DIVERSITY OF GAG GENE SEQUENCE ENCODING IMMUNODOMINANT EPITOPE ON CAPSID PROTEIN OF LENTIVIRUSES FROM SHEEP IN POLAND

MONIKA OLECH, MARZENA ROLA-ŁUSZCZAK, BOŻENNA KOZACZYŃSKA, PIOTR KUBIŚ, ANETA PLUTA, ANNA GIL, AND JACEK KUŹMAK

Department of Biochemistry, National Veterinary Research Institute, 24-100 Pulawy, Poland

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Abstract

In the study, a 122 bp fragment of gag gene encoding immunodominant epitope on capsid protein of small ruminant lentiviruses (SRLVs) found in sheep was amplified by PCR and analysed by SSCP and sequencing. Out of 30 DNA samples, five showed different migration patterns, demonstrating the individual variations within gag sequences, which were confirmed afterwards by sequence analysis. In two samples nucleotide changes yielded amino acid substitutions highlighting the conservative nature of gag encoded immunoreactive epitope but also potential insensitivity of a single-strain-based immunoassay.

Key words: small ruminant lentiviruses, SSCP, gag gene sequence, Poland.

Small ruminant lentiviruses (SRLVs), for whose prototype are maedi visna virus (MVV) and caprine arthritis-encephalitis virus (CAEV), are a highly heterogeneous group of infectious agents affecting sheep and goats. Infections are widespread and cause several economic losses due to chronic mastitis, pneumonia, arthritis and early culling (2). The high heterogeneity of nucleotide and amino acid sequences in SRLVs may determine their antigenicity, virulence and may affect their persistence, and escape from the immune system. Although sheep and goats lentiviruses have been considered as species specific for a long time, now it is clear that natural cross-species infection may occur and the eradication of infection in one animal species cannot exclude the SRLVs infection in the other (10, 12). Thus a new phylogenetic classification of SRLVs was proposed and MVV-related viruses are now referred to group A and CAEV prototypes, originally isolated from goats, belong to group B. Group A is the most heterogeneous group and can be divided into several subtypes, whereas group B can be divided into three subtypes B1- B3 (1, 16).

The genome of SRLVs consists of gag and env genes open reading frames encoding structural proteins and among gag encoded proteins, capsid antigen (CA) is considered to be the first antigen recognised by host humoral response (7, 8). It was demonstrated that gag encoded protein is responsible for main antigenic cross-reaction between MVV and CAEV and p25 capsid protein is considered as one of the immunodominant antigens in SRLVs infection (5). Mapping studies revealed the presence of 17 amino acid sequence LNNEAERWVRQNPAPN in the N’-terminal half of p25 protein and the motif –NPP- was the most important to preserve a spectrum of cross-reactivity (14). In fact, many ELISAs based on a single strain are usually applied in the detection of antibodies in both species (4). However, when aforementioned antigenic variability within SRLVs was taken into account, substantial differences within C-terminal part of 17 aa epitope were noted between two Italian isolates belonging to subtypes A3 and B2 (6). This also affected the sensitivity of the ELISA when homologous and heterologous sera were tested.

Recent serological surveys revealed that SRLVs infections are widely distributed among sheep in Poland (11) and analysis of gag sequences clearly showed that SRLVs groups A and B also circulate in sheep flocks (9, 10). The aim of this study was to investigate the genetic diversity of gag gene sequences encoding immunodominant epitope on capsid protein of lentiviruses found in sheep in Poland.

Material and Methods

DNA samples. Initially, blood samples were drawn from 102 sheep, randomly selected from six flocks in which infection with SRLVs was recognised on the basis of previous serological survey. None of the animals demonstrated apparent clinical signs of maedi-visna. Peripheral blood leukocytes (PBLs) were isolated.
from 10 ml of blood by centrifugation at 1,500 g for 25 min. Erythrocytes were haemolysed by osmotic shock with H2O and 4.5% NaCl. After two washes in PBS, the supernatant was discarded and the cells pellet (5×10^5 cells) was used for extraction of genomic DNA with the BLOOD Mini DNA kit (A&K Biotechnology, Poland), according to the manufacturer’s protocol. DNA concentration was estimated by measuring the optical density at 260 nm. Samples were stored at −20°C until PCR amplification.

**PCR amplification.** At first, semi-nested PCR was used to detect the 375 bp fragment of *gag* gene (3). Then, all samples indicating the presence of proviral DNA were subjected to two rounds nested PCR allowing amplification of a 122 bp fragment of *gag* gene, encoding immunodominant epitope on p25 capsid protein. All primers were selected on the basis of published sequence of pLV1KS1 plasmid, according to the previously published data (17). PCR primers were designed within conserved sequences allowing amplification highly divergent prototypic K1514, CAEV-Co, and SRLVs field strains, respectively (3). The primers MGAG1 919- CCTATTGTGAATTTGCAA-936 and MGAG2 1564- CACATCTGCACATG-1564 were used for the first amplification, while primers GAGEp11098- GCCTGGGATTAGCAGCAAG-1119 and GAGEp2 1220-CCACATCCTGATGATCCA-1198 were used for the second amplification. Amplification was carried out with the use of a Biometra Thermal Cycler under the following conditions: 5 min at 94°C, 1 min at 56°C, 1 min at 72°C. After the last cycle, the samples were incubated at 70°C for 15 min. The first and the second rounds of PCR were performed in 35 cycles using the same conditions. The PCR mix consisted of: 2.5 μl 10x buffer; 1.5 mM MgCl2; 0.2 μM of each primer; 200 μM of each dNTP; 1 U of EuroBlue Taq polymerase (Euro Bio) and 1 μg of DNA. For each amplification, a positive control (pLV1KS1 plasmid) containing a full-length insert of MVV 1514 and negative control (without DNA) were run in parallel with all specimens. After amplification, PCR products from each sample were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide (1 μg/mL) in 1X TAE buffer.

**SSCP analysis.** Ten microlitres of the PCR-amplified DNA fragments were diluted 1:2 in a denaturing loading mix (95% formamide; 0.05% bromophenol blue; 0.05% xylene cyanol) and heated at 95°C for 5 min and then chilled on ice for at least 5 min. Fifteen microlitres of each diluted sample were loaded onto a non-denaturing 8% polyacrylamide gel containing 8% glycerol. Electrophoresis was performed using a Protean II apparatus (BioRad). Samples were run in TBE (53 mM Tris-HCl; 53 mM boric acid; 1.5 mM EDTA2Na) buffer at 25 mA for 6 h at 6°C. After electrophoresis, the gels were stained with silver stain.

**DNA sequencing and sequence analysis.** Amplification products were purified with the use of the Geneclean II kit (Bio 101) and were ligated to the pDRIVEplasmid vector (Promega). The ligation products were used to transform EZ Competent E.coli cells (Promega) and the resulted plasmid were isolated and digested with EcoR I endonuclease to identify the correct insert size. The nucleotide sequence of cloned PCR fragments was determined by fluorescent dye deoxy-terminator cycle sequencing kit (Amersham Biosciences) using T7 and SP6 primers (Promega) and analysed with by an ABI Prism 377 DNA sequencer (Applied Biosystems). Sequence data were analysed using the Chromas version 1.45 and GeneDoc Multiple Sequence Alignment Editor 7 Shading Utility, version 2.6.002.

**Results**

To investigate genetic heterogeneity of immunodominant epitope of SRLVs capsid protein at first large-scale molecular screening was conducted to select animals infected by SRLVs. For this purpose, semi-nested PCR was used to amplify a 375bp fragment of the SRLVs *gag* gene. Out of 102 DNA samples tested, proviral DNA was successfully amplified from 34 (33%) samples. When all these samples were subjected to amplification of a 122 bp fragment of *gag* gene, encoding major immunodominant region of the capsid protein, lentiviral sequences were found in 30 samples. In order to determine the genetic variability within a 122 bp *gag* encoding region all these samples were analysed by SSCP. As shown on Fig. 1, the upper part of each lane represents the number of single strands (ss) DNA that reflects the heterogeneity of the predominant sequence of *gag* gene, whereas the double-stranded (ds) bands were located in the lower part of the gel. Mobility shift of ssDNA was clearly observed on electrophoresis at 6°C but not prominently at 16°C (data not shown), thus revealing that a higher temperature might have destroyed some conformations of the analysed ssDNA. The extended sample heterogeneity was analysed on one gel, and the results of SSCP reaction of all samples analysed were compared to those obtained from pLV1KS1 plasmid DNA, which were used as a standard. SSCP analysis of the PCR products representing five samples showed unequivocal electrophoretic patterns as a comparison of controlling plasmid DNA, thus demonstrating the individual variations within *gag* encoding sequences. Migration patterns identical to these noted for control plasmid were seen in remaining 25 samples.

In order to prove whether SSCP analysis can predict sequence differences between the PCR products, all DNA samples were sequenced and then aligned among themselves and with the sequence of pLV1KS1 plasmid. The sequence analysis (Fig. 2) revealed a total of 13 nucleotide changes, in five samples, representing those with distinct migration patterns. Most of them were point mutation, mainly located in the third base position, and they predominantly concerned A → G transitions (58%), while remaining G → A, C → T, and C→A changes occurred at low frequency. Insertion of cytosome in the position 1200 of nucleotide sequence was noted in sample 18. All 25 samples, representing
identical migration patterns, were grouped similarly by the sequence analysis. These data demonstrated that SSCP was a reliable predictor of genetic heterogeneity in the fragment of gag gene. Furthermore, SSCP showed ability to discriminate between samples differentiated by one nucleotide change only. Within these variations nucleotide changes from A to C and from A to G yielded amino acid substitutions of lysine (K) to glutamic acid (E) at position 90 and asparagine (N) to threonine (T) at position 92 in samples 11 and 169, respectively (Fig. 3). In sample 18, insertion of a single cytosine in position 106 resulted in shift of an open reading frame and change of terminal amino acid sequence DQIMGV for ASNNGS. Remaining nucleotide changes were non-synonymous substitutions.

**Discussion**

Genetic heterogeneity of gag gene of many retroviruses, including SRLVs, was extensively studied; however, the studies were based mostly on a simple sequence analysis (18, 13). In the study, SSCP was applied to demonstrate the diversity among gag gene sequences encoding immunodominant epitope on capsid protein of lentiviruses found in sheep, in Poland. Since the sensitivity of detection of a single-basis polymorphism by SSCP primarily depends on the size of the DNA fragment, a 122 bp fragment was selected to maximise the sensitivity of the assay. As it was confirmed by sequence analysis, SSCP enables us to detect a single nucleotide changes within the analysed fragment of gag gene.

![PCR-SSCP analysis of the gag gene encoding epitopes of p25 protein (122 bp) of MVV. Lane: M- Ladder 100 bp, PL- DNA of plasmid pLV1KS1, DNA of samples: 10, 169, 11, 18, and 3 have different pattern, DNA of samples: 1, 5, 6, 7, 8, 12, 13, and 20 have the same pattern as PL. Single stranded DNA and double stranded DNA indicate ssDNA and dsDNA, respectively.](image1)

![Nucleotide sequences alignment of the 122 bp fragment of gag gene encoding epitopes of p25 protein. Dots indicate nucleotides that are identical to the reference sequence depicted on the top line. Dashes indicate gaps in sequence.](image2)

![Amino acid sequence alignment of the 122 bp fragment of gag gene encoding epitopes of p25 protein. Dots indicate nucleotides that are identical to the reference sequence depicted on the top line. Dashes indicate gaps in sequence.](image3)
A high degree of similarity among sheep SRLVs sequences was found in the immunodominant epitope on p25 protein. Till now, the conservation of capsid immunodominant epitopes was essential for the development of ELISA based on p25 protein (15, 6). However due to very plastic nature of lentiviral genome, a search for epitopes of local value is still needed in different areas (4). In the study, the amino acid substitution found in the gag gene in two sheep might be a critical value for the sensitivity of serological tests. Especially, there were point mutations in the codon for the first and third aa at N-terminal part of epitope, where lysine (K) and asparagine (N) were substituted by glutamic acid (E) and threonine (T), respectively. Similar analysis performed with SRLVs in Spanish sheep and goats showed single nucleotide mutations within the sequence encoding N-terminal part of the epitope, resulting in substitution of glutamic acid (E) to aspartic acid (D) in one sheep and two goats (13). In both studies, the aa changes were identified in the N-terminal epitope (sequence LNEEAERW); however, in the previously mentioned study (6) all amino acid substitutions were found in C-terminal part of the epitope leading to creation of distinct C-terminal epitopes (group A-VRQNPPGP and group B-RRNPPPPPA). The possible infection of Italian sheep with CAEV-related viruses would possibly explain the differences between both studies. It is worth to mention that all sequences from Polish sheep were quite similar to K1514 isolate, thus it finds confirmation the fact that the animals were infected with MVV-related viruses. However, the last study (10) clearly showed that SRLVs found in sheep population in Poland were highly heterogenous and can be affiliated into subtypes A1, A12, A13, as well as, B1 and B2, confirming the cross-species transmission event.

To sum up, the study demonstrated a high degree of sequence conservation within sequences encoding immunodominant epitope on p25 protein among SRLVs sheep found in Poland. Whether a few amino acid changes found in the study, especially leading to the substitution of basic aa (lysine) to acidic aa (glutamic acid), can change the immunodominant epitope and affect the diagnostic sensitivity of ELISA, remain to be verified.

References


