Asymmetric PCR-SSCP: a Useful Tool for Detection of OLA-DRB1 (MHC Class II) Gene Polymorphism in Slovak Improved Valachian Sheep

Ľ. TKÁČIKOVÁ[1], M. R. BHIDE[1], I. MIKULA[1,2]

[1]Laboratory of Biomedical Microbiology and Immunology, University of Veterinary Medicine, Košice, Slovakia; [1,2]Institute of Neuroimmunology, Slovak Academy of Sciences, Bratislava, Slovakia

Received November 22, 2004
Accepted June 6, 2005

Abstract

Tkáčiková Ľ., M. R., Bhide I. Mikula: Asymmetric PCR-SSCP: a Useful Tool for Detection of OLA-DRB1(MHC class II) Gene Polymorphism in Slovak Improved Valachian Sheep. Acta Vet. Brno 2005, 74: 275-278.

Detection of OLA-DBR1 (exon 2) gene polymorphism is presented in the paper. Rapid and inexpensive polymorphism detection method, namely, single stranded conformation polymorphism (SSCP) was assessed. Modification of the SSCP to asymmetric PCR-SSCP enabled a more simplified assay for the clustering the individuals into distinct groups (profiles) on the basis of band patterns, as only single stranded amplicons were detected. A total of 400 Valachian sheep were included in the study. In this cohort, 25 distinct clusters were noticed. Among 25 groups the frequency of *k* profile was the highest (28%), followed by profile *e* (21%), *p* (16 %), *w* (9%) and *d* (9%). The homologous SSCP as well as asymmetric-PCR-SSCP patterns were observed among the twins: This finding has shown the sensitivity of both methods to segregate the individuals on the basis of their allelic forms.

MHC, OLA- DRB1 gene, SSCP, polymorphism, sheep

The major histocompatibility complex (MHC) plays a central role in the immune response of vertebrates. The extreme polymorphism in MHC genes enables the host to recognize enormous numbers of foreign peptides to trigger an immune reaction. The class II gene region of the sheep MHC (OLA) has an organization similar to that of humans (Scott et al. 1987). Within this region, two sub-regions, namely, DR and DQ exhibit higher polymorphism (Amills et al. 1998). Among OLA class II genes, the DRB1 locus is highly polymorphic. In particular the polymorphism is present in exon 2, which encodes the antigen-binding site. To date nearly 160 OLA-DRB1 alleles have been recorded from various sheep breeds (Konnai et al. 2003).

In the population genetics, analysis of large number of samples is a prerequisite. Though the DNA sequencing is gold standard for most of the phylogenetic studies, the cost requisite for such analysis is quite high as well as it is time consuming. Recently, simple and rapid techniques like PCR-RFLP (restriction fragment length polymorphism) and DGGE (denaturing gradient gel electrophoresis) have been used by different researchers for the detection of MHC gene polymorphism (Aldridge et al. 1998; Konnai et al. 2003). These techniques enable to group the individuals into clusters on the basis of gene polymorphism. However, the amount and cost of restriction enzymes required for analyzing large numbers of samples may jeopardize the use of RFLP.

SSCP (single stranded conformation polymorphism) offers a simple and inexpensive method for genotyping. Hitherto, SSCP has been extensively used in biomedical research, especially for rapid bacterial genotyping. In this study we used simple SSCP as well as modified asymmetric PCR-SSCP for DRB1 genotyping in autochthonous Valachian sheep breed. To our knowledge, no report is as yet available elaborating the OLA-DBR1 alleles present in the Valachian breed. Considering the recent interest in Valachian sheep breeding

Address for correspondence:
Doc. MVDr. Ľ. Tkáčiková, PhD.
Laboratory of Biomedical Microbiology and Immunology
University of Veterinary Medicine
Komenského 73, 041 81 Košice, Slovakia

Phone: + 421 556 336 614
Fax: +421 556 323 666
E-mail: tkacikov@uvm.sk
http://www.vfu.cz/acta-vet/actavet.htm

in Middle and Eastern European countries it is necessary to know the natural disease resistance ability of this sheep breed. The results presented in this study create a benchmark for further gene polymorphism study influencing the ability of disease resistance or susceptibility of this breed.

Materials and Methods

Animals

A total of 400 autochthonous Valachian sheep (including 20 twins) were included in the study from different farms located in Eastern Slovakia. Genomic DNA was isolated from blood leukocytes and DNA was stored at -20 °C until used.

PCR amplification

Two PCR primers forward (5'-TCT CTG CAG CAC ATT TCC TGG-3') and reverse (5'-CTC GCC GCT GCA CAG TGA AAC-3') (Ammer et al. 1992) were used to amplify entire exon 2, with flanking intron. The total product length was 279 bp. Reactions were performed by using 0.6 – 1.3 µg of genomic DNA in a 50 µl final volume. The PCR reaction mixture contained 100 µM of each dNTP, 0.5µM of each primer, 1.5 mM $MgCl_2$, and 1U of *Taq* polymerase. The conditions for PCR amplification were as follows: 94 oC for 5 min, followed by 35 cycles of 94 °C for 90 s, 60 °C for 30 s and 72 °C for 90 s with final extension at 72 °C for 10 min.

To produce single stranded amplimers, asymmetric PCR was performed. In short, the PCR reaction mixture was maintained as described previously except the concentration of DRB1R primer was reduced by 100 folds. For amplification of asymmetric amplimers the same PCR cycles were used as described above.

Single-Strand Conformational Polymorphism Analysis

Five 5 µl of each amplified product was mixed with 3 µl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). Double stranded amplimers were subjected to denaturation at 95 °C for 8 min and samples were rapidly cooled on ice. Denaturated as well as single stranded amplimers derived form asymmetric PCR were loaded on acrylamide isacrylamide (37.5:1; Bio-Rad) gels. In the study we tried 6, 8, 10 and 14% polyacrylamide gels. The concentration of glycerol in gel was kept either 5% or 10% to compare the effect on resolution. Electrophoresis was performed using 200 V at 5 °C in 0.5 × TBE buffer. Gels were silver-stained according to the method of Bassam et al. (1991). Different electrophoresis runs (14, 16, 18 and 20 hours) were also tried in the study.

Results

Genotyping of OLA-DRB1-exon 2 revealed a total of 25 groups on the basis of PCR-SSCP. Genogroups obtained by the SSCP of double stranded amplimer were correlated with the results of SSCP performed by using the products of asymmetric PCR (amplified single stranded amplimers). However, the results obtained from asymmetric PCR-SSCP were much easier for further analysis and clustering of the profiles. Fig. 1 and Fig. 2 (Plate XIV) present the PCR-SSCP and asymmetric PCR-SSCP profiles representative for each cluster.

Among different concentrations of polyacrylamide used in the study 14% gel yielded the most satisfactory results. In case of lower percentages diffused bands were observed (data not presented). The use of 10% glycerol improved the band resolution of the polyacrylamide gel. Similarly, 20 hours of electrophoresis run separated the band more distinctly.

Among 25 clusters the frequency of *k* profile was the highest (28%) followed by groups *e* (21%), *p* (16 %), *w* (9%) and *d* (9%) (Fig. 3). SSCP patterns for the twins were identical except in 3 twin pairs.

Discussion

The Valachian sheep is the westernmost of Zackel sheep breeds, which came from the Romanian Southern Carpathians to the High Tatra and the Low Tatra mountains in Slovakia. The Improved Valachian breed is the well-represented sheep breed in Slovakia along with other breeds like Tsigai and Merino. Valachian sheep are mainly used for milk production. During the last five years the interest in sheep breeding has revived in Slovakia, with emphasis on the improved Valachian breed.

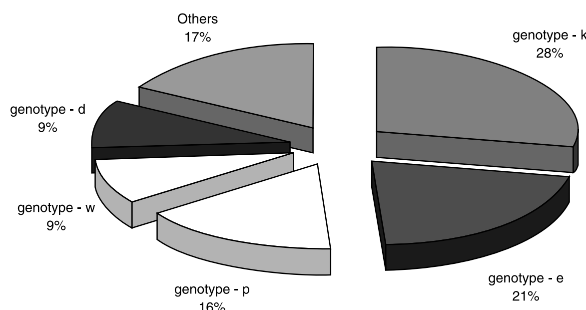

Fig. 3. Frequencies of MHC-DRB1 allelic profiles in Slovak Valachian sheep breed. Others (17%) were constituted by 20 different profiles reveled from PCR-SSCP analysis.

Table 1. SSCP profiles showing either homozygosity or heterogygosity in the Valachian sheep population

Zygosity	**SSCP profile**	**Total Percentage**
Homozygotes	b, f, i, j l, s	24 %
Heterozygotes	a, c, d, e, g, h, k, m, n, o, p, q, r, t, u, v, w, x, y	76 %

Homo- and heterozygosity was confirmed on the basis of asymmetric PCR- SSCP.

MHC gene region is the most important because of its relationship with disease resistance or susceptibility. Several studies (Schwaiger et al. 1995; Buitkamp et al. 1996; Charon et al. 2002) have shown the association of OLA-DRB1 alleles with reduced faecal egg counts in parasitic infestations. Antibody production was also influenced by MHC microsatellite alleles in the nematode infestation (Outteridge et al. 1996). DRB1-exon 2 is particularly associated with parasitic infestations. Nonetheless, ovine MHC class II DRB1 alleles were also reported to be associated with resistance or susceptibility to development of bovine leukemia virus-induced ovine lymphoma (Nagaoka et al. 1999).

The extreme polymorphism in DR region was confirmed in our experiment recording 25 different SSCP patterns. Heterozygosity was significantly higher (96%; $P < 0.05$, *t*-test) among the Valachian sheep as described in Table1. Different SSCP patterns of DRB1 gene have been reported earlier (Kostia et al. 1998; Jugo and Vicario 2000; Jugo and Vicario 2001). The frequency of the *k* genogroup was the highest (28%) followed by genogroup *e* (21%). The sequencing of alleles from each SSCP group can help to determine the possible natural disease resistance of Valachian sheep. Also the asymmetric PCR-SSCP coupled with allele sequencing can prove as a prominent molecular marker system. From 20 twins pairs 17 pairs had similar SSCP patterns confirming the sensitivity of segregation ability of SSCP.

Because of the multiple polymorphism and heterogeneity, the results obtained in DGGE, RFLP as well as SSCP analysis are difficult to analyze and distinguish them in to the clusters. The use of asymmetrical PCR-SSCP proved to be the challenging modification to ease the data processing and cluster analysis. Moreover two distinct bands (in case of heterozygotes) and single band (in case of homozygote) appeared in asymmetrical PCR-SSCP can be dissected, purified and sequenced for pin-point sequenced based identification of alleles. Kostia et al. (1998) also reported the simplicity and speedy nature of SSCP method to accelerate DRB1 genotyping of sheep breeds.

In short, the application of asymmetric PCR-SSCP technique can facilitates the good molecular marker system for gene polymorphism detection in a large number of individuals, which is usually difficult by using traditional typing techniques.

Asymetrická PCR-SSCP ako užitočný nástroj na detekciu polymorfizmu v OLA-DRB1(MHC trieda II) géne slovenských oviec plemena Zošľachtená valaška

V práci je prezentovaná detekcia polymorfizmu v 2 exóne OLA-DBR1 génu. Bola použitá rýchla a málo náročná metóda stanovenia jednovláknového konformačného polymorfizmu (Single Stranded Conformation Polymorphism, SSCP). Modifikácia SSCP na asymetrickú PCR-SSCP umožní oveľa jednoduchšie rozdelenie jedincov do jednotlivých genetických skupín na základe rozdielnych pruhových profilov, keďže sú analyzované iba jednovláknité amplikóny. Do tejto štúdie bolo zahrnutých 400 oviec plemena Zošľachtená valaška, pričom bolo zaznamenaných 25 rozdielnych genetických skupín. Najčastejšie boli zastúpené skupiny *k* (28 %), *e* (21%), *p* (16 %), *w* (9%) a *d* (9%). U dvojičiek bol pozorovaný výskyt homológnych vzorov tak v SSCP ako aj v asymetrickej PCR-SSCP, čo poukazuje na senzitivitu daných metód pri segregácii jednotlivých zvierat na základe prítomnosti jednotlivých alel.

Acknowledgements

This research was supported by Slovak Grant Agency VEGA 1/0433/03.

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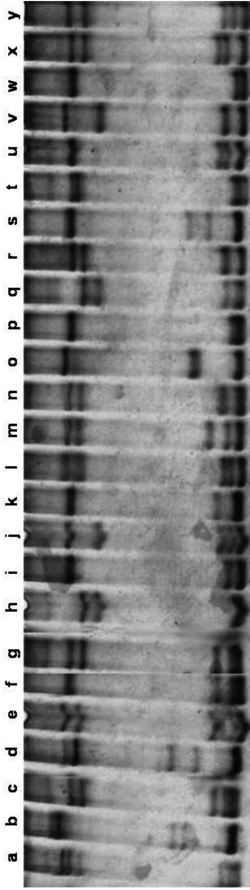

Fig. 1. Polymorphism patterns obtained by SSCP analysis of amplimers (DRB1 exon 2 gene)

Sheep genomic DNA isolation followed by amplification of DRB1 gene (exon 2) and SSCP analysis of double stranded amplicons; the letters indicate genotype based on SSCP profile

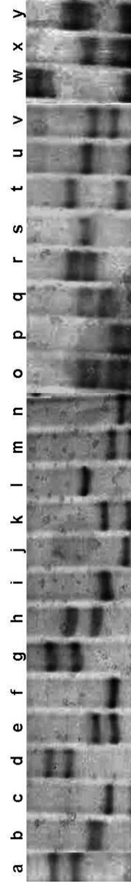

Fig. 2. Asymmetric PCR-SSCP profiles of DRB1 exon 2 gene

Sheep genomic DNA isolation followed by amplification of DRB1 gene (exon 2) and SSCP analysis of double stranded amplicons; the letters indicate genotype based on SSCP profile