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DLA-DRB1 histocompatibility genotyping using RT-nested PCR and cycle sequencing

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Abstract

Class-II histocompatibility genes are associated with predisposition to autoimmune diseases in many mammal species. We have developed a technique using reverse transcriptase and nested-PCR for amplification from blood samples of expressed sequences encoded by canine DLA-DRB1 loci. In the first polymerase chain reaction (PCR), we utilize primers DR-SP and DR-STOP as developed by Sarmiento et al. (1990). In the nested PCR, we utilize two additional primers, namely primer 57 [5′-TCTTGGAGGCTCCTGGATGACAGC-3′] and primer 367 [5′-CACAACTACGGGGTGATT-GAGAGC-3′] to produce a 334 bp amplified product. After digestion with restriction endonucleases, some of the alleles can be identified by restriction fragment length polymorphism (RFLP). The increasing information on new DLA-DRB1 alleles over the last two years renders the DLA-DRB1 too diverse for convenient use of RFLP. However, the expressed sequences amplified by our protocol can be conveniently identified by cycle sequencing. This RT *n*-PCR protocol will suffice for the genotyping of individual dogs at the DLA-DRB1 locus.

1. Introduction

Autoimmune reactions are complex and can appear capricious, appearing and disappearing with little apparent cause. In many species, these diseases run in families and genetics is assumed to be an underlying major risk factor (Nepom and Erlich, 1991; Ebers et al., 1995). Identification of genetic predispositions in canine patients could aid diagnoses and, thus, better guide veterinary practitioners in their choice of treatments.

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We are interested in identifying the genes which predispose dogs to autoimmune maladies, especially lymphocytic thyroiditis (Happ, 1995). In humans, predispositions to autoimmune diseases have been associated with specific genotypes of the polymorphic major histocompatibility complex (MHC) (Nepom and Erlich, 1991; Schwartz, 1993; Nepom, 1995). The usual associations involve the human HLA-DQ and HLA-DR class II loci. The protein products of these genes are plasma membrane heterodimers, consisting of α and β polypeptide chains which are encoded either by HLA-DRA and HLA-DRB or by HLA-DQA and HLA-DQB loci. The α/β heterodimers present peptide fragments to T-cell receptors (TCR) on $CD4^+$ lymphocytes.

Most MHC loci are polymorphic, with major diversity concentrated in the hypervariable regions that encode the sites which bind antigenic peptides during their presentation to the TCR. The link between specific class II MHC alleles and disease predispositions is rooted in the tendency of some 'presentation platforms' to bind self-antigen fragments (e.g. Kwok et al., 1995; Nepom et al., 1996; Wickler et al., 1997).

Dogs have provided experimental models for research on organ and bone marrow transplants (Deeg and Storb, 1994) and, thus, considerable effort has been devoted to understanding canine MHC genotypes. Histotyping of canine class II MHC loci traditionally utilized mixed lymphocyte reactions (Third International Workshop on Canine Immunogenetics: Bull et al., 1987; Deeg et al., 1986) but since 1990, more precise molecular techniques are available (Sarmiento et al., 1990; Kennedy et al., 1999a; Wagner, 1998; Wagner et al., 1998). In the dog, major class II diversity lies in the DLA-DQA1, DLA-DQB1, and DLA-DRB1 loci (Kennedy et al., 1999b).

At the onset of our research on potential associations between DLA genotypes and autoimmune disease, we chose to focus first on developing a convenient technique for routinely identifying alleles at the DRB1 locus, for which the most polymorphisms had been described (Deeg et al., 1986; Sarmiento et al., 1990). On the basis of the sequence information provided by Sarmiento et al. (1990) for cDNAs, we attempted to develop PCR-based techniques for convenient and unambiguous genotyping of the nine known DLA-DRB alleles, in homozygotes and heterozygotes. Our aim was to identify the alleles by the differences in the second exon. Several papers describing new alleles have been appeared since we began this work (e.g. Francino et al., 1997; Polvi et al., 1997; Kennedy et al., 1998; Wagner et al., 1998) and recently the terminology for the DLA alleles has been revised recently and standardized by the ISAG DLA Nomenclature Committee (Kennedy et al., 1999b). In the present paper, we will employ the allele designations set according to the Nomenclature Committee report.

2. Materials and methods

Our animal materials were mixed-breed Alaskan huskies from dog lots in Fairbanks, Alaska and purebred Doberman Pinschers from dog shows and kennels in Florida. We simplified the templates for PCR amplification by employing reverse transcriptase (RT-PCR). Total RNA was isolated either by the Tri-Reagent BD kit (Molecular Research Center, Cincinnati, Ohio) or by RNAeasy columns (Qiagen, Valenica, California). RNA was reverse transcribed into cDNA using the Gibco-BRL Superscript II enzyme and PCR

reagents. The reaction mixture consisted of 5× RT (SSI) buffer (4 μl), 10 mM dNTP mix (1 μl), cloned RNase inhibitor (Gibco-BRL) (1 μl), random hexamer primers (1 μl), 50% DMSO (1 μl) (Sidhu et al., 1996), RNA (10 μl), and Superscript II RT (2 μl). The reaction was allowed to run for 60 min at 37°C, and the reverse transcriptase inactivated by heating for 5 min at 95°C.

To make cDNAs of the expressed sequences in blood cells, we employed the polymerase chain reaction (PCR). The 20 μl reaction mixture from the reverse transcriptase step was added to a PCR reaction mix which included 10× Taq Polymerase buffer (Gibco-BRL) (8 μl), 50 mM $MgCl_2$ (4 μl), 10 mM dNTP mix (4 μl), primers (DR-SP [5′] (2 μl) and DR-STOP [3′] (2 μl, Sarmiento et al., 1990), sterile nanopure water to reach a total volume of 100 μl and an overlayer of 100 μl mineral oil (Sigma, sterilized) was added. After one minute of initial denaturation (94°C), Taq polymerase (1 μl) (5 U/μl, Gibco-BRL) was added to effect a hot start. The initial denaturation continued for a total time of 4 min and the PCR mix was subjected to 30 cycles of annealing (55°C, 2 min), DNA polymerization (72°C, 4 min), and denaturation (94°C, 1 min), allowed to polymerize for an additional 10 min, and finally cooled to 4°C. When run on an agarose gel, this reaction mixture sometimes contained faint bands at the desired 819 bp (data not shown).

To increase the amplification of the second exon, we used nested PCR, akin to that employed by Bein et al. (1992) for human DRB alleles and for Amills et al. (1996) for caprine DRB alleles. The primers for the N-PCR amplification, designated ‘57’ and ‘367’, were designed to produce a 334 bp product. Primer 57 spanned codons −24 through −16 in the sense strand [5′-TCTTGGAGGCTCCTGGATGACAGC-3′]. Primer 367 spanned codons 81 through 88 in the complementary strand [5′-CACAAC-TACGGGGTGATTGAGAGC-3′]. For the nested PCR, an aliquot (3–10 μl) from the first PCR reaction was subjected to a nested PCR reaction with increased magnesium (4 mM), the utilization of primers 57 and 367, and an annealing step at 61°C for 2 min. The length of the final product was confirmed as 334 bp by electrophoresis in 1–2% agarose gels.

We chose a panel of eight restriction enzymes to unambiguously identify a unique pattern of fragments for all potential genotypes of the nine alleles described by Sarmiento et al. (1990) (Table 1). Four enzymes were scored on the basis of presence or absence of a single cut (HinfI, ThaI, MboI, XmnI), while the other four enzymes (AvaI, BstyI, Bsp1286I, RsaI) yielded more complex patterns with different alleles. To confirm each identification, we purified the amplified products with the Qiagen QIAquick PCR purification kit and cycle sequenced with a Perkin–Elmer 373A Automated DNA Sequencer in the Core Facilities at the University of Vermont or at the University of Alaska Fairbanks. The resulting sequences were analyzed using ABI Sequence Navigator software.

3. Results and discussion

The nested PCR amplification consistently produced a single 334 bp product. The results from two representative digests of these products are shown in Fig. 1. The left

Table 1
For HinfI, MboI, ThaI, and XmnI with a single site for digestion

DRB1 allele	HinfI	MboII	ThaI	XmnI	AvaI			Bsp1286I					BstyI				RsaI									
	245, 89	228, 106	290, 44	268, 68	225	120	109	227	180	107	64	42	267	177	90	67	249	209	179	129	125	114	95	86	39	30
*0101	1	0	0	0+	+	−	+	+	−	+	−	−	+	−	−	+	−	+	−	−	+	−	−	−	−	−
*0102	1	0	0	0+	+	−	+	+	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−
*0201	1	0	1	0+	+	−	+	+	−	+	−	−	−	+	+	+	−	+	−	−	+	−	−	−	−	−
*0401	1	0	0	0	−	+	+	+	−	−	+	+	−	−	−	−	−	−	−	+	−	+	+	−	−	−
*0601	0	0	1	0+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	+	−	−	−	−	+	+	+
*0701	0	0	1	0+	+	−	+	+	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	+	+	−
*0801	0	1	1	0+	+	−	+	+	−	+	−	−	+	−	−	+	+	−	−	−	−	−	−	+	−	−
*0901	1	0	1	1+	+	−	+	+	−	+	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−
*1501	0	0	0	0+	+	−	+	−	+	−	+	+	−	−	−	−	−	+	−	−	−	−	−	+	+	−

Note: 1 indicates a cut and 0 indicates no cut. For the fragments from digestion by AvaI, Bsp1286I, BstyI, and RsaI, + indicates the presence of a fragment and − indicates no fragment of that size.

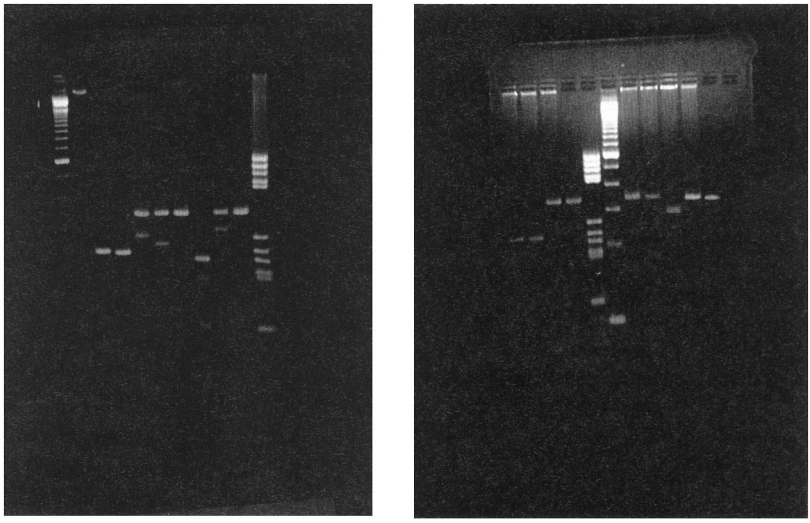

Fig. 1. RFLP from the DNA isolated from two Alaskan huskies after RT-*n*PCR. The lanes for the dog on the left (DRB1*0101/*0601) are AvaI, Bsp1286I, BstyI, HinfI, MboII, RsaI, ThaI, and XmnI followed a pBR322-HaeIII standard. The lanes for the dog on the right (DRB1*0601/*0801 variant) are AvaI, Bsp1286I, BstyI, HinfI, pPR322-HaeIII, 100bp ladder, MboII, RsaI, ThaI, and XmnI.

panel shows the cDNA from a DRB1*0101/*0601 heterozygote after digestion with the eight enzyme panel. The right panel shows an RFLP pattern of a dog with DRB1*0601 and another DRB1 allele not among those nine sequenced by Sarmiento et al. (1990). The presence of the unexpected allele was confirmed by cycle sequencing. This allele appeared to be a variant on what is now designated as DRB1*0801 (Kennedy et al., 1999b). We subsequently found two more unexpected RFLP profiles in other dogs.

Our RFLP technique distinguished among the nine previously known DRB1 alleles and also provided evidence for the presence of new alleles. With the likelihood of more and more alleles for DRB1, we judged that RFLP was not likely to remain robust as a discriminator. Therefore, we opted to make our identifications of alleles by cycle sequencing the 334 bp product of the nested PCR amplification.

The practical utility of this RT-*n*PCR amplification followed by cycle sequencing technique was demonstrated by its application to blood from 30 Doberman Pinschers. Within this sample of dogs, DLA-DRB1*0102, DRB1*0201, DRB1*0601, DRB1*0701, and DRB1*1901 are represented. The genotype frequencies are shown in Fig. 2.

A variety of protocols are now available for DLA histotyping at the molecular level. While our work using RFLP to histotype the DRB1 locus was in progress, an alternative RFLP technique was reported for DRB1 alleles (Francino et al., 1997). Both the Francino technique and our RT-*n*PCR reported in the present paper suffice for histotyping the previously known alleles (Sarmiento, 1996) and also detect additional alleles. However, as demonstrated in recent papers subsequent to the benchmark study by Sarmiento et al. (1990), the class II DLA loci are highly polymorphic and many alleles have been reported within the last year (Kennedy et al., 1999b). At the present time, the number of known

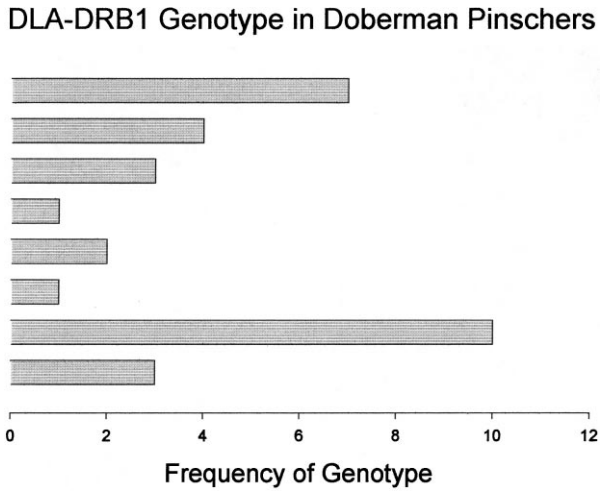

Fig. 2. The frequency of DLA-DRB1 genotypes in Doberman Pinschers as determined by RT-*n*PCR and cycle sequencing.

sequences for the DQA1, DQB1, and DRB1 loci, are 11, 25, and 36, respectively (Kennedy et al., 1999b). Notable among them are PCR-SSCP (single-stranded conformational polymorphism) developed by Wagner et al. (1998) and PCR-SSOP (sequence-specific oligonucleotide probe) developed by Kennedy et al. (1999a).

The amplified *n*-PCR product we describe in this paper is quite suitable for cycle sequencing to identify known alleles and to detect new ones and, thus, this RT *n*-PCR technique can serve to genotype individual dogs. However, the RFLP technique we developed is not efficient or adequate for convenient typing of the increasingly large number of alleles of DLA-DRB1 and reverse transcriptase followed by two-stage PCR is cumbersome for large numbers of samples. Other recently proposed alternatives (PCR-SSCP as proposed by Wagner et al. (1998) and PCR-SSOP as proposed by Kennedy et al., 1999a) are likely to be more efficient for broad screening.

MHC polymorphism can be used for population studies. For example, the HLA-DRB*201 allele found at high incidence in Italians and Rumanians but was not found in Singapore–Chinese, Thais, Indians, Koreans, French, Germans, and Spanish (Sujirachato et al., 1994). In dogs, similar techniques could be used to catalog the diversity within a breed or a strain and, thus, to examine the importance of particular associations between breed-predispositions to specific diseases and genetics. As well, these MHC loci might allow investigators to draw inferences about relatedness of different gene pools. The small sample examined in the present study is insufficient to draw any conclusions about breeds or strains, but work is in progress with larger sample sizes.

MHC polymorphism has been linked to diverse idiopathic medical syndromes other than those usually identified as autoimmune conditions. For example, certain HLA Class II alleles are associated with recurrent fetal loss in Danish women (Christiansen et al., 1994). The potential for use of animal histotyping in the evaluation of analogous reproductive conditions in companion and agricultural animals is intriguing.

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