Typing of *Ovar-DRB1* second exon with PCR-RFLP technique in Iranian Shaul Sheep

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(Received 3 Nov 2008; revised version 7 Mar 2009; accepted 11 Apr 2009)

Summary

Several studies have focused on polymorphisms of major histocompatibility complex (MHC) in sheep, *Ovar-MHC*. This molecule plays a pivotal role in antigen presentation for eliciting immune responses against invading pathogens. The best-characterized genetic control of disease resistance and immune response in animals is associated with MHC. Numerous molecular genetic investigations have been undertaken to detect polymorphisms of MHC genes and their association with resistance to infectious diseases. We have examined *Ovar-DRB1* in DNA samples of 82 Shaul Sheep using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method. Identities of 8 different patterns and 5 distinct DRB1 alleles among Iranian Shaul Sheep have been determined. PCR-RFLP analysis allows rapid identification of *Ovar-DRB1* types and enables rapid discrimination between homozygotes and heterozygotes. Data obtained from the present study have revealed that the exon 2 region of *Ovar-DRB1* was highly polymorphic in sheep. So PCR-RFLP can be applied to the analysis of this locus.

Key words: MHC, *Ovar-DRB1*, PCR-RFLP, Shaul

Introduction

Major histocompatibility complex (MHC) plays a key 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 major histocompatibility complex (MHC) of sheep is known as Ovar, located on chromosome 20. The Ovar class II genes encode polymorphic glycoprotein composed of non covalently linked α and β-subunits that play a pivotal role in the initiation of the immune response to pathogen-derived peptide antigens. Among Ovar MHC class II genes, the expressed *DRB1* locus has been found to be highly polymorphic (Ballingall *et al*., 1992; Fabb *et al*., 1993; Schwaiger *et al*., 1994; Ballingall *et al*., 1995; Schwaiger *et al*., 1995; Kostia *et al*., 1998; Paterson, 1998; Jugo and Vicario, 2000; Konnai *et al*., 2003b). In particular, a high polymorphism level is present in exon 2, which encodes the antigen-binding site (Outteridge *et al*., 1996; Escayg *et al*., 1997; Konnai *et al*., 2003a, b). Variation in these genes may impact immune responses to pathogens, which may lead to variation in disease susceptibility (Tizard, 2003). In studies of variation in disease susceptibility, it is important to have the ability to characterize MHC polymorphism and determine its relationship to immune responsiveness. This requires reliable and sensitive typing methods. Several methods are now available to analyze MHC polymorphism in domestic animals. In sheep, the polymorphism of *Ovar-DRB1* has been defined using several PCR-based methods including sequence-specific oligonucleotide probe analysis

(Schwaiger *et al*., 1993; Schwaiger *et al*., 1994), single-strand conformational polymorphism (SSCP) (Kostia *et al*., 1998; Jugo and Vicario, 2000), RFLP analysis with identification using Southern blot analysis (Dutia *et al*., 1994) and cloning and sequencing (Nagaoka *et al*., 1999). DNA sequencing is the gold standard for most of the phylogenetic studies, and the cost requisite for such analysis is quite high and time consuming as well (Konnai *et al*., 2003b).

Shaul Sheep are the best meat-type breed in Iran and have a high number of lambs per ewe. The current study was aimed at analysing the genetic diversity of the Ovar-*DRB1* locus in this breed, by amplification of the exon 2 region of *Ovar-DRB1* alleles by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method.

Materials and Methods

Sampling and DNA extraction

Eighty two blood samples were collected from Shaul sheep populations raised in the Research Institute of the Faculty of Veterinary Medicine (Amin-Abad, Tehran, Iran). The whole blood was preserved in acid citrate dextrose solution and stored at -20°C. DNA was prepared from whole blood cells as described by Sambrook and Russell (2001) with some modifications. For each sample, three hundred micro liters of whole blood was added to 500 µl red blood cell lysis buffer (20 mM Tris-HCl, pH = 7.6) mixed and incubated at room temperature. After 10 min tubes were centrifuged at 12000 g for 20 sec and supernatant was discarded. Pellet was resuspended in 600 µl of cell lysis buffer (10 mM Tris-HCl, pH = 8; 1 mM EDTA, pH = 8; 0.1% sodium dodecyl sulphate) and homogenized. 300 µl denaturing solution (4.0 M guanidinium thiocyanate, 25 mM sodium citrate pH = 7, 0.5% N-lauroylsarcosine) was added and incubated at room temperature for 10 min. Then three hundred micro liters of potassium acetate (3.0 M potassium and 5.0 M acetate) was added and mixed for 30 sec. After centrifugation for 3 min at 12000 g, supernatant was transferred to a fresh tube containing 600 µl of cold isopropanol to precipitate the DNA. The pellet was washed with 70% ethanol and resuspended in 50 µl of TE buffer (pH = 7.6).

DRB1.2 amplification and RFLP

Nested PCR was used for the amplification of the second exon (308 bp) of the *DRB1* gene. Briefly, the first PCR stage was performed in a final volume of 25 µl containing template DNA, 20 pmole of each primer (5'-AGGAGTCCGCTCCTGTGAC TA-3' and 5'-ACTCACAGTCGTACACAC TCG-3' (Konnai *et al*., 2003b)), PCR buffer (20 mM Tris-HCl pH = 8.4, 50 mM KCl), 1 mM $MgCl_2$, 0.25 mM of dNTPs, and 1 U of *Taq* DNA polymerase. This solution was initially denatured at 94°C for 4 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (60°C for 2 min), and elongation (72°C for 1 min) and a final extension at 72°C for 5 min. Subsequently, 2 µL of the first-stage PCR product was used as template DNA for the second-stage PCR in a final volume of 50 µl containing 20 pmole of each primer (5'-ATCCTCTCTCTG CAGCACATTTCC-3' and 5'-TTTAAAT TCGCGCTCACCTCGCCGCT-3' (Konnai *et al*., 2003b)), 1 U of *Taq* DNA polymerase, and the remaining components in the concentrations stated above. The initial denaturation (95°C for 2 min) was followed by 14 cycles of denaturation (95°C for 1 min), annealing (68°C decrease 0.5°C per cycle for 30 s), and elongation (72°C for 30 s), and 19 cycles of denaturation (95°C for 30 sec), annealing (61°C for 30 sec), elongation (72°C for 30 sec), and a final extension (72°C for 5 min). Contamination and self-priming controls were included in each PCR round and 5 µl of the last PCR stage was electrophoresed on 1.5% agarose gels in order to check the quality and specificity of DNA fragment amplification. To examine the nucleotide sequence variability at the second exon of *DRB1*, locus RFLP protocol was carried out using *Rsa*I digestion. Aliquots (10 µl) of the second-stage PCR product (308 bp) were digested with the *Rsa*I restriction enzymes according to the manufacturer's instructions (Fermentas, Germany). The resulting DNA fragments were separated on 12% polyacrylamide gels, using *Msp*I-digested *pBR322* (Fermentas, Germany) as a

molecular marker. Samples were run in TBE buffer (53 mM Tris-HCl; 53 mM boric acid; 1.5 mM EDTA2Na) at 150 V for 1 h at room temperature. After ethidium bromide staining, the gels were photographed under UV light with a Gel Doc system (Vilber Lourmat Inc. Cedex, France).

Population genetic analysis

Gene and genotypic frequencies were estimated by direct counting. Expected homozygosity and heterozygosity were computed using the Levene method (Levene, 1949). Deviations from Hardy-Weinberg (HW) equilibrium were estimated by the *F*IS parameter (Weir and Cockerham, 1984). The amount of gene diversity in the Shaul breed was measured by the number of alleles (*n*a) and the unbiased expected heterozygosity (*h*e) according to the formula proposed by Nei (1973). The Ewens-Watterson neutrality test was estimated using the method described by Manly (1987). The Popgene 1.32 software was used to estimate the parameters mentioned above.

Results

DRB1 polymorphism was analyzed by PCR amplification and RFLP of *DRB1* second exon in Iranian Shaul Sheep. DNA bands of the expected size (308 bp) were obtained after PCR amplification.

When each amplified product was cleaved by the restriction enzyme, 8 different patterns were observed in *Rsa*I digests (Table 1, Fig. 1). 5 *Rsa*I alleles were similar to those previously recognized and reported by Konnai *et al*. (2003a). The *Rsa*I *a, b, c, f and g* restriction patterns identified here have been previously reported for *Ovar-DRB1* in other breeds. A number of bands in different restriction patterns was from 1 to 7. The size of bands in the identified alleles are illustrated in Table 2.

Table 2: Allelic frequencies of the second exon of MHC-DRB1 gene in Iranian Shaul Sheep

Alleles	Size of bands in identified alleles	Allelic frequency
RsaI a	308 bp	0.2866
RsaI b	240 bp/68 bp	0.0671
RsaI c	117 bp/69 bp/68 bp/54 bp	0.0061
RsaI f	84 bp/68 bp/54 bp/39 bp/33 bp/30 bp	0.0305
RsaI g	117 bp/68 bp/54 bp/39 bp/30 bp	0.6098

The numbers of animals that belonged to each of the different patterns by RFLP analysis are summarized in Table 1. Allelic frequencies were determined by direct counting for each *RsaI* restriction pattern separately. In Shaul Sheep 4 homozygous genotypes: *aa*, *bb*, *ff*, *g*g and 5 distinct alleles: A, B, C, F, G were observed. Genotype *ag* was the most common and the most frequent pattern exhibited (43 of a total 82 samples) and allele G had the highest allelic frequency (0.6098) in this population. Heterozygous genotypes in Shaul Sheep were: *ag*, *bg*, *ac* and *af*. Observed homozygosity and heterozygosity rates were 0.3659 and 0.6341, respectively. Expected homozygosity and heterozygosity rates were also 0.4561 and 0.5439.

The HW test showed that the studied population deviate significantly from the theoretical proportions in patterns of RsaI (*F*IS = 0.3795; P = 0.0005).

Discussion

Polymorphism in the second exon

Table 1: Frequency distribution, genotypic frequencies and size of observed bands after RsaI digests in different patterns of the second exon of MHC-DRB1 gene in Iranian Shaul Sheep

Genotype	Size of bands in *Rsa*I patterns	Number	Genotypic frequency (%)
RsaI aa	308 bp	1	1.21
RsaI bb	240 bp/68 bp	2	2.43
RsaI ff	84 bp/68 bp/54 bp/39 bp/33 bp/30 bp	2	2.43
RsaI ac	308 bp/117 bp/69 bp/68 bp/54 bp	1	1.21
RsaI gg	117 bp/68 bp/54 bp/39 bp/30 bp	25	30.48
RsaI ag	308 bp/117 bp/68 bp/54 bp/39 bp/30 bp	43	52.43
RsaI af	308 bp/84 bp/68 bp/54 bp/39 bp/33 bp/30 bp	1	1.21
RsaI bg	240 bp/68 bp/117 bp/68 bp/54 bp/39 bp/30 bp	7	8.53
total		82	

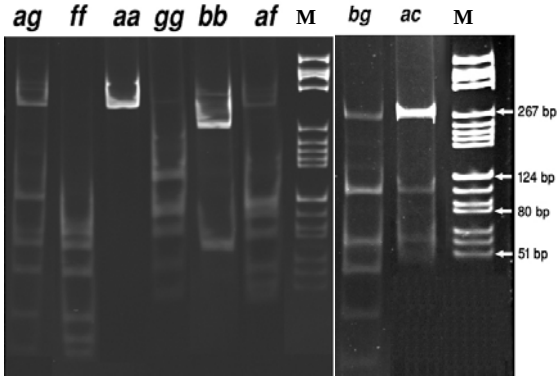

Fig. 1: PCR-RFLP analysis of the second exon of *Ovar-DRB1* gene (308 bp) from Iranian Shaul Sheep. Each line represents a haplotype (ag, ff, aa, gg, bb, af, bg, ac) which belongs to 5 different alleles (a, b, c, f, g) observed in this study. M is *Msp*I digest of *pBR*322 DNA fragment size marker

sequence of DRB locus in Iranian Shaul Sheep was studied. Compared to cattle and other ruminant species, *Ovar-DRB1* locus is poorly characterized and no data on the types and the extent of polymorphism in this species have been reported from Iran.

Different methods have been employed for typing *Ovar-DRB1* genes in various sheep breeds and have revealed extensive polymorphism at these loci. Among different methods PCR-RFLP analysis has been suggested for typing DRB1 alleles of farm animals (Amills *et al*., 1996; Rasool *et al*., 2000; Konnai *et al*., 2003b; Dongxiao and Yuan, 2004; Gruszczynska *et al*., 2005).

In the current study, PCR-RFLP was used to identify DRB1 second exon polymorphism in Shaul Sheep. We reported here a series of 8 DRB1 second exon types based on PCR-RFLP. Within the group described here there are a number of frequent *RsaI* patterns, including *a, b, c, f, g*. These patterns are in accordance with the published data for several recent studies concerning polymorphisms in exon 2 of *Ovar-DRB1* gene employing PCR-RFLP (Konnai *et al*., 2003a; Dongxiao and Yuan, 2004; Gruszczynska *et al*., 2005).

In contrast, patterns D, E and F, present in the Konnai *et al*. (2003a) study were not found in Shaul Sheep. Considering the digestion patterns of the studied exon with the *RsaI* enzyme, pattern *g* was the most frequent (0.2902) in Shaul Sheep, similar to Polish Sheep reported by Gruszczynska *et al*. (2005) (~ 0.40).

The HW test showed that the studied population deviate significantly from the theoretical proportions (*F*IS = 0.3795; P = 0.0005). A possible explanation for these results is the single origin of the founder population of Shaul Sheep in Iran. Genetic improvement programs conducted on this Iranian Sheep could also lead to the same results. At least inbreeding and frequency dependent selection might be the main reason for inadequate maintenance of MHC diversity in this ecotype.

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