DEVELOPMENT OF A DNA MICROARRAY FOR DETECTION OF EXPRESSED EQUINE CLASSICAL MHC CLASS I ALLELES IN A DEFINED POPULATION

By

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To the Faculty of Washington State University:

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DEVELOPMENT OF A DNA MICROARRAY FOR DETECTION OF EXPRESSED EQUINE CLASSICAL MHC CLASS I ALLELES IN A DEFINED POPULATION

Abstract

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Development of an accurate and efficient molecular-based equine major histocompatibility complex (MHC) class I typing method is necessary to advance the study of T lymphocyte immune responses to intracellular pathogens. A DNA microarray is one means by which multiple MHC class I genes can be detected simultaneously and discriminated on the basis of as little as a single nucleotide difference. In this study, expressed classical MHC class I genes from six Arabian horses, homozygous and heterozygous for serologically defined equine leukocyte antigen (ELA)-A1, A4, and W11 haplotypes, plus a serologically undefined haplotype, were amplified by reverse transcription (RT)-PCR using conserved primers, cloned, and sequenced. A total of nine different expressed classical MHC class I alleles were detected, with between two and five alleles identified in each horse. Allele-specific, conserved (positive control), and random nucleotide (negative control) 23- to 27-mer oligonucleotide microarray probes were designed based on these sequences, and spotted onto an epoxy-coated masked slide using a robotic arrayer. Bulk RT-PCR products from each horse were biotinylated by nick translation, hybridized to the array and detected using tyramide signal amplification. In all horses it was possible to accurately detect the alleles associated with each haplotype with greater

efficiency than cloning and sequencing. The microarray was therefore an effective MHC class I typing method for the horses in this defined population, and will be useful for screening experimental subjects for studies evaluating T cell vaccines and immunotherapeutic strategies against a variety of equine infectious diseases.

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CHAPTER ONE

INTRODUCTION

Classical MHC class I molecules are highly polymorphic membrane-bound glycolproteins that present self and non-self endogenous peptides to CD8+ T lymphocytes. The extreme polymorphism observed in this group of proteins is predominantly due to nonsynonymous substitutions within the exons that encode the peptide-binding site (Beck and Trowsdale 2000). The structural changes in the peptide-binding site that result from these amino acid differences have a significant effect on peptide binding affinity and dictate the conformation of the MHC/peptide complex presented to cytotoxic T lymphocytes (CTL). The observation that a single amino acid difference within the α-2 domain of two MHC class I molecules (7-6 and 141) can alter or abolish recognition of equine infectious anemia virus CTL epitopes (Mealey et al. 2006) highlights the immunologic importance of MHC polymorphism in the horse.

MHC class I polymorphisms have been historically assessed by serologic typing using reagents derived from multiparous mares and horses alloimmunized with horse blood cells (Bailey 1980). With this method approximately 15 distinct specificities could be identified, designated ELA-A1 through ELA-A19 (Lazary et al. 1988). More recently, MHC class I alleles have been characterized at the molecular level through cDNA library screening, reverse transcriptase polymerase chain reaction (RT-PCR) experiments, and genomic sequencing from BAC libraries, with a total of approximately 60 equine MHC class I molecules being reported to date (Barbis et al. 1994;Carpenter et al. 2001;Chung et al. 2003;Ellis et al. 1995;McGuire et al. 2003;Tallmadge et al. 2005). Despite these efforts, serotyping remains the standard for equine MHC class I typing primarily due to the lack of a uniform, efficient, and accurate nucleic-based ELA typing method.

In humans, serotyping has largely been replaced with nucleic-acid based typing methods because these techniques have significantly greater accuracy and reproducibility (Elsner and Blasczyk 2004), and they have the capacity to detect alleles not distinguished by available antisera (Williams 2001). The three molecular methods predominantly used for human leukocyte antigen (HLA) typing are sequence-specific oligonucleotide probe hybridization (SSOPH), sequence-specific primer PCR (SSP-PCR), and direct DNA sequencing of PCR products (Williams 2001). The method of choice is dictated by the level of resolution required, turn-around time, and the volume of samples to be processed. In addition to these techniques, DNA microarrays provide a rapid and economical molecular-based screening method that is ideally suited for leukocyte antigen typing because of their capacity to screen for multiple polymorphic sequences simultaneously, and to distinguish between genes with as little as a single nucleotide difference (Letowski et al. 2004;Warsen et al. 2004). The goal of this study was to develop a high resolution microarray capable of identifying expressed classical MHC class I alleles comprising serologically defined ELA-A haplotypes with known inheritance patterns in a defined population of Arabian horses.

CHAPTER TWO

RESULTS AND DISCUSSION

Arabian horses selected for inclusion in this study were part of the Washington State University research breeding herd and were chosen based on their ELA-A haplotype and consanguinity (Table 1). ELA-A haplotyes were determined serologically by lymphocyte microtoxicity (Bailey 1980;Terasaki et al. 1978) using reagents provided by Dr. E. Bailey (University of Kentucky, Lexington, KY). Four of the horses (mares 162, 172, 174, and 184) had only a single haplotype detected serologically, indicating that these horses were either homozygous for that haplotype or heterozygous with a haplotype not recognized by available antisera. All other horses were heterozygous for the ELA-A1, A4, or W11 haplotypes.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Histopaque-1077 (Sigma-Aldrich) and cryopreserved. An RNeasy Kit (Qiagen, Valencia, CA) was used to isolate RNA from these archived PBMC and RT-PCR was performed using a One-Step RT-PCR Kit (Invitrogen, Carlsbad, CA). Conserved primers were used as described (Chung et al. 2003;Tallmadge et al. 2005) with slight modification to amplify classical equine MHC class I genes (forward: 5'-GTG GAC GAC ACG CAG TTC-3'; reverse: 5'-CAG CAA GGA AGC AAA TGA TC-3'). RT-PCR was carried out with SuperScript II RT/Platinum *Taq* mixture (Invitrogen) using the following conditions: 1 cycle at 50ºC for 50 min, 1 cycle at 94ºC for 2 min, 30 cycles at 94ºC for 30 sec, 60ºC for 30 sec, and 68ºC for 2 min, and a final cycle at 68ºC for 7 min. Amplified RT-PCR products were gel-purified, cloned into the pCR2.1 TA TOPO cloning vector (Invitrogen), and sequenced as described (Chung et al. 2003). Most MHC class I sequences were identified in multiple clones, verifying the accuracy of those sequences. Only sequences that were cloned multiple times in an individual horse or in more than one horse were

considered for analysis. Sequences were analyzed with Vector NTI software (Invitrogen) while alignments and sequence identity tables were generated with ClustalW2 and Boxshade programs.

A total of nine different classical MHC class I alleles were detected, with between two and five alleles identified in each horse (Table 2). In some cases, as many as 48 clones had to be sequenced to detect a single allele sequence. Each of the ELA-A1 haplotypes represented in this herd were inherited from one of three dams (mares 162, 169, or 172). In horses with the ELA-A1 haplotype originating from mare 162 or 172, alleles 113 and 7-6 were repeatedly identified. In contrast, the ELA-A1 haplotype from mare 169 was only associated with a single allele, 141. ELA-A4 and W11 haplotypes were inherited from a common sire (Sire B) in all horses. Comparison of the sequence data from horses heterozygous for these alleles indicated that the W11 haplotype was associated with alleles 11.a, 11.b, and 106, and the A4 haplotype was associated with alleles 7-1 and 7-4. The full complement of alleles associated with the W11 haplotype could not, however, be detected in all horses bearing this haplotype (i.e. horse 176). This finding reflects the fact that certain alleles were cloned with disproportionate frequency in some of the horses, either due to increased expression of that allele or bias in the RT-PCR or cloning reaction. It was not necessary to overcome this bias with exhaustive sequencing in all horses because identification of any alleles with known haplotypic association (i.e. 11.a) in conjunction with the horse's pedigree was sufficient to confirm the serologic typing. Lastly, cloning and sequencing provided the information required to determine if horses with only a single serologically detectable haplotype were indeed homozygous for that haplotype or heterozygous with a haplotype not defined by available antisera. For horse 184, only alleles associated with the serologically defined ELA-A4 haplotype were detected, indicating that this horse was an MHC class I homozygote. For horses 172 and 174 we detected a common set of

alleles associated with an inherited haplotype that was not identified by available antisera (116, 106, 11.b) (Table 2). The only detectable difference between the serologically undefined haplotype and the W11 haplotype was the substitution of allele 116 for allele 11.a. This suggested that at least in these horses the W11 serotype was defined by the presence of allele 11.a while the absence of 11.a resulted in the lack of serologic reactivity. The inability to detect all haplotypes by serologic means has also been observed in other species and is presumed to be due to the absence of the serologically undefined alleles in the population from which the antisera was derived, or due to low surface expression of the encoded class I molecules (Hurley et al. 1999). In addition to this limitation, serologic reagents lack the specificity to resolve minor nucleotide polymorphisms (i.e. SNPs) that can have significant functional implications, such as the presentation of CTL epitopes (McGuire et al. 2003;Mealey et al. 2006). Therefore, more robust nucleic acid-based MHC class I typing methods are needed to advance the study of equine T cell immunology, autoimmunity, and tissue transplantation in the horse. As observed in this study and others, RT-PCR followed by cloning and sequencing represents one method to detect expressed MHC class I alleles in the horse. The cost and inefficiency of cloning RT-PCR products and sequencing a large number of clones however, limits the utility of this technique as a routine screening tool.

To construct a microarray capable of detecting the expressed classical MHC class I alleles in this group of horses, twenty four oligonucleotide probes (23 to 27 nucleotides in length) were designed to conserved (positive control probes) and allele-specific regions within α1, α2, α3, transmembrane, and cytoplasmic domains (Table 3). Included in this set were two probes specific for an allele (8-5) that had not been identified by cloning and sequencing. Although cloning and sequencing described above did not identify allele 8-5 in any horses, these

probes were included in the array based on the previous identification of allele 8-5 (by cDNA library screening) in horse 2152 (McGuire et al. 2003). All probes had an average melting temperature of 63ºC (range: 61-65 ºC), 60.5% GC content (range: 51-69%) and were assessed for secondary structure using Oligo Design and Alalysis tools (Integrated DNA Technologies). Independent arrays consisting of two replicate spots of each probe and a biotin pseudoprobe (Call et al. 2003) were spotted on each individual well of a 10-well, Teflon/epoxy-silane masked/coated microscope slide by an S3 SciflexArrayer (Scienion AG, Berlin, Germany), as previously described (Call et al. 2003). Following spotting, slides were baked for 1 hr at 130ºC (<21 in Hg) and then stored at room temperature until used.

As positive controls for the specificity of the microarray, ten plasmid clones representing each allele were biotynilated by nick translation and hybridized to the microarray at 64 ºC. Previously described procedures and chemistry detection steps, which included tyramide signal amplification, were carried out as previously described (Call et al. 2003). Arrays were imaged using an arrayWoRx Auto*e* scanner (Applied Precision, Issaquah, WA) and the images were processed with softWoRx Tracker software (Applied Percision). The threshold for positive detection was determined using a receiver operator characteristic (ROC; NCSS 2004 Statistical software) curve generated from the binary classification of a core set of probes. For eighteen of twenty allele-specific probes there was a one-to-one correspondence with the cloned control allele (Table 3). The remaining two probes, which were designed to be specific for the 11.a allele (probes 23 and 24), cross-reacted with multiple alleles. In all cases the alleles identified by the cross-reactive probes only differed from the complimentary sequence of the probe by a single nucleotide. Altogether however, the array was sufficiently specific to differentiate the two cloned alleles that differed by only one nucleotide (7-6 and 141).

To determine the utility of the array for identifying these alleles in individual horses, conserved primers were used to amplify expressed classical MHC class I genes and the bulk RT-PCR products from each horse were hybridized to the array. For all horses bearing the ELA-A1 haplotype the microarray detected allele 113 on at least one probe (Table 3). The microarray also consistently detected either allele 141 or 7-6 in horses with the ELA-A1 haplotype, depending on the mare from which the ELA-A1 haplotype was inherited. Thus, the array indicated that the A1 haplotype comprised a minimum of two loci. In horses homozygous and heterozygous for the serologically defined ELA-A4 haplotype, the 7-1 allele was specifically detected with all complimentary probes and the 7-4 allele was consistently detected with one of two complimentary probes (Probe 3). Therefore, the A4 haplotype also comprised a minimum of two loci. Microarray detection of alleles associated with the ELA-W11 haplotype was similarly in agreement with that observed by cloning and sequencing; however, with the microarray it was possible to identify the full complement of ELA-W11 alleles (i.e. 106, 116, and 11.a) in all horses with this haplotype. It should be noted that under these conditions, probes 23 and 24 were 100% specific, as they only hybridized with target DNA generated from horses with the ELA-W11 haplotype. This increased specificity as compared to that obtained with the cloned RT-PCR products indicated that the concentration of each allelic target was low enough in the bulk RT-PCR products to limit non-specific hybridization to these probes. Based on these results it appeared that the W11 haplotype comprised a minimum of three loci. The observation that the number of loci differed dependent on haplotype was consistent with the results of others (Tallmadge et al, unpublished).

In bulk RT-PCR products from both horses with the serologically undefined haplotype, mare 172 (A1/Undefined) and mare 174 (A4/Undefined), alleles 106, 116 and 11.b were detected

(Table 4). This result agreed with the previous cloning and sequencing results and confirmed that at least in this group of horses the presence of these three alleles along with the absence of 11a conferred the undefined haplotype, which like the W11 haplotype, comprised at least three loci. Interestingly, the microarray detected target DNA complimentary to one of the 8-5 specific probes (probe 20) in both of these horses even though 8-5 had not been detected by cloning and sequencing. This likely reflected the increased sensitivity of the array in this group of horses. The 8-5 allele was previously detected in ELA-A1/A4 heterozygous horse 2152 by screening a cDNA library (McGuire et al. 2003), but a subsequent study did not identify 8-5 in this same horse by cloning and sequencing RT-PCR products (Chung et al. 2003). The latter finding was consistent with our results, and indicate that some alleles may not be detected in all horses with these methods. Surprisingly, the 8-5 allele has also been independently detected in an unrelated ELA-A3 homozygous horse by screening a genomic DNA BAC library and by RT-PCR using allele-specific primers (Tallmadge et al. 2005). Thus, the haplotypic association for the 8-5 allele is not entirely clear and 8-5 may represent a poorly expressed allele occurring in more than one serologically defined haplotype.

Probe 17 also yielded positive results in horse 172. This probe was designed to promiscuously bind either the 7-1 or 8-5 alleles (Tables 3 & 4). Because the other three 7-1-specific probes (probes 9, 13, and 16) were negative, it is unlikely that probe 17 was detecting the 7-1 allele in horse 172 (consistent with the absence of the ELA-A4 haplotype). Consistent with the observation above for probe 20, probe 17 was likely detecting the 8-5 allele in horse 172 (or an allele not yet characterized in this group of horses containing shared sequence complimentary to the probe). The same may have been true in horse 174, but because this horse had the 7-1 allele (all 7-1-specific probes positive, consistent with 174's ELA-A4 haplotype),

determining whether probe 17 was binding 7-1 or 8-5 (or both) was not possible given the absence of supporting cloning and sequencing data.

In summary, the microarray designed in this study consistently identified expressed classical MHC class I alleles in this defined group of horses. The microarray has a significant advantage in throughput, with the potential to screen 30 or more horses in a 24-hour period. In the future this array could be expanded to include alleles associated with less common ELA-haplotypes in this herd, as well as alleles present in the larger outbred equine population. Optimization of the array for use with genomic DNA would improve its utility for larger scale ELA typing because fewer steps would be required and viable cells would not be needed for RNA recovery. For T cell immunology and transplantation applications however, the initial RT-PCR step imparts greater functional relevance by limiting detection to expressed alleles. Detection methods based on the amplification of genomic DNA in humans can mistype non-expressed variants such as null alleles, which is clearly a significant problem for tissue transplantation applications (Elsner and Blasczyk 2004). Use of RT-PCR in these cases would be beneficial because many expression variant alleles are not transcribed (Elsner and Blasczyk 2004). Regardless, the microarray described herein will be useful in the design of future equine T cell vaccine and immunotherapeutic studies by allowing rapid selection of horses with known alleles of functional significance, or alternatively, to ensure that individual alleles are not over represented in experimental groups. In the case of T cell vaccine development, the microarray will greatly facilitate identification of CTL epitopes that can be presented by a diverse set of MHC molecules, and should be useful in characterizing differential immunologic responses between individuals at the class I allelic level.

Table 1. Pedigree of ELA-A1, A4, and W11 horses

Horse	ELA-A haplotype*	Dam	Dam ELA-A haplotype	Sire	Sire ELA-A haplotype
172	A1	Not Available	Not typed	Not Available	Not typed
174	A4	172	A1	Sire B	A4/W11
176	A1/W11	169	A1/A4	Sire B	A4/W11
184	A4	174	A4	2135†	A4/W11
2140	A1/W11	172	A1	Sire B	A4/W11
2152	A1/A4	162	A1	Sire B	A4/W11
2244	A1/W11	181†††	W11	2152††	A1/A4

* All ELA-A haplotypes were determined serologically by lymphocyte microtoxicity. It was not known if horses with only a single detectable haplotype were homozygous or heterozygous with a haplotype not recognized by available antisera.

† ELA-A4 of Sire 2135 inherited through Sire B.

†† ELA-A4 of Sire 2152 inherited through Sire B.

††† ELA-W11 of Dam 181 inherited through Sire B.

Table 2. Classical MHC class I alleles identified by RT-PCR cloning and sequencing

Horse	ELA-A haplotype	Allele designation	GenBank accession/ Citation	Number of clones with each sequence/ No. sequenced
172	A1	7-6	AY225155/ McGuire et al. 2003	1/48
		113	AY176101/ Chung et al. 2003	11/48
	Undefined	116	AY176104/ Chung et al. 2003	13/48
		106	AY176094/ Chung et al. 2003	2/48
		11.b	EF577070/ Tallmadge et al. unpublished	9/48
174	A4	7-1	AY225151/ McGuire et al. 2003	22/48
		7-4	AY225153/ McGuire et al. 2003	9/48
	Undefined	116	See above	2/48
		106	See above	3/48
		11.b	See above	2/48
176	A1	141	AY374512/ Chung et al. 2003	25/28
	W11	106	See above	3/28
184	A4/A4	7-1	See above	69/90
		7-4	See above	6/90
2140	A1	7-6	See above	9/40
		113	See above	2/40
	W11	11.a	EF577068/ Tallmadge et al. unpublished	13/40
		106	See above	1/40
		11.b	See above	4/40
2152	A4	7-1	See above	30/48
		7-4	See above	6/48
	A1	7-6	See above	1/48
		113	See above	4/48

Table 3. Nucleotide sequences of microarray probes and specificity for detection of classical MHC class I alleles

Probe ID	Probe sequence	Complimentary allele	Cloned controls detected	Horses in which positive result obtained
1*	TACTACAACCAGAGCGAGGCCGGGT	All	All	All
2†	GAGAAGGCACGCTACGAGCTTCACG	None	None	None
3	AGAGGAGACACGGAGCGTCAAGGAC	7-4	7-4	174, 184, 2152
4	TTTCCGAGGGAACCTGCGGACCG	116/11.a	116, 11.a	172, 174, 176, 2140, 2244
5	GCCCCTCTTCCGCGGGTACAGTC	7-4	7-4	None
6	GCGGGTATGAACAGTTCGCCTACGA	113	113	172, 176, 2244
7	GGCGCGTTCTCCGTGGGTACAGT	106	106	174, 176, 2244
8	GTGTGGCGGAGAAAGAAAGGAACTACC	11.b	11.b, 11.a	172, 174, 176, 2140, 2244
9	ACTACCTGGATGGGGAGTGCGTGGA	7-1	7-1	174, 184, 2152
10*	TTCCAGAAGTGGGCGGCTGTGGTG	All	All	All
11†	TTCCAGAACTGGTCGGCTGTGGTGG	None	None	None
12	CCTGGCACCAAGATGAAGAGGACCT	113	113	172, 176, 2140, 2152, 2244
13	AGCTTGTGGACACTAGGCCTGCAGG	7-1	7-1	174, 184, 2152
14	TGCAGGGGACCGAACCTTCCAGAAG	7-6/141	7-6, 141	172, 176, 2140, 2152, 2244
15	GGTGAGGCGGAGCAGTGCAGGAA	7-6	7-6	172, 2140, 2152, 2244
16	GCAGACATACACATGCCGTGTGCAG	7-1	7-1	174, 184, 2152
17	AAGAGGGAGCTACGTGCAGACTGCA	7-1/8-5	7-1, 8-5	172, 174, 184, 2152
18	CGCGTGGATCCCCCAGAGACACA	11.b	11.b	172, 174, 176, 2140, 2244
19	GGTGTGGCGGAGCAGTGCAGGAA	141	141	176
20	GAGATCGCCCTAACCTGGCAGCGT	8-5	8-5	172,174
21	AGCGCACGGATCCTCCAAGGACAC	8-5	8-5	None
22	GGACCGCGCTCAGCTACTACAACCA	116/11.a	116, 11.a	172, 174, 176, 2140, 2244
23	GTGGAGGGGCTCCGCAGATACCT	11.a	11.a, 106, 116	176, 2244
24	TGAAGGAAGCCACACAGACTTTCCGAG	11.a	11.a, 8-5, 7-6, 141	176, 2140, 2244

* Positive control probes complimentary to all classical MHC class I alleles

† Negative control probes were designed by random rearrangement of conserved probes or arbitrary substitution of individual nucleotides

Table 4. Comparison of cloning/sequencing and microarray methods for detection of classical MHC class I alleles

Horse	Serologic haplotype	Alleles identified by cloning and sequencing	Alleles detected by microarray
172	A1	7-6, 113	7-6, 113
	Undefined	116, 106, 11.b	116, 11.b, 106, 8-5*
174	A4	7-1, 7-4	7-1, 7-4
	Undefined	116, 106, 11.b	116, 11.b, 106, 8-5*
176	A1	141	141, 113
	W11	106	11.a, 11.b, 106
184	A4/A4	7-1, 7-4	7-1, 7-4
2140	A1	7-6, 113	7-6, 113
	W11	11.a, 106, 11.b	11.a, 11.b, 106
2152	A1	7-6, 113	7-6, 113
	A4	7-1, 7-4	7-1, 7-4
2244	A1	Not sequenced	7-6, 113
	W11		11.a, 11.b, 106

* Haplotypic association unclear.

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