Immunogenetics (2006) 58:995–1001
DOI 10.1007/s00251-006-0164-8

BRIEF COMMUNICATION

MHC class I allele frequencies in pigtail macaques of diverse origin

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Received: 15 August 2006 / Accepted: 21 September 2006 / Published online: 10 November 2006

Abstract Pigtail macaques (*Macaca nemestrina*) are an increasingly common primate model for the study of human AIDS. Major Histocompatibility complex (MHC) class I-restricted $CD8^+$ T cell responses are a critical part of the adaptive immune response to HIV-1 in humans and simian immunodeficiency virus (SIV) in macaques; however, MHC class I alleles have not yet been comprehensively characterized in pigtail macaques. The frequencies of ten previously defined alleles (four *Mane-A* and six *Mane-B*) were investigated in detail in 109 pigtail macaques using reference strand-mediated conformational analysis (RSCA). The macaques were derived from three separate breeding colonies in the USA, Indonesia and Australia, and allele frequencies were analysed within and between these groups. *Mane-A*10*, an allele that restricts the immunodominant SIV Gag epitope KP9, was the most common allele, present in 32.1% of the animals overall, with similar frequencies across the three cohorts. Additionally, RSCA identified a new allele (*Mane-A*17*) common to three Indonesian pigtail macaques responding to the same Gag $CD8^+$ T cell epitope. This broad characterization of common MHC class I alleles in more than 100 pigtail macaques further develops this animal model for the study of virus-specific $CD8^+$ T cell responses.

Keywords Pigtail macaques · MHC class I · Reference strand-mediated conformational analysis (RSCA) · $CD8^+$ T cell

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There is an urgent need for the development of an effective vaccine against human immunodeficiency virus (HIV). Major Histocompatibility complex (MHC) class I-restricted $CD8^+$ T cell responses are a vital component of immune control of HIV infection in human and simian immunodeficiency virus (SIV) infection of macaques (Lifson et al. 2001). Accordingly, successful HIV vaccine strategies will likely rely on harnessing the potential of MHC-restricted T cell-mediated immunity. It is therefore an increasingly important task to better understand the natural mechanism (s) by which $CD8^+$ T cells function to fight diseases like HIV. This, in turn, requires a detailed study of MHC class I alleles present in our current animal models.

There are a number of macaque models commonly used in HIV vaccine research, primarily in the SIV and chimeric simian/human immunodeficiency virus (SHIV) infection of rhesus (*Macaca mulatta*), cynomolgus (*M.*

fascicularis) and pigtail macaques (*M. nemestrina*). However, there is a decreasing and geographically limited supply of Indian rhesus macaques, which is the most well-studied model (Cohen 2000). Indian origin rhesus macaques expressing the *Mamu-A*01* allele are in even shorter supply, as MHC tetramers are commonly available to study $CD8^+$ T cell immunity to *Mamu-A*01*-presented epitopes (Miller et al. 1991; Mothe et al. 2002). Cynomolgus macaques are also widely used, but many SIV and SHIV strains are less pathogenic in this species (Reimann et al. 2005). This problem has recently led to the emergence of important and reliable alternative models for studying the role of $CD8^+$ T cells in HIV infection. Pigtail macaques, in particular, have become an increasingly studied model of SIV/SHIV pathogenesis, which is suitable for vaccine studies (Batten et al. 2006; Dale et al. 2004; Kent et al. 2001).

For the pigtail macaque model to be of maximum usefulness in HIV vaccine development, our understanding of the T cell immunity and MHC genetics of these animals must be greatly improved (Lafont et al. 2004; Smith et al. 2005a,b). To date, research has shown that pigtail macaques possess MHC-A, -B and -I loci, and, as in rhesus macaques, the MHC-A and -B loci are duplicated (Lafont et al. 2004). However, few animals have been studied in detail. Thus far, 16 *M. nemestrina Mane-A* and 22 *Mane-B* MHC class I alleles have been characterized from only 11 pigtail macaques (Lafont et al. 2004; Smith et al. 2005a). Work is also being done to determine which SIV/SHIV epitopes are restricted by these 38 MHC alleles. For example, *Mane-A*10* has been shown to restrict the immunodominant SIV Gag epitope KP9 and is associated with lower viral loads after SIV infection (Smith et al. 2005a,b).

Two areas of further exploration include assessing *Mane* class I allele frequencies and identifying additional MHC alleles. This study aims to enhance our current understanding of *Mane* class I genetics by: (1) exploring the frequencies of MHC class I alleles in a large sample of pigtail macaques from diverse origins and (2) identifying previously uncharacterized MHC alleles that are shared between multiple animals.

Reference strand-mediated conformational analysis (RSCA) is well suited to MHC typing animals where reagents or primers for large numbers of specific alleles are not available. RSCA can identify the presence of multiple class I cDNAs within a single sample and has been used for MHC classes I and II typing in humans (Arguello and Madrigal 1999), cynomolgus macaques (Krebs et al. 2005) and various other species (Baquero et al. 2006; Drake et al. 2004; Kennedy et al. 2003). To assess the MHC allele frequencies for ten previously characterized MHC class I alleles in 109 pigtail macaques, RSCA was employed using a modified short fragment method previously described (Smith et al. 2005a,b).

Briefly, pigtail macaque class I sequences spanning 200 base pairs (bp) of the polymorphic peptide binding regions were amplified using Phusion DNA polymerase (Finnzymes, Espoo, Finland), 1 μl of a 25 μM phosphate-labelled forward primer (5′Phos-shtRSCA; 5′-[phos]-AggggCCggAgTATTggg-3′) and 1 μl of a 25 μM unlabelled reverse primer (3′sht-RSCA; 5′-TTCAggRC gAWgTAATCC-3′) for an initial 30-s 98°C step followed by 35 cycles of 5 s at 98°C, 1 s at 55°C and 20 s at 72°C, then a final extension at 72°C for 5 min. Five reference strands were generated using FAM-labelled rhesus macaque reference clones *Mamu-A*0705*, *Mamu-A*15*, *Mamu-A*20*, *Mamu-B*05* and *Mamu-B*07*. Single-stranded cDNA amplicons were then heteroduplexed with each of the fluorescently labelled reference strands (Smith et al. 2005a). The labelled heteroduplexes were run on a non-denaturing gel (Long Ranger gel mix, Cambrex, Mt. Waverly, Victoria, Australia) on an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA), where each heteroduplex displays a characteristic mobility. The pigtail macaque cDNA heteroduplex mobilities were then compared to the mobilities of ten previously characterized, sequence-verified *Mane* MHC class I clones (*Mane-A*03*, *Mane-A*10*, *Mane-A*14*, *Mane-A*16*, *Mane-B*02*, *Mane-B*03*, *Mane-B*10*, *Mane-B*12*, *Mane-B*15* and *Mane-B*11/22*, which differ by one nucleotide/one amino acid in the cytoplasmic tail; Smith et al. 2005a). Peaks were analysed using DAx data acquisition and analysis software (Van Mierlo Software, Eindhoven, The Netherlands).

Each pigtail macaque MHC class I allele of interest has a characteristic mobility with each of the five rhesus macaque reference strands. *Mane-A*10*, for example, has a typical mobility of 261.4 bp with *Mamu-A*0705*, 310.6 bp with *Mamu-A*15*, 298.9 bp with *Mamu-A*20*, 245.3 bp with *Mamu-B*05* and 310.2 bp with *Mamu-B*07*. In this study, the majority of the animals were assessed by RSCA twice with five reference strands, although a small number were assessed once with four reference strands and once with five. To define the presence of an allele, all the cDNA heteroduplexes had to mobilize very closely to the target allele heteroduplexes. Specifically, the following criteria were employed in all analyses: If five reference strands were used, three of the reference strands must have generated a cDNA heteroduplex mobility within 1.5 bp of the target allele value, one reference strand could generate a heteroduplex within 2.0 bp and one reference strand within 2.5 bp. If only four reference strands were used, three must have generated a cDNA heteroduplex mobility within 1.5 bp of the target allele value, and the fourth could generate a mobility within 2.5 bp of the target.

Table 1 MHC class I typing of pigtail macaques

Animal	*A*03*	*A*10*	*A*14*	*A*16*	*B*02*	*B*03*	*B*10*	*B*11/22*	*B*12*	*B*15*
Indonesian origin										
1.3731		✓	✓		✓				✓	
2.3308										
4236									✓	
4240	✓								✓	
4241				✓						
4253	✓									
4293			✓				✓	✓		✓
4295		✓							✓	
4296		✓	✓				✓	✓		
4299		✓								
4301	✓									
4532			✓							
4648			✓				✓	✓		
4658		✓	✓				✓	✓		
5023							✓	✓		
5350					✓					
5614		✓	✓							
5912										
6167		✓								
6169			✓							
6238					✓					
6255						✓				
6259					✓					
6262	✓			✓			✓	✓		
6263			✓				✓	✓		✓
6274										
6279		✓			✓					
6349		✓								✓
6366						✓				
6597							✓	✓		
6804							✓			
7992	✓				✓		✓	✓		
8012	✓									
8014		✓								✓
8020		✓			✓		✓	✓		
8240		✓							✓	
8241	✓	✓							✓	
8244		✓								
8247										
8251					✓					
8252										
8436					✓	✓				
8454		✓					✓	✓		
8673				✓						
8676										✓
8680					✓					
8682			✓							✓
8868	✓									
8873										
8881										
8883	✓									
9017		✓			✓		✓	✓		
9018										
9019									✓	
9020		✓			✓					✓

Table 1 (continued)

Animal	*A*03*	*A*10*	*A*14*	*A*16*	*B*02*	*B*03*	*B*10*	*B*11/22*	*B*12*	*B*15*
9021	✓	✓							✓	
9175		✓								
9176		✓								
9180	✓								✓	
9182			✓			✓			✓	✓
9183		✓							✓	
9196	✓					✓				
Frequency (%)	19	34	18	5	19	8	21	19	18	13
US origin										
387			✓							
389			✓							
394										
708										
713										
715	✓								✓	
17834		✓	✓							✓
17850	✓									✓
18031										
18033	✓					✓				
18242										
18292		✓								
A1P003		✓								
A1P005			✓				✓	✓		
A1P014										
A1P016		✓							✓	
A1P018		✓	✓			✓				
A2P005		✓								
BI55	✓				✓				✓	
BK09										
BM03	✓									
BP33		✓								✓
BP41									✓	
CC33										
O1P005		✓							✓	
O1P006										
O2P004	✓									
O3P003										
T4363		✓			✓					
T4364					✓					
Frequency (%)	20	30	17	0	10	7	3	3	17	10
Australian origin										
086B		✓								✓
1335	✓	✓				✓			✓	
2374	✓	✓				✓				
2621										
3B14										
3C7D		✓								
3117										
3773	✓				✓					
5A3F										
5504									✓	
5773	✓									
6115										
6870					✓					
8060							✓	✓		
8305		✓			✓					
9532										

Table 1 (continued)

Animal	*A*03*	*A*10*	*A*14*	*A*16*	*B*02*	*B*03*	*B*10*	*B*11/22*	*B*12*	*B*15*
H21	✓									
Frequency (%)	29	29	0	0	18	12	6	6	12	6

One hundred and nine pigtail macaques were typed for ten class I MHC alleles (four Mane-A and six Mane-B) by RSCA. Each animal's specific genotype with respect to the ten MHC alleles is described. Animals are grouped according to their breeding colony origins into three subsets: (1) Indonesian, n=62; (2) US, n=30; and (3) Australian, n=17. The frequencies of each of the ten MHC alleles within the three separate groups are shown directly beneath the genotyping data for each group.

The 109 pigtail macaques studied were obtained from three different breeding colonies located at the Primate Research Centre in Bogor, Indonesia (Pamungkas et al. 2005), the National Macaque Facility in Werribee, Australia (De Rose et al. 2005) and Johns Hopkins University in the USA, which obtained the macaques from four US primate breeding facilities (Oregon and Tulane National Primate Research Centres, Covance and Alpha Genesis; Carruth et al. 2005). All macaques were screened for the presence of each of the ten *Mane* MHC class I alleles of interest (Table 1; Fig. 1). *Mane-A*10*, an allele previously shown to restrict the immunodominant SIV Gag epitope KP9 (Smith et al. 2005b), was overall the most common allele, with a frequency of 32.1%. The frequencies of the nine other alleles ranged from 2.8 (*Mane-A*16*) to 21.1% (*Mane-A*03*). Almost all alleles typed for were present in macaques bred in the three different locations (i.e. Indonesia, USA and Australia; Fig. 1). This suggests that RSCA is likely to prove a broadly useful tool for MHC typing pigtail macaques from geographically diverse breeding facilities. *Mane-A*10* had a similar frequency across all three subsets, with no statistically significant differences in allele frequencies across the different breeding colonies. *Mane-A*14* was more common in macaques from the Indonesian and US breeding colonies than the Australian colony (17 vs 0%), but this did not reach statistical significance (p=0.1, Fisher exact test).

Most macaques (78%) analysed in this study possessed one to four of the ten MHC class I alleles for which we typed (Table 1). For example, macaque 1.3731 was positive for three class I alleles, *Mane-A*10*, *Mane-B*02* and *Mane-B*12* (Fig. 2). Macaque 6169 was positive for only one allele of interest, *Mane-A*14* (Fig. 2). Despite characterising alleles in most macaques, 24 of the 109 macaques (22%) studied did not possess any of the ten MHC alleles we evaluated in detail. Further study of the RSCA profiles in 18 of these 24 macaques indicated that 11 of these 18 animals expressed at least one of the 15 additional *Mane* alleles for which we had clones and were able to analyse. Macaque 6115, for example, was positive for *Mane-B*19*, which was not among the ten alleles we studied (Fig. 2). Those animals lacking all 25 pigtail macaque MHC class I alleles for which we could analyse by RSCA likely have MHC genotypes that include uncharacterized alleles and/or characterized alleles for which we do not yet have reagents.

A new *Mane-A* allele was identified from three Indonesian pigtail macaques responding to the same Gag $CD8^+$ T cell epitope (AF9, SIV_{mac239} Gag amino acids 371–379). Cloning and sequencing of the three animals' class I MHC alleles was performed as previously described (Smith et al.

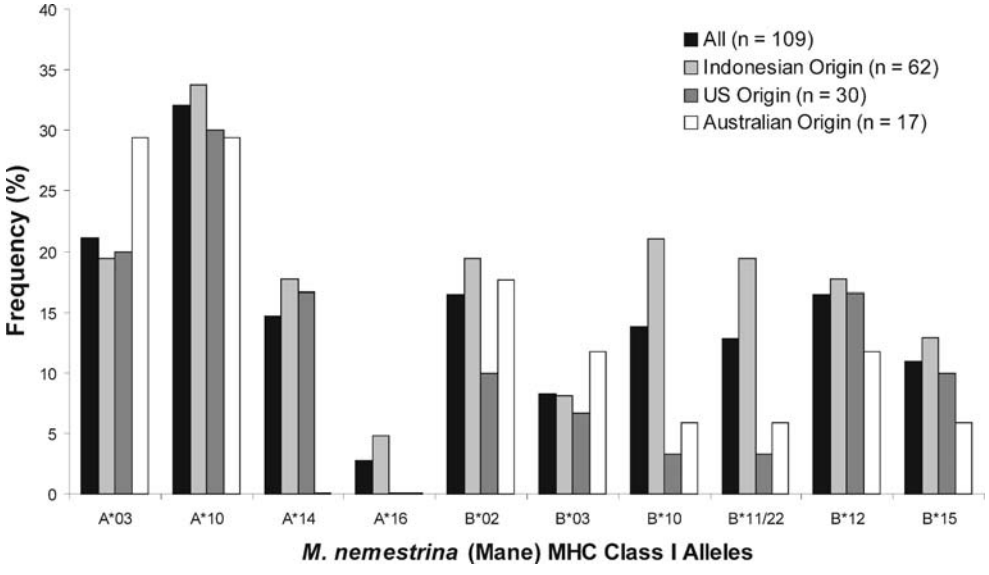
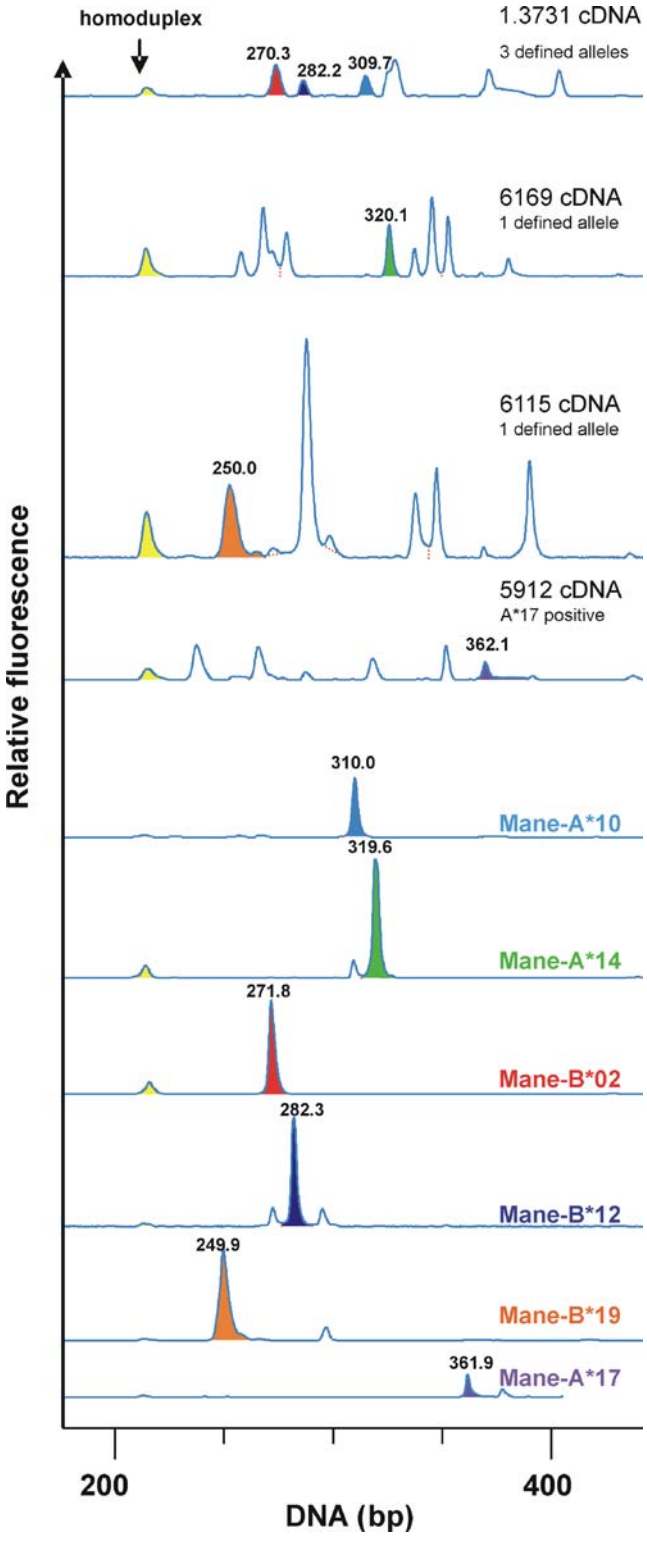


Fig. 1 Frequencies of ten class I MHC alleles in pigtail macaques. The frequencies of ten class I MHC alleles were analysed in a sample of 109 pigtail macaques. Macaques were divided into three groups on the basis of their breeding colony origins (i.e. Indonesia, USA and Australia)



◀ **Fig. 2** RSCA analysis of pigtail macaque cDNA for the presence of class I MHC alleles. RSCA data from four pigtail macaque cDNA samples (*upper four traces*) and six pigtail macaque MHC class I clones, *Mane-A*10*, *Mane-A*14*, *Mane-B*02*, *Mane-B*12*, *Mane-B*19* and *Mane-A*17* (*lower six traces*), are depicted. Macaque 1.3731 generated heteroduplex peaks characteristic of three class I alleles, *Mane-A*10* (*light blue*), *Mane-B*02* (*red*) and *Mane-B*12* (*dark blue*). Macaque 6169 generated a heteroduplex peak characteristic of *Mane-A*14* (*green*). cDNA isolated from macaque 6115 did not generate heteroduplex peaks characteristic of any of the ten MHC alleles that we studied in detail. It did, however, generate a peak characteristic of *Mane-B*19* (*orange*). cDNA isolated from macaque 5912 generated a heteroduplex peak characteristic of the newly identified allele, *Mane-A*17* (*purple*). In all samples, the homoduplex peak is highlighted in *yellow*. Only RSCA profiles with the *Mamu-B*07* reference strand are shown, although each trace was confirmed with four other reference strands (not shown)

2005b). All three animals had more than three MHC class I clones each, which had identical nucleotide sequences but had not previously been described. We defined this allele as *Mane-A*17* (GenBank accession no. DQ886026). The mobility of *Mane-A*17* was then characterized by RSCA in comparison to the three macaques' cDNA (Fig. 2). The RSCA analysis confirmed that all three macaques share this allele.

These results indicate that RSCA is a reliable and useful tool to simultaneously type numerous pigtail macaques for multiple MHC class I alleles. The RSCA technique can easily be expanded to type for newly discovered alleles, as clones of new alleles become available. Animals' cDNA heteroduplex mobility profiles can then be examined to see if a match exists between them and the new allele's RSCA mobility profile, using the criteria described above. RSCA is also a helpful technique in the preliminary determination of epitope restriction by *Mane* MHC alleles. Multiple animals that respond to the same specific disease epitope (in our case, an SIV epitope) can be genotyped by RSCA. Shared alleles represent likely candidates for MHC restriction, which can then be confirmed by folding candidate MHC polypeptides around the putative T cell epitope.

The sensitivity of our RSCA is difficult to determine in the absence of a larger scale sequencing project. In a recent SIV infection of 36 Indonesian pigtail macaques, 13 were identified by RSCA as *Mane-A*10* positive. When the same 36 animals were evaluated by polymerase chain reaction using sequence-specific primer, as previously described (Smith et al. 2005b), 12 animals were identified as *Mane-A*10* positive. After SIV infection, the 36 animals were analysed for a KP9-specific response using a KP9/*Mane-A*10* tetramer (Smith et al. 2005b), and all 13 animals typed as *Mane-A*10* positive by RSCA demonstrated a clear response. These results suggest that our RSCA technique reliably detects the presence of a functional *Mane-A*10* allele in pigtail macaques.

A limitation with the current RSCA technology is that animals can only be typed for alleles for which an RSCA profile exists. Currently, our laboratory has the capacity to type for 25 of the 38 reported pigtail macaque class I MHC alleles. The presence of numerous pigtail macaques lacking all or the majority of those 25 alleles suggests that many

other *M. nemestrina* class I alleles exist that have not yet been characterized. It is therefore necessary to continue identifying and characterizing new class I alleles through cloning and sequencing, and subsequently by RSCA.

An additional limitation of the RSCA technique involves inherent but infrequent difficulties in using it to classify animals as having particular alleles. Although the criteria described in this paper provide a stringent framework for determining whether or not animals have an allele, cases do arise where the data are difficult to interpret. To resolve most ambiguities, animals should be analysed at least twice by RSCA.

The typing of numerous macaques for ten class I MHC alleles allowed us to look for patterns of shared inheritances that are suggestive of gene linkage. In 14 of 15 cases, animals that possess the allele *Mane-B*10* also have *Mane-B*11/22*. There is a high probability that these two MHC genes are co-inherited and are thus part of the same MHC class I haplotype. Similarly, recent RSCA studies of Mauritian cynomolgus macaques have identified common class I MHC haplotypes (Krebs et al. 2005). Further study is necessary to define MHC class I haplotypes in pigtail macaques and to confirm instances of allele linkage.

In conclusion, this study typed 109 pigtail macaques for ten common MHC class I alleles by RSCA. We identified the frequencies of these alleles in pigtail macaques of diverse origin, were able to confirm the presence of a new shared allele and were able to identify a potential haplotypic linkage. The ability to genotype pigtail macaques for MHC class I alleles further advances the utility of this model for HIV/SIV vaccine research and other applications.

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