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A simplified method for typing DRB alleles in deer

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ABSTRACT

Major histocompatibility complex (MHC) genes present foreign antigens to the immune system and polymorphism in these genes may have a key role in the variability of the host response to pathogenic challenge. The DRB genes are part of the MHC class II family whose proteins present foreign antigenic peptides on the surface of the macrophage for recognition by the CD4⁺T cells of the immune response. The genes are highly variable with over 100 different variants found in some species. The goal of this study was the development and testing of a simplified system for differentiating between the different DRB alleles already identified in red deer. The typing method chosen used DNA hybridisation to discriminate between the DRB alleles. The RNA from each deer was extracted from cultured leukocytes. Reverse transcription followed by PCR was used to amplify the DRB region. Amino-linked oligonucleotides representing variable regions of the DRB region were covalently bound to a nylon membrane and probed with end-labeled PCR product. The degree of binding of the labelled PCR products to the membrane-bound oligonucleotides was used to discriminate between the different DRB genes amplified. This proved to be a simple and robust method of typing which eliminated any requirement for electrophoresis.

Keywords: Major histocompatibility complex, Deer, DRB gene typing.

INTRODUCTION

The major histocompatibility complex (MHC) is a gene complex whose role is the presentation of foreign antigens to the immune system. These cell surface proteins are composed of two peptide chains, A and B, that present intracellularly degraded antigenic peptides of 8-25 residues (Brown *et al.*, 1993) to CD4⁺ T lymphocytes. This study examines the class II subset of the MHC genes, which present antigens from foreign particles engulfed by the cells and degraded within liposomes. The class II genes are expressed by B-lymphocytes, macrophages, activated T lymphocytes and dendritic cells. The human class II region is 1100 kilobases long and expresses six related gene groups DM, DN DO, DP, DQ and DR (Bodmer *et al.*, 1994).

The polymorphisms are located in the region of the protein that binds and presents the peptides, the sequence of which is encoded in exon 2 (Miyada *et al.*, 1985). Of these class II gene families, the DR and DQ are the most highly expressed and the most highly polymorphic.

The aim of this study was the development and testing of a simplified system for differentiating between the 48 different DRB exon 2 alleles already identified in red deer, Swarbrick *et al.*, (1995). The method presented in this paper uses DNA hybridisation of sequences amplified from exon 2 to membrane bound oligonucleotides with the presence or absence of binding to the oligonucleotide used to discriminate between DRB alleles.

METHODS

White blood cells were isolated from 10 ml of blood and resuspended in tissue culture media (RPMI 1640;

Gibco BRL, Gaithersburg, MD) as described by Griffin *et al.* (1991). Approximately 10 million cells were incubated at 37°C for 18-48 hours in tissue culture medium containing 21 µg/ml of Concanavalin A. Total RNA was isolated following the method of Chirgwin *et al.* (1979).

Synthesis of first strand cDNA was performed using Superscript II Reverse Transcriptase (Gibco BRL). Approximately 1 µg of total RNA was incubated at 42°C for 50 minutes in a 20 µl reaction volume containing 2 pmol of oligo (dT)15, 5 mM deoxynucleotides, 4 mM dithiothreitol, 1x Reverse Transcriptase buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl and 3 mM MgCl₂), 200 units Superscript II Reverse Transcriptase, and 40 units of RNasin (Promega).

The DRB exon 2 was amplified by PCR directly from the first-strand cDNA prepared as described above. Primers for PCR were designed from cattle sequence information (Groenen *et al.*, (1990)). The primers were selected on the basis that they had been previously used by Swarbrick *et al.* (1995) to amplify the DRB exon 2 region in deer for sequencing and there was no co-amplification of other regions.

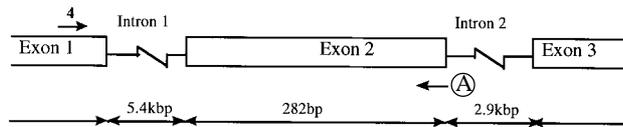
Primer BoLA4 (5' AGCTCTGACAGTGATACTGATGG-3') was designed from the 3' region of cattle DRB exon 1 and Primer BoLAA (5'-CTCGCCGCTGCACAGTGAAAC-3') from the 3' region of DRB exon 2 (Figure 1). Primer A (20 pmol/µl) was end-labeled using [³²P] ATP and T4 polynucleotide kinase, as described by Sambrook *et al.* (1989). The reaction contained: 1 µl of first-strand cDNA reaction mixture (as above), 20 pmol of unlabeled primer, 0.5-5 pmol labeled primer, 0.2 mM deoxynucleotides, 1x Jeffreys buffer (Jeffreys *et al.*, 1990) in a total volume of 50 µl.

PCR products of 320 nucleotides were obtained from amplification at: thirty cycles of 94°C for 30 seconds, 57°C

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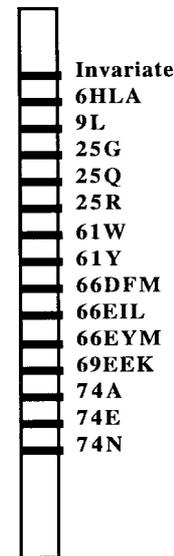
FIGURE 1: The arrangement of the DRB exons 1-3 in the genome showing the position and size of Introns 1 and 2. 4 is primer BoLA4. A is primer BoLAA



for 1 minute, and 72°C for 1 minute. Designing primers to amplify across intron 1 (5.4 kbp) effectively selected against any amplification from any genomic DNA likely to be contaminating the cDNA preparation.

Oligonucleotides (Table 1) with a 5' terminal amino linker (150 pmol) were covalently bound in 2mm wide arrays to an activated negatively charged Biodyne C membrane (PALL Europe Limited) using a miniblotted as described by Kamerbeek *et al.* (1997). The membrane with bound oligonucleotides was cut, perpendicular to the oligonucleotides, into 2mm wide strips. Each strip therefore contained an array of the 15 bound oligonucleotides. This is illustrated in Figure 2. Initially the strips were washed for 15 minutes with 20 mM EDTA, pH 8, at room temperature, and then with 2x SSPE (3 M NaCl, 0.2 M NaH₂PO₄·2H₂O, 0.02 M disodium EDTA, pH 7.4)/ 0.1% SDS for five minutes at 60°C. Each strip was then hybridized in a 1.5 ml microfuge tube with an aliquot (20-30 l) of the endlabeled PCR reaction (denatured for 10 minutes at 95°C) and 1.2 ml of hybridization buffer (Church and Gilbert, 1984) (0.263 M phosphate buffer (0.0684 M Na₂HPO₄, 0.0316 M NaH₂PO₄) pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA, 1% Dextran Sulphate) at 52°C for two hours. The membranes were washed in 6x SSC (3 M NaCl, 0.3 M Trisodium citrate, pH 7) for twenty minutes at room temperature, then stringency washed at 57°C for

FIGURE 2: Schematic diagram of the Biodyne C membrane with bound amino-oligonucleotides representing the DRB alleles of exon 2 hybridized to an end-labeled PCR product. The amino-linked oligonucleotides were bound to the membrane in the order shown in the figure.



ninety seconds with 6x SSC. Membranes were exposed to X-Omat™ AR-5 film (Eastman Kodak Company, Rochester, NY, USA) for up to two days at -80°C to obtain an autoradiogram.

RESULTS

An example using the new typing method is shown in Figure 3. The animals typed comprise a small deer pedigree of one sire and two dams, with one offspring from each dam. The autoradiograph for each individual is

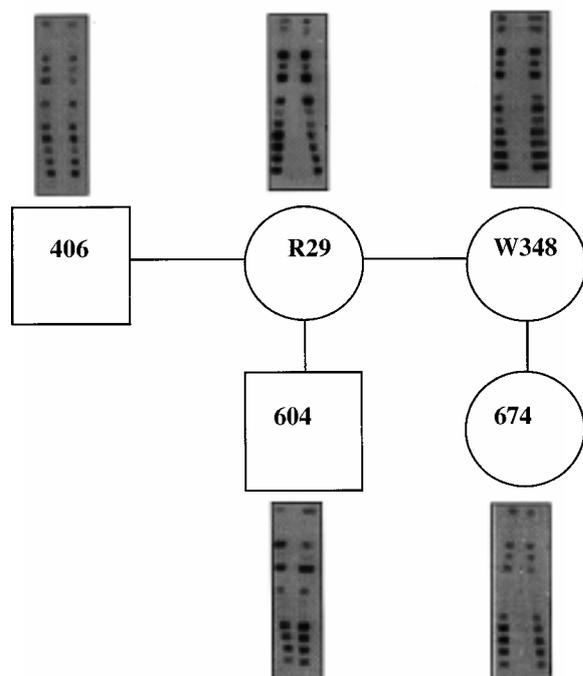
TABLE 1: DNA sequence of the fifteen amino-linked oligonucleotides used to type the DRB exon 2 sequences. The left column shows the name of the oligonucleotide, the next column shows the nucleotide sequence of the oligonucleotide (I = Inosine). The third column shows the amino acid codons of exon 2 that the oligonucleotide was designed to detect.

Name	Oligonucleotide Sequence	Target Amino Acid Codons*
Invariate	5' amino GCGGTGACIGAGCTGGGCGG 3'	49** AVTELGR
6HLA	5' amino CACATTTCTGGAGCATCTTAAGG 3'	5 PHFLEHLK
9L	5' amino CACATTTCTGCTGTATIITAAG 3'	5 PHFLLY(A/S/T)K
25G	5' amino CAGCGGGTGGGGIICCTG 3'	21 TQRVG(F/L/Y)L
25Q	5' amino CAGCGGGTGCGGIICCTG 3'	21 T(D/E/Q)RVQ(F/Y)L
25R	5' amino CAGCGGGTGCGGIICCTG 3'	21 TQRVR(F/L/Y)L
61W	5' amino GCCAAGIICTGGAACAGCCAG 3'	58 AK(G/L/Y)WNSQ
61Y	5' amino CGCCAAGTACTATAACAGCCA 3'	58 AKYYNSQ
66DFM	5' amino CCAGAAGGATTTTCATGGAGCAG 3'	63 QKDFMEQ
66EIL	5' amino GCCIGAAGGAGATCCTGGAG 3'	63 SQKEILE
66EYM	5' amino AGCCAGAAGGAGTACATGGAG 3'	63 SQKEYME
69EEK	5' amino CCTGGAGGAGAAGCGGGCC 3'	68 LEEKRA
74A	5' amino CGGGCCGCGGTGGACACG 3'	72 RAAVDT
74E	5' amino CGGGCCGAGGTGGACACG 3'	72 RAEVDT
74N	5' amino CGGGCCAATGTGGACACGTA 3'	72 RANVDTY

*The one letter code for amino acids is used.

**The number refers to the position of the amino acid in amino acid sequence of exon 2.

FIGURE 3: The inheritance of DRB alleles in a small pedigree of deer. The pedigree is presented as a sire (406) and two hinds (R29 and W348), and the offspring of each hind (604, 674). Represented with each animal is an autoradiograph. This shows the result of hybridization of the individuals amplified exon 2 region with the bound oligonucleotides representing the different DNA sequences of exon 2. The oligonucleotides were bound to the membrane in the order shown in Figure 2.



shown. Two strips are shown for each individual representing two separate PCR reactions. This was done to ensure that the typing method was reproducible. Also included on the strip is a control oligonucleotide containing sequence from a region that doesn't vary. This acts as a positive control for PCR amplification. Because each deer can have up to 6 different expressed DRB alleles, (Swarbrick, *et al.*, 1995), this method is only able to identify the presence or absence of a particular sequence in an individual and not determine how many copies of the sequence are expressed when it is present. As expected, the invariable sequence was found in all individuals. The sequences 9L, 61W and 74N which were not present in sires and dams were not found in the offspring. Variation in inheritance was observed with other sequences; 6HLA, present in the dams but not the sire was not inherited by either offspring and 61Y present in both sire and dams was inherited by only one of the offspring.

DISCUSSION

To completely define all the expressed DRB alleles of individual deer would require a major cloning and sequencing effort well beyond the scope and cost of a routine typing method. The virtue of this method is its simplicity. To achieve this simplicity we have compromised our ability to quantify the number of expressed exon

2 sequences of a particular type. The positive results we get indicate the presence of a sequence but give no indication of the number of copies present. This feature means that the method will be most useful for allele association studies where the presence or absence of a particular sequence can be associated with response to a particular disease. In the results presented here, a low stringency hybridization is used to detect the presence or absence of an expressed sequence. The method cannot, however, be used in genetic linkage studies where inheritance is studied as each individual gene must be identified. The first use of the method will be in to determine whether any of these DRB sequences is associated with resistance or susceptibility to *Mycobacterium bovis* in red deer.

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