	4.76 (3.52-6.44)^{B}

Cq – quantification cycle; C1qA – gene of interest, subcomponent A of complement C1q; UCHL5 – reference gene, ubiquitin carboxyl-terminal hydrolase L5; ΔCq=C1qA - UCHL5; ΔΔCq=(ΔCq BLV-infected - ΔCq BLV-non-infected; AI – BLV-infected aleukemic stage; PL – BLV-infected persistent lymphocytotic.
^{AB}Fold changes among groups in comparison indicated with the same capital letter differ at $p \leq 0.01$.

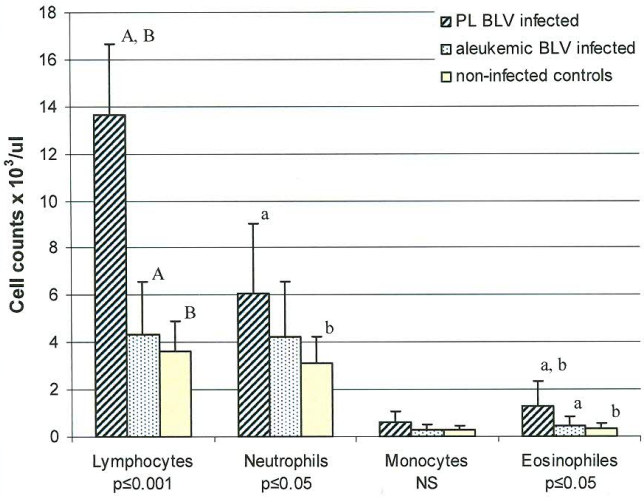

Fig. 1. Hematological profile of animals analysed in the study. Statistical differences in differential cell counts are indicated by the same letters (A, B – $p \leq 0.001$; a, b – $p \leq 0.05$) and were verified by one-way Anova and Scheffe's test. NS – non-significant.

the monocyte numbers. It is known that although $CD5^+$ IgM^+ B cells are the main cell type targeted by BLV [Panai *et al.* 2013], other blood cell types (including monocytes and macrophages) could also be infected [Heeney *et al.* 1992, Werling *et al.* 1998], becoming the reservoir of the virus. It was reported that monocytes from BLV-infected cattle were characterised by an altered expression of some surface antigens such as CD11b, CD32 (FcγRII) and MHC class II [Werling *et al.* 1998] and were functionally less efficient [Blagitz *et al.* 2017]. The decreased expression of C1qA mRNA in monocytes could also contribute to its functional disturbance, although it needs to be confirmed in further studies.

Quantification of serum C1qA content in naturally BLV-infected and uninfected cows

The serum C1qA protein level was examined using the competitive ELISA technique and it was also observe to decrease in BLV-infected animals (Fig. 2). The average content of C1qA in serum (mean±SE) was estimated at 31.6±3.78 µg/ml and 54.5±5.94 µg/ml in PL and BLV non-infected groups, respectively ($p \leq 0.05$). The average content of C1qA in the BLV-infected aleukemic group was estimated at 41.88±4.6 and the differences in comparison to both BLV non-infected and BLV-infected PL animal groups were not significant, probably due to the small sample size and large variation within each group to be compared. It is known that the bulk of C1q serum content is released from differentiated tissue macrophages and bone marrow [Lu *et al.* 2008] and due to the potential compensation the observed correlation between the blood cell C1qA mRNA and the serum protein level in BLV-infected animals need to be regarded with caution. In mice and humans a wide variation in complement

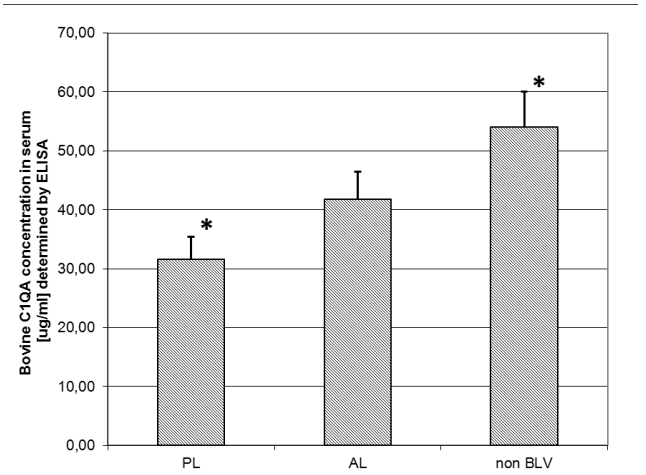

Fig. 2. Bovine C1qA average concentration in serum [μg/ml] ± SE determined by competitive ELISA in groups of BLV-infected cows including PL and AL stages of disease and BLV non-infected cows. The differences between means were verified by one-way Anova and Scheffe's test. * - significant at $p \leq 0.05$.

component levels in serum, including C1q between different individuals, was reported in the case of C1q depletion, due to the causative mutations found in patients with autoimmune diseases or recurrent infections [Mitchell *et al*. 2002, Walport 2002].

Analysis of polymorphism within 5'regulatory sequence of bovine C1qA gene

As gene expression changes among individuals are frequently driven by DNA polymorphisms within non-coding regulatory sequences, it was decided to identify the causative DNA polymorphism within the bovine C1qA regulatory sequence. Three SNPs were identified, all of them previously recorded in the dbSNP database. The potential effect of the SNP to generate or abolish a transcription factor binding site was analysed using the MatInspector programme (Tab. 3). In this study the attempt to link the differential expression of the C1qA gene with DNA polymorphism in the regulatory sequence of the gene was unsuccessful due to insufficient data. According to the dbSNP database, more than 70 SNPs with rs numbers were observed within the 576bp region analysed in this study as a putative bovine C1qA promoter. It is of interest that in Polish Holstein-Friesian Black-and-White cattle this region seemed to be rather conservative with only three SNPs found. Unfortunately, due to the low minor allele frequency detected in our group of animals no association studies could be performed. Therefore the contribution of the identified SNPs to the observed C1qA gene differential expression should be considered as negligible. However, it needs to be emphasised that the effect of a more distantly located, but causative DNA polymorphism may not be ruled out. Further research is required to clarify this issue.

It was reported that the interaction of specific peptide antagonists with the evolutionarily conserved coiled-coil structural motif of envelope transmembrane

glycoprotein (TM) in both BLV and HTLV-1 inhibits their membrane fusion and entry into the cell [Lamb *et al.* 2008]. As human C1q inhibits HTLV-1 infectivity through direct binding to TM known as gp21 [Ikeda *et al.* 1998], a question arises whether bovine C1q could bind to the BLV gp30 transmembrane glycoprotein counterpart and mediate a similar effect. To our best knowledge it is not yet been clarified.

In conclusion, the differential expression of complement subcomponent C1qA in the monocyte/macrophage lineage of BLV-infected and BLV non-infected animals observed in this study and the premise from the related C1q-HTLV1 system suggest that the bovine C1qA gene should be considered as a potential candidate gene associated with BLV resistance or pathogenesis. Failure or subversion of an appropriate complement system response may be pivotal to BLV infection and disease progression.

Table 3. Characteristics of SNPs identified within the 576bp 5'-flanking region of the bovine C1qA gene and their predictive effect on binding of transcription factors using the bioinformatic approach (MatInspector, Genomatix Software Suite). MAF calculations were based on genotypes of 36 Polish Holstein-Friesian cows included in the study

dbSNP	BTA2 NCBI position AN: AC_000159	Position within the C1qA gene	MAF	Allele change	TF binding site lost or generated due to SNP	TF	DNA strand with binding sequence
rs380181974	130795774	-31 from transcription start	0.04 (T)	**C>T**	new	REST/NRSF	-
					lost	ZIC2	+
rs385047543	130795702	5'UTR	0.12 (A)	**G>A**	new	p53	+
rs381968173	130795669	intron 1	0.04 (T)	**G>T**	lost	E2F	+
					new	HSF1	-
					new	PAX6	-

SNP – single nucleotide polymorphism; AN – accession number, BTA – *Bos taurus* autosome; MAF – minor allele frequency; TF – transcription factor.

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