Immunogenetics (2002) 54:348–352
DOI 10.1007/s00251-002-0465-5

ORIGINAL PAPER

**Lorna J. Kennedy · Ruth Ryvar · Rosalind M. Gaskell
Diane D. Addie · Kim Willoughby · Stuart D. Carter
Wendy Thomson · William E.R. Ollier Alan D. Radford**

# Sequence analysis of MHC DRB alleles in domestic cats from the United Kingdom

Received: 10 January 2002 / Revised: 1 May 2002 / Published online: 6 July 2002


**Abstract** The DRB gene of the domestic cat MHC appears to be highly polymorphic, with 71 alleles provisionally reported, based on exon 2 sequence. However, these alleles were reported prior to the adoption of strict criteria for allele identification. In this study, we investigated FLA-DRB exon 2 polymorphisms in a cohort of 33 British domestic cats by polymerase chain reaction (PCR) and clonal sequence analysis. Applying the strict criteria for assigning new alleles as used by the established mammalian MHC nomenclature committees, we defined 13 FLA-DRB alleles, including four previously unreported alleles. We identified many sequences that were one or two base pairs different from these 13 defined alleles, and have shown that these are most likely artefacts of PCR amplification. When the same criteria for allele acceptance were applied to the remaining previously reported sequences, 11 further alleles were confirmed. This suggests that to date there is good evidence for 24 FLA-DRB alleles fulfilling nomenclature criteria. Analysis of these 24 alleles reveals a similar pattern of MHC polymorphism to that seen in other mammals, with three regions of hypervariability.

**Keywords** Cat · Major histocompatibility complex · Feline leucocyte antigen · DRB

EMBL accession numbers: AJ428209 for rr2; AJ428210 for rr3; AJ428211 for rr6; AJ428212 for rr7

L.J. Kennedy (✉) · R. Ryvar · R.M. Gaskell · K. Willoughby
S.D. Carter · W. Thomson · W.E.R. Ollier · A.D. Radford
Mammalian Immunogenetics Research Group,
Faculty of Veterinary Science, University of Liverpool,
Crown Street, Liverpool, L69 7ZJ, UK
e-mail: Lorna.Kennedy@man.ac.uk
Tel.: +44-161-2757316, Fax: +44-161-2751617

D.D. Addie
Department of Veterinary Pathology, University of Glasgow,
Bearsden Road, Bearsden, Glasgow, G61 1QH, UK

## Introduction

All higher species examined to date have within their genome a major histocompatibility complex (MHC), containing three regions of tightly linked genes (class I, II and III). These genes are central to the control of the immune response, and influence susceptibility/resistance to disease and vaccine response. Furthermore, in human, matching of donor and recipients for MHC polymorphisms is beneficial for transplant survival. The human (Rhodes and Trowsdale 1999) and mouse (Amadou et al. 1999) MHC are the best characterised. Although less is known for other species, it is clear that the number of genes in the MHC of different species varies, as may the extent of any polymorphism.

Molecular characterisation of the domestic cat MHC [feline leucocyte antigen (FLA)] has only recently begun. The FLA system is known to contain class I, II and III genes (Yuhki and O'Brien 1988), although the precise number of genes and their relative positions remains uncertain. Genes within the class I and II regions appear to be highly polymorphic, but the full extent of this polymorphism has not yet been determined.

To date, most studies have concentrated on FLA-DRB exon 2 polymorphisms. A total of 71 FLA-DRB alleles have been provisionally reported in studies of domestic cats from around the world (Kuwahara et al. 2000; Yuhki and O'Brien 1997). Within these 71 FLA-DRB sequences, the hypervariable antigenic recognition domains appear to be present, but less clearly defined than those of other mammalian MHCs (Bontrop et al. 1999; Davies et al. 1997; Kennedy et al. 2001; Marsh et al. 2001), with many amino acid polymorphisms throughout exon 2. The number of DRB genes present in individual cats is not yet clear. The presence of two genes has been confirmed (Beck et al. 2001). However, there is some evidence, based on the number of polymorphic alleles identified within individual cats, to suggest that further DRB genes may be present in some animals (Beck et al. 2001; Kuwahara et al. 2000; Yuhki and O'Brien 1997). However, previous studies have not used recognised criteria

to assign new alleles, and the possibility exists that some previously reported alleles might represent PCR artefacts rather than genuine alleles.

In this paper we studied FLA-DRB polymorphisms in a group of British domestic cats. We compare our results with those of previous studies using the strict criterion for defining new alleles adopted by existing nomenclature committees.

## Materials and methods

Blood samples (EDTA) were obtained from 33 cats from three different breed groups within the United Kingdom (11 Korats, 10 Burmese and 12 domestic short hair). Total DNA was extracted from 100 µl of blood according to the manufacturer's protocol (QIAamp DNA Mini Kit; Qiagen). Primers for initial PCR amplifications were based on those used to amplify exon 2 of human HLA-DRB genes (DRB219 and DRB61a), as used previously by Yuhki and O'Brien (1997). However, the forward primer could not amplify all the published alleles due to a polymorphic position at its 3′ end; thus, one nucleotide was removed in order to increase the number of alleles that could be detected. Therefore, the primer sequences used for subsequent PCRs in this study were DRB219 (modified), 5′-CCACACAGCACGTTTC[T/C]T-3′, and DRB61a, 5′-CCGCTGCACTGTGAAGCT-3′. This modification was also used by Kuwahara (2000).

Initial PCRs used a standard non-proof reading *Taq* DNA polymerase (ABgene). In later PCRs an alternative enzyme, *Pfu* (Stratagene), was used, to reduce PCR-associated substitutions. Each PCR was performed using 100 ng of each primer and 1 µl of template DNA in a final volume of 50 µl, according to the manufacturer's instructions. Cycling conditions for PCR consisted of 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by a final extension stage of 72 °C for 10 min.

All amplicons were cloned using appropriate commercial vectors (TOPO TA and Zero Blunt TOPO PCR cloning kits for sequencing; Invitrogen). Recombinant plasmids were selected and purified according to manufacturer's instructions (Wizard Plus Minipreps, Promega) and standard techniques. Multiple clones of each amplicon were sequenced according to conditions specified by the manufacturer (ABI prism dye terminator cycle sequencing ready reaction kit; Perkin Elmer). All clones were sequenced in one direction only, since similar work in the dog has shown no differences between forward and reverse sequences in over 800 clones (Kennedy unpublished data).

In order to determine the approximate error rate associated with the PCR amplification protocol used in this study, we amplified a known template (previously cloned FLA-DRB*0107 in pCR4-TOPO; Invitrogen) using the standard PCR protocol. Multiple clones of this amplicon were made in a second vector (pCR-XL-TOPO; Invitrogen) and sequenced as described. In order to minimise template plasmid carry-over through the entire PCR and transformation protocol, the template plasmid was first digested using *Pvu*II. By using a distinct vector for the second round of cloning, sequence analysis was able to confirm that all sequenced products had been through the entire reamplification protocol rather than representing carry-over of the original template plasmid.

Sequence alignments were performed using programs PILEUP and PRETTY from version 8 of the Wisconsin package, Genetics Computer Group (Deveraux et al. 1984). Comparative sequence alignments were generated by Format_Aln.cgi, which was written by James Robinson for the IMGT/HLA Database. Phylogenetic analysis was performed using programs SEQBOOT, DNADIST (Kimura) and NEIGHBOR from the Phylip package (Felsenstein 1989). Sliding window analysis (window size 5) of the observed synonymous (dS) and non-synonymous substitutions (dN) within the sequence were determined based on the method of Nei and Gojobori (1986), as implemented in SNAP (http://hiv-web.lanl.gov/SNAP/WEBSNAP/SNAP.html).

**Table 1** Results of clonal sequence analysis of an amplicon derived from one cat in this study

| Clone number | Sequence |
|---|---|
| 1,2 | DRB*0501 |
| 3 | DRB*0501 except C-28→T |
| 4 | DRB*0501 except A-122→G |
| 5 | DRB*0501 except A-242→G |
| 6, 7 | DRB*0107 |
| 8 | DRB*0107 except G-231→A and T-233→G |
| 9 | Recombinant 5′-DRB*0501 and 3′-DRB*0107. Cross-over occurs somewhere between 111 and 124 |

## Results

Initial sequence analysis of amplicons generated using primers DRB219 and DRB61a suggested these primers only amplified a subset of possible FLA-DRB alleles. Therefore, the forward primer was modified. Using these modified primers (DRB219 modified and DRB61a), amplicons were generated from all 33 cats in this study.

Multiple clones were sequenced for amplicons obtained from each cat. Table 1 shows a set of nine clones derived from one of the cats. Of these nine clones, two were an exact match with the previously published DRB*0501 allele (Yuhki and O'Brien 1997) and three had single base changes from DRB*0501. Two others were an exact match with the previously published DRB*0107 allele (Yuhki and O'Brien 1988) and one had two base changes from DRB*0107. The ninth clone appeared to be a recombinant of DRB*0501 at the 5′ end with the 3′ part of DRB*0107. In ongoing studies, this recombination has not been found in four other family members (data not presented). Several other instances of apparently new alleles in other cats were later revealed to be recombinations between two alleles identified in the same cat (data not presented). Similar PCR error rates and formation of a variety of PCR artefacts have been observed in MHC DLA-DRB1 cloning experiments in the dog, where upwards of 800 clones have been sequenced (Kennedy, unpublished data). In order to determine the approximate error rate associated with our PCR, sequence was obtained from seven clones derived from a known template (FLA-DRB*0107). Five of these clones were identical to the template. However, two clones (28.5%) each had single base discrepancies compared with the original template (data not presented).

Because of the likely error rates associated with PCR amplification, individual sequences from this study were not taken as new alleles until they had been identified in a second independent PCR. Using this criterion, a total of thirteen FLA-DRB alleles were identified among the 33 cats studied. Table 2 lists the 13 alleles identified, the number of cats in which each allele was found, and the number of clones sequenced that gave exact matches with these alleles (range per cat per allele: 1–10). Four of the 13 alleles were new, having not previously been reported (local nomenclature DRB rr2, rr3, rr6 and rr7).

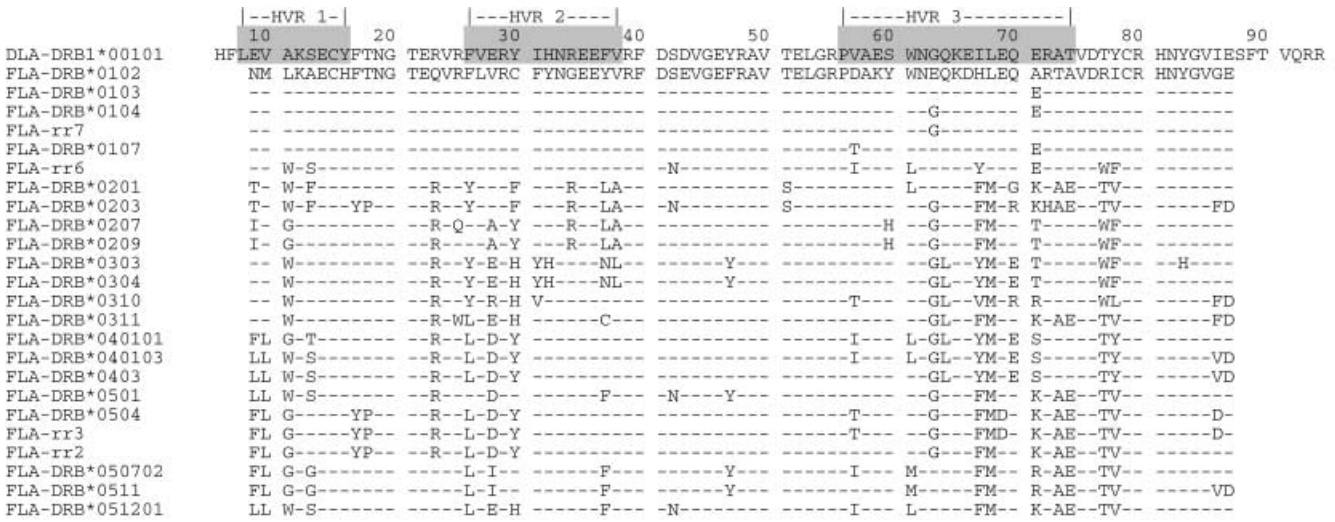


**Fig. 1** FLA-DRB partial exon 2 amino acid alignments. The HVR are indicated as defined for canine DRB1, and codons are numbered according to alignment with dog and human DRB1 (Kennedy et al. 1999)

**Table 2** FLA-DRB alleles found in this study

| FLA-DRB allele | No. of cats in which allele found | No. of clones sequenced which gave exact match | No. of clones with 1- to 2-bp mismatch |
|---|---|---|---|
| 0102 | 3 | 3 | 1 |
| 0103 | 2 | 7 | 1 |
| 0104 | 2 | 10 | 2 |
| 0107 | 18 | 42 | 9 |
| 0203 | 4 | 17 | 1 |
| 0501 | 18 | 72 | 25 |
| 0511 | 5 | 11 | 5 |
| 051201 | 5 | 31 | 10 |
| 040103 | 4 | 17 | 7 |
| rr2 | 4 | 13 | 0 |
| rr3 | 3 | 14 | 7 |
| rr6 | 4 | 9 | 0 |
| rr7 | 4 | 10 | 1 |
| Totals | 76 | 256 | 69 |

**Table 3** Numbers of FLA-DRB alleles found in each cat

| No of DRB alleles found per cat | No of cats (*n*=33) |
|---|---|
| 1 | 10 |
| 2 | 10 |
| 3 | 6 |
| 4 | 7 |

Five alleles were the same as those previously reported as being present in more than one cat [DRB*0102, *0103, *0104, *0107 and *0501 (Yuhki and O'Brien 1997)]. Four alleles were identical to sequences previously found in only single cats [DRB*0203, *0511, *040103 and *051201 (Kuwahara et al. 2000; Yuhki and O'Brien 1997)]. In total, from the 33 cats studied, there were many sequences that were one or two base pairs different from defined FLA-DRB alleles identified in the same cat [69 clones from a total of 328 (21%), see Table 2]. These were assumed to be due to mis-incorporation of bases during PCR amplification. Table 3 shows the numbers of alleles found per cat. In this study, the maximum number of alleles found in any single cat was four.

For further analysis, the 13 alleles identified in this study were compared with 11 other alleles from previous studies that also satisfy the strict criterion of being identified in more than a single PCR (Kuwahara et al. 2000; Yuhki and O'Brien 1997). Figure 1 shows the amino acid alignment for these 24 alleles, together with dog DLA-DRB1*00101 for comparison. Numbers refer to codon positions according to alignment with human and dog sequences (Kennedy et al. 1999). The position of the three hypervariable regions (HVRs), as defined for the dog (Kennedy et al. 1999), are generally conserved in the 24 cat alleles, although the position of HVR3 may be somewhat further from HVR2 in the cat. (The nucleotide alignment can be obtained from our web site: http://www.pcweb.liv.ac.uk/ALANRAD/Mirg/MIRGhome.htm or from http://www.ebi.ac.uk/imgt/mhc/fla). Phylogenetic analysis of these 24 allele sequences is shown in Fig. 2, where three clusters of sequences are identified [a cluster has

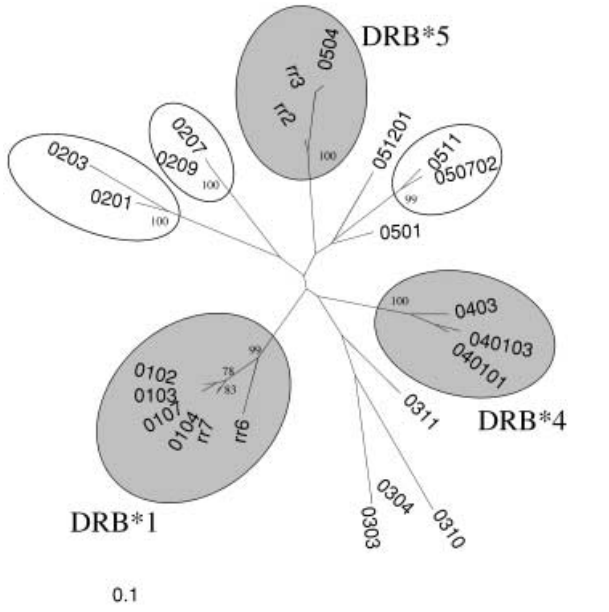


**Fig. 2** Phylogenetic analysis of the 13 FLA-DRB partial exon 2 sequences in this study, plus 11 others previously reported (Kuwahara et al. 2000; Yuhki and O'Brien 1997). *Grey bubbles* correspond to groups previously reported (Yuhki and O'Brien 1997), and are indicated only where greater than two sequences cluster together supported by a high bootstrap value (>75). *Clear bubbles* surround other potential groups, which are considered provisional, being supported by high bootstrap values, but that currently contain only two sequences

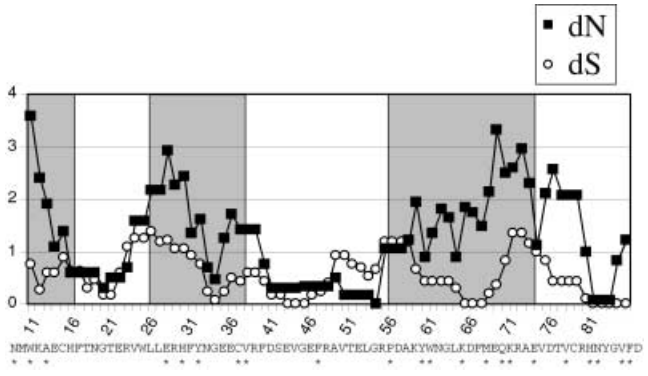


**Fig. 3** Sliding window analysis of synonymous (dS) and non-synonymous (dN) mutations within exon 2 of FLA-DRB based on a total of 24 alleles (window size 5). Codons are numbered according to alignment with dog and human DRB1. The consensus sequence for the alleles is aligned beneath the graph. *Asterisks* correspond to the residues identified as important for antigen binding by X-ray crystallography in HLA (Brown et al. 1993). *Shaded areas* correspond to HVRs as defined for canine DRB1 (Kennedy et al. 1999)

been defined as a group of more than two sequences supported by a high (>75) bootstrap value]. These correspond to the three previously characterised groups: Feca-DRB*1, *4 and *5 (Yuhki and O'Brien 1997). The pattern of synonymous (dS) and non-synonymous (dN) nucleotide substitutions along the feline DRB locus based on these 24 alleles is shown in Fig. 3. In general, dN is greater than dS within the HVRs and in regions corresponding to mapped antigenic recognition sites within human DRB.

## Discussion

In this study, we have identified 13 FLA-DRB alleles in 33 cats by PCR and clonal sequence analysis. These alleles meet the criteria used by the human, cattle and dog nomenclature committees to define new alleles (Davies et al. 1997; Kennedy et al. 1999; Marsh et al. 2001). The criteria are that when using DNA cloning and sequencing there have to be at least three identical clones, identified in either two separate PCRs from the same individual, or from PCRs from at least two different individuals. Of the 13 alleles identified in this study, four are new (DRB rr2, rr3, rr6 and rr7), and five were previously reported and already fulfilled the criteria for new alleles (DRB*0102, 0103, 0104, 0107 and 0501). The remaining four alleles (DRB*0203, 0511, 040103 and 051201) were previously found in single cats and single clones only. A total of 11 further alleles from previous studies also fulfil these criteria (Kuwahara et al. 2000; Yuhki and O'Brien 1997). This gives a total of 24 alleles that have been found in more than one cat and/or more than one laboratory.

In both previous studies and this paper, a proportion of identified sequences were only found in clones from individual PCRs. As such, these sequences fail to meet the criteria for allele identification and some are likely to represent artefacts of PCR (Ennis et al. 1990). Indeed, many of these sequences were only one or two base pairs different from accepted alleles that were also identified in the same PCR (e.g. in Table 1, clones 3 and 4 are each only one nucleotide different from DRB*0501, which was also found in the same cat). Another source of sequencing artefacts in studies based on clonal sequence analysis of PCR products is intra-PCR recombination. Such artefacts would appear very different from other alleles. Such an event may well explain hybrid sequences identified in this study (Table 1, clone 9) and previous studies [e.g. DRB*0101 could be a recombination between DRB*051201 and DRB*0107, both of which are relatively frequent alleles (Yuhki and O'Brien 1997)]. Although errors during PCR may mean that many of the other alleles previously reported turn out to be artefacts of methodology, it is likely that some of these alleles will eventually be confirmed as more data become available. The authors are currently in the process of establishing a feline MHC nomenclature committee, to ensure standard and rigorous criteria for the identification and naming of new alleles. In this paper we have used local laboratory names for new alleles (rr2, rr3, rr6 and rr7). To avoid confusion, these are deliberately unlike the official names that will be assigned to these alleles by the newly established feline nomenclature committee.

In our study, the maximum number of alleles found in any individual cat varied from one to four, suggesting

the presence of a minimum of two DRB genes per haplotype. However, it is clear that DNA cloning and sequencing does not necessarily identify all the alleles in a cat, and we have not sequenced enough clones from each cat to be certain that all alleles have been found. In previous studies, up to six alleles have been found in single cats (Kuwahara et al. 2000; Yuhki and O'Brien 1997). However, this may represent an overestimate due to PCR artefacts. Also, different FLA haplotypes may carry different numbers of expressed (and unexpressed) DRB genes, as is the situation in many other mammals including humans, chimpanzees and marmosets (Bontrop et al. 1999), cattle (Lewin et al. 1999) and horse (Fraser and Bailey 1996). Ultimately, in order to define the precise number of DRB genes in the cat, it will be necessary to develop non-clonal molecular typing methods.

The patterns of polymorphism in these 24 FLA-DRB alleles appear to match those found in other species, including human (Marsh et al. 2001), cattle (Davies et al. 1997) and dog (Kennedy et al. 2001), having three HVRs, interspersed with largely conserved regions. These data suggest that the cat MHC is not different from the MHC of other mammals. The further study of the feline MHC will be important both for comparative studies and for understanding individual variation in response to vaccination, infection and disease in cats. It may be particularly beneficial to the improved success of feline kidney transplantation (Kuwahara et al. 2001).

**Acknowledgements** This study was kindly supported by a grant from the Pet Plan Charitable trust. All the experiments performed for this study comply with the current British laws.

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