Technical Note: DNA Typing for Ovine MHC *DRB1* Using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

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ABSTRACT

The ovine major histocompatibility complex (Ovar) class II *DRB1* second exon was amplified by polymerase chain reaction (PCR) from DNA samples of 52 Suffolk sheep. Polymerase chain reaction products were characterized by the restriction fragment length polymorphism (RFLP) technique using nine restriction enzymes, *RsaI*, *HaeIII*, *SacI*, *SacII*, *DdeI*, *NciI*, *Hin1I*, *EcoRI*, and *BstNI*, yielding 13 types. Sequencing of cloned PCR products identified 16 *Ovar-DRB1* alleles. Collectively, all PCR-RFLP patterns exactly matched those predicted from DNA sequences. These findings strongly indicate that the PCR-RFLP method using a combination of nine restriction endonucleases is a very powerful tool in Ovar typing.

(**Key words:** *Ovar-DRB1* exon 2, PCR-RFLP, genetic polymorphism, restriction enzyme)

Abbreviation key: MHC = major histocompatibility complex, **Ovar** = ovine MHC, **SSCP** = single-strand conformational polymorphism.

The major histocompatibility complex (MHC) of sheep is known as **Ovar** and is located on chromosome 20. The Ovar class II genes encode polymorphic glycoproteins composed of noncovalently 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. To date, 106 *Ovar-DRB1* alleles have been identified by DNA sequencing of exon 2 from various breeds of

sheep (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; Nagaoka et al., 1999; Jugo and Vicario, 2000; Konnai et al., 2003; GenBank, accession numbers: M73984, Z92726 to Z92735, AF324840 to AF324861).

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; and 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). On the other hand, in other domestic species, such as the cow (van Eijk et al., 1992), goat (Amills et al., 1996), pig (Shia et al., 1995), dog (Francino et al., 1997), cat (Kuwahara et al., 2000), and mouse (Peng and Craft, 1996), PCR-RFLP provides a simple method for the rapid analysis of class II polymorphisms. However, there is very little information available about PCR-RFLP methods for Ovar typing. Here, we describe the development of PCR-RFLP method that allows identification of polymorphisms in exon 2 of *Ovar-DRB1* for large numbers of animals and discuss the relationship between the results of PCR-RFLP and the identification of *Ovar-DRB1* alleles determined by cloning and sequencing of genomic DNA.

To develop the PCR-RFLP method, we received blood samples from 52 1-yr-old Suffolk sheep donated from JAPAN LAMB Co., Ltd., and two associated farms, Hiroshima farm and Otofuke farm. Samples of genomic DNA were obtained from whole blood as described by Takeshima et al. (2001, 2002). The second exon of *Ovar-*

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DRB1 was amplified by PCR technique in two stages because it was difficult to amplify the alleles from all samples by a single PCR. Therefore, to avoid mistakes due to PCR errors, we amplified alleles from five separate PCR per animal and confirmed whether these sequences were identical among five clones. The first round of PCR was performed with primers OLA-ERB1 (GC) 5'-CCG GAA TTC CCG TCT CTG CAG CAC ATT TCT T-3' and HL031 5'-TTT AAA TTC GCG CTC ACC TCG CCG CT-3' (van Eijk et al., 1992). We subjected 100 ng of genomic DNA to amplification by PCR in a total volume of 20 μ l of PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X 100, 1.5 mM MgCl₂, and 120 μ M dNTP], to which 0.2 mM each primer, and 2.5 U of rTaq polymerase (Toyobo Biochemicals, Tokyo, Japan) had been added. Reactions were performed in a thermocycler (TGRADIENT 96; Biometra, Goettingen, Germany) under the following conditions: one cycle of incubation for 5 min at 94°C, followed by 15 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s, with final extension at 72°C for 10 min. We used 3 μ l of the resulting mixture, plus primers OLA-ERB1 (GC) and OLA-XRBI (5'-AGC TCG AGC GCT GCA CAG TGA AAC TC-3') for the second round of PCR. The conditions for the second round of PCR were one cycle for 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s with final extension at 72°C for 10 min. Ten microliters of the mixture after the second round of PCR were digested for 4 h at 37°C with 5 U of *RsaI* (Toyobo Biochemicals), *HaeIII* (Toyobo Biochemicals), *SacI* (Toyobo Biochemicals), *SacII* (Toyobo Biochemicals), *DdeI* (New England Biolabs Inc., Beverly, MA), *NciI* (New England Biolabs Inc.), *Hin1I* (Takara Biomedicals, Otsu, Japan), *EcoRI* (Toyobo Biochemicals) or at 60°C with 5 U of *BstNI* (New England Biolabs Inc.) in a total volume of 20 μ l. Samples were resolved by a 3 or 5% agarose gel electrophoresis.

When each amplified product was cleaved by the restriction enzymes, we observed the following numbers of patterns in each of the digests: *RsaI*, 8; *HaeIII*, 6; *SacI*, 2; *SacII*, 2; *DdeI*, 3; *NciI*, 2; *Hin1I*, 2; *EcoRI*, 2; and *BstNI*, 2 (Table 1). Collectively, using PCR-RFLP, 13 *Ovar-DRB1* PCR-RFLP types (01-13) were identified in 52 Suffolk sheep. Examples of the PCR-RFLP analysis are shown in Figure 1. We verified the predicted RFLP profiles of *Ovar-DRB1* alleles by sequencing cloned amplified products as described elsewhere (Nagaoka et al., 1999), and all the observed patterns of fragments matched exactly those predicted from DNA sequences (Figure 2). Sequencing of cloned PCR allowed the identification of 16 *Ovar-DRB1* alleles among the 52 animals. These findings indicate that PCR-RFLP with nine restriction enzymes is a very powerful tool for Ovar typing.

Figure 1. The PCR-RFLP analysis of exon 2 of the *Ovar-DRB1* gene (296 bp) from seven Suffolk sheep, B.0248, W.199, W.194, B.0240, IW.279, Or.104, and W.196. The PCR-amplified fragments of DNA from the second-round of PCR were cleaved by *RsaI*, *HaeIII*, *SacI*, *SacII*, *DdeI*, *NciI*, *Hin1I*, *EcoRI*, and *BstNI*. M is *MspI* digest of *pBR322* DNA fragment size marker. Two animals, Or.104 and W.196, each yield a single pattern with all nine restriction enzymes (homozygosity), while five animals, namely, B.0248, W.199, W.194, B.0240, and IW.279, yielded multiple heterozygous patterns.

Table 1. Definition of 16 ovine major histocompatibility complex class II exon 2 alleles by PCR-RFLP using nine restriction enzymes.

PCR-RFLP types	Patterns									<i>Ovar-DRB1</i> alleles
	<i>RsaI</i>	<i>HaeIII</i>	<i>SacI</i>	<i>SacII</i>	<i>DdeI</i>	<i>Hin1I</i>	<i>NciI</i>	<i>EcoRI</i>	<i>BstNI</i>	
01	a	a	a	a	b	a	a	a	a	*0801
02	b	b	a	b	b	a	b	a	a	*0702, *0703
03	b	d	a	b	a	b	b	a	b	*1301
04	c	d	a	b	c	b	b	a	b	*1202
05	c	f	a	a	a	a	b	a	a	*03411
06	d	c	b	a	a	a	b	b	a	*03012
07	e	e	a	a	a	a	b	a	a	*0332
08	f	e	a	a	a	a	a	a	a	*0323, *0333
09	f	e	b	a	a	a	a	a	a	*0605
10	f	f	b	a	c	b	a	a	a	*0109
11	g	d	a	a	c	b	a	a	a	*01072, *0141
12	g	d	a	a	a	b	a	a	a	*0201
13	h	f	a	a	a	b	a	a	a	*0203

In cattle, 56 of the 106 sequenced MHC (*BoLA*)-DRB3 alleles from exon 2 could be distinguished as 56 PCR-RFLP types (<http://www.projects.roslin.ac.uk/bola/bolafram.html>). Moreover, using PCR-RFLP typing, many new *BoLA-DRB3.2* allele types were found in Jersey cows, European cattle, and African cattle that have not been reported previously (Gelhaus et al., 1995; Gillespie et al., 1998). We also investigated the predicted RFLP patterns in 106 previously described *Ovar-DRB1* alleles using our system. Furthermore, the 106 se-

quenced *Ovar-DRB1* alleles could be distinguished as 63 restriction fragment patterns by PCR-RFLP (data not shown). Thus, PCR-RFLP typing may be useful for the characterization of polymorphism in exon 2 of the *Ovar-DRB1* gene as well as for PCR-RFLP typing for *BoLA-DRB3.2* alleles. On the other hand, in this study, we have not yet verified the PCR-RFLP patterns of many *Ovar-DRB1* alleles. It should, however, be emphasized that the predicted PCR-RFLP patterns of endonucleases used have been individually verified, be-

Figure 2. The RFLP patterns (A) and sizes (B) of *Ovar-DRB1* alleles exon 2 with the enzymes *RsaI*, *HaeIII*, *SacI*, *SacII*, *DdeI*, *Hin1I*, *NciI*, *EcoRI*, and *BstNI*. The full length of the second-round products of PCR for alleles without a deletion is 296 bp (237 + 59 bp). Numbers indicate the sizes of DNA fragments in base pairs.

cause in this study all the observed PCR-RFLP patterns of fragments matched exactly those predicted from DNA sequences. The reliability and sensitivity of the PCR-RFLP method is attributed to strict enzyme substrate specificity of endonucleases that can reproducibly resolve a one-base mismatch in sequence. Thus, based on the published exon 2 sequences, we anticipate that our chosen panel of endonucleases will yield the predicted PCR-RFLP profiles for as yet unverified *Ovar-DRB1* alleles.

In sheep, the polymorphism of *Ovar-DRB1* has been defined by PCR-sequence-specific oligonucleotide probe analysis (Schwaiger et al., 1993; Schwaiger et al., 1994) and PCR-SSCP (Kostia et al., 1998; Jugo and Vicario, 2000), and these methods are relatively more time-consuming and expensive than the PCR-RFLP method. Furthermore, this method enables us to establish a molecular typing method for identifying *Ovar-DRB1* polymorphisms in large cohorts of animals, and the PCR-RFLP method might be a useful tool for analysis of frequencies of alleles in sheep. Therefore, the PCR-RFLP method may be used either independently or to complement *Ovar-DRB1* typing by PCR-SSO and PCR-SSCP methods in order to define or confirm allele identity.

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