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BRIEF COMMUNICATION

Development of an *ELA-DRA* gene typing method based on pyrosequencing technology

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Abstract

The polymorphism of equine lymphocyte antigen (ELA) class II *DRA* gene had been detected by polymerase chain reaction–single-strand conformational polymorphism (PCR-SSCP) and reference strand-mediated conformation analysis. These methodologies allowed to identify 11 *ELA-DRA* exon 2 sequences, three of which are widely distributed among domestic horse breeds. Herein, we describe the development of a pyrosequencing-based method applicable to *ELA-DRA* typing, by screening samples from eight different horse breeds previously typed by PCR-SSCP. This sequence-based method would be useful in high-throughput genotyping of major histocompatibility complex genes in horses and other animal species, making this system interesting as a rapid screening method for animal genotyping of immune-related genes.

Single nucleotide polymorphisms (SNPs) had been widely used to characterize allelic sequences to perform haplotype identification and to analyse the level of expression of major histocompatibility complex (MHC) genes. Sequence-specific primers (SSP), sequence-specific oligonucleotide probes (SSOP), sequencing-based typing (SBT), polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), polymerase chain reaction–single-strand conformational polymorphism (PCR-SSCP) and reference strand-mediated conformation analysis (RSCA) were molecular methods traditionally and currently used for MHC typing (1–12). Improvement of high-throughput methods based on sequencing, pyrosequencing, real-time PCR, TaqMan assay and microarrays have been developed in recent years (13–17).

In view of the fact that several polymorphisms at MHC genes have been associated to resistance/susceptibility to infectious diseases in domestic animals (18–24), the availability of high-throughput genotyping methods is a valuable tool to perform a rapid polymorphism screening in animal genotyping of immune-related genes in horses and other animal species.

Pyrosequencing™ technology (25, 26) is a real-time DNA sequencing technique that is based on the detection of released pyrophosphate (PPi) during DNA synthesis and has been successful for both confirmatory sequencing and *de novo* sequencing (27–32). After an oligonucleotide is hybridized to a single-stranded DNA template, a cascade of enzymatic reactions starts with the nucleic acid polymerization reaction

primed by an internal primer. Each of the four dNTPs is then individually added to the reaction mixture, and inorganic PPi is released as a result of nucleotide incorporation by polymerase. Visible light is generated proportionally to the number of incorporated nucleotides (33), detected by a CCD camera and seen as peaks in a pyrogram™.

In domestic horses, equine lymphocyte antigen (*ELA*)-*DRA* class II MHC gene have been examined for polymorphism by PCR-SSCP and RSCA methodologies (10, 11, 34). Eleven DRA exon 2 sequences were reported among the existing equid species, three of them have been found widely distributed among horse breeds and a fourth one had been detected in some horse breeds (11) by using the RSCA method. This study describes the development of an SBT method applicable to high- or medium-throughput of *ELA-DRA* typing, based on pyrosequencing technology. We have applied this methodology to screen *ELA-DRA* polymorphism in a sample that includes animals from eight horse breeds previously genotyped by PCR-SSCP methodology.

A total of 97 horses were analysed in this study. Genomic DNA was isolated by the DNAzol® method (Invitrogen, Carlsbad, CA) from blood lymphocytes from 78 horses belonging to the following breeds: Criollo Argentino ($n = 8$), Thoroughbred (n = 8), Argentine Peruvian Paso ($n = 10$), Arabian ($n = 10$), Silla Argentino ($n = 39$), and Quarter Horse ($n = 3$). DNA samples from Spanish Pure Breed horses ($n = 8$) as well as Asturcón, Losino, Mallorquín and Menorquín breeds ($n = 11$) were provided by the Applied Research Laboratory, Service of Horse Breed and Remonta, from Córdoba, Spain. Because of their low number, the later were pooled as Spanish native breeds for this study.

Horse DNA samples were typed for *ELA-DRA* second exon polymorphism by the PCR-SSCP methodology described by Albright-Fraser *et al.* (34) by using DRABe1 (forward) and DRABe2 (reverse) primers. Amplification products from different SSCP band patterns were cloned into pCR®2.1-TOPO® (TOPO TA Cloning®; Invitrogen). Recombinant plasmid DNA isolation was performed by using S.N.A.P. Miniprep Kit (Invitrogen) and three clones of each variant were sequenced using DYEnamic ET Terminator Kit and universal primers with a MegaBACE 1000 automated sequencer (GE Healthcare, Sunnyvale, CA). Sequence was accepted if all reactions produced identical results at a given base. The obtained sequences showed 100% identity to one of the three *ELA-DRA* horse reported alleles *ELA-DRA*0101*, *ELA-DRA*0201* and *ELA-DRA*0301* (accession numbers L47174, M60100 and L47172, respectively). *ELA-DRA*JBH11* allele (accession number AJ575295) described in Brown et al. (11) was not found in this study. The clones corresponding to the mentioned alleles were used as reference samples for pyrosequencing typing.

ELA-DRA exon 2 nucleotide sequences were aligned by using Clustal W1.8 (Baylor College of Medicine; http://searchlauncher.bmc.tmc.edu). An internal sequencing primer (DRA-int 5′-GAGCCTCAAAGCTGG-3′) complementary to positions 147–161 on the forward strand of exon 2 was designed using Pyrosequencing Primer SNP Design 1.01 software (http://www.pyrosequencing.com). This primer is located upstream to the SNPs selected to differentiate horse DRA alleles.

For *ELA-DRA* exon 2 pyrosequencing, a number of horse samples that showed different genotypes by SSCP typing were PCR amplified with the same oligonucleotide primers (34), except for forward primer that was biotinilated for the subsequent purification step. PCR was performed with 2 μl DNA in a 25 μl reaction mixture containing 1× PCR buffer (Invitrogen), 0.15 μM for each primer, 200 mM each dNTP, 2 mM $MgCl_2$ and 0.5 units of Taq polymerase (Invitrogen), in a PTC-100 thermocycler (MJ Research, Boston, MA; Bio-Rad Laboratories Inc., Hercules, CA). PCR conditions consisted of an initial denaturalization step of 2 min at 95°C, followed by 45 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min, plus a final extension at 72°C for 10 min.

After generation of the template by PCR, the product was purified by capturing the biotinylated strand with streptavidin-coated Sepharose beads (Streptvidin Sepharose™ High Performance; GE Healthcare). This immobilized biotinylated strand was used as pyrosequencing template (26, 33). Pyrosequencing was carried out with internal sequencing primer diluted to 0.3 μM in the annealing buffer provided by the supplier, using the Pyro Gold Reagent Kit (Biotage AB, Uppsala, Sweden). A PyroMark Prep Workstation (Biotage AB) was used for all steps other than bead addition and transfer. Samples were run on a PSQ™96 System instrument, and outgoing results were analysed using pyrosequencing software (Biotage AB).

ELA-DRA exon 2 sequences were well characterized and conserved in the areas that surround the SNPs of interest, so it was possible to use a programmed nucleotide delivery for sequencing. The specific order of nucleotide dispensation keeps the extension of different alleles synchronized after the polymorphic region and avoided the constraints of *de novo* sequencing of polymorphic regions in heterozygous DNA material (32). In addition, a specific nucleotide dispensation order reduces the number of pyrosequencing cycles required and thereby increase the pyrosequencing quality. Homozygous and heterozygous genotypes were unambiguously detected, showing to be more sensitive than SSCP typing and having advantages in terms of throughput. In this study, the application of the pyrosequencing technique allows the validation of the genotypes detected by SSCP typing in almost all samples. By this approach, we could determine that some SSCP homozygous genotypes were in fact heterozygous.

The pyrosequencing genotyping allowed us to detect the two SNPs at positions 140 and 144 that defined the *Equus caballus*-specific alleles *ELA-DRA*0101*, *ELA-DRA*0201*

and *ELA-DRA*0301*. The pyrograms obtained for six horses representing homozygous (Figure 1A–C) and heterozygous genotypes (Figure 1D–F) of *ELA-DRA* exon 2 alleles are shown in Figure 1. Nucleotide addition, according to the order of nucleotides, is indicated below the pyrogram and the corresponding *DRA* genotype is indicated above the pyrogram. As shown in Figure 1, the two analysed SNPs can be unambiguously identified. The signal intensity of polymorphic sites in the homozygote doubled the peak intensity observed in the heterozygote genotypes. Although in same samples background was observed, noise intensity was irrelevant because it was twofold or threefold lower than the signal corresponding to the addition of a unique nucleotide.

In this study, we did not detect new *DRA* alleles. As mentioned above, *DRA*JBH11* allele was not observed in this study, but the method detected nucleotide positions that are not variable between **JBH11* and *DRA*0101* sequences. However, a second internal primer would be incorporated to detect the nucleotide differences present in other positions of the second exon sequence, even those species-specific ones.

Previously published allele frequencies for the equine *DRA* gene were determined by PCR-SSCP and RSCA

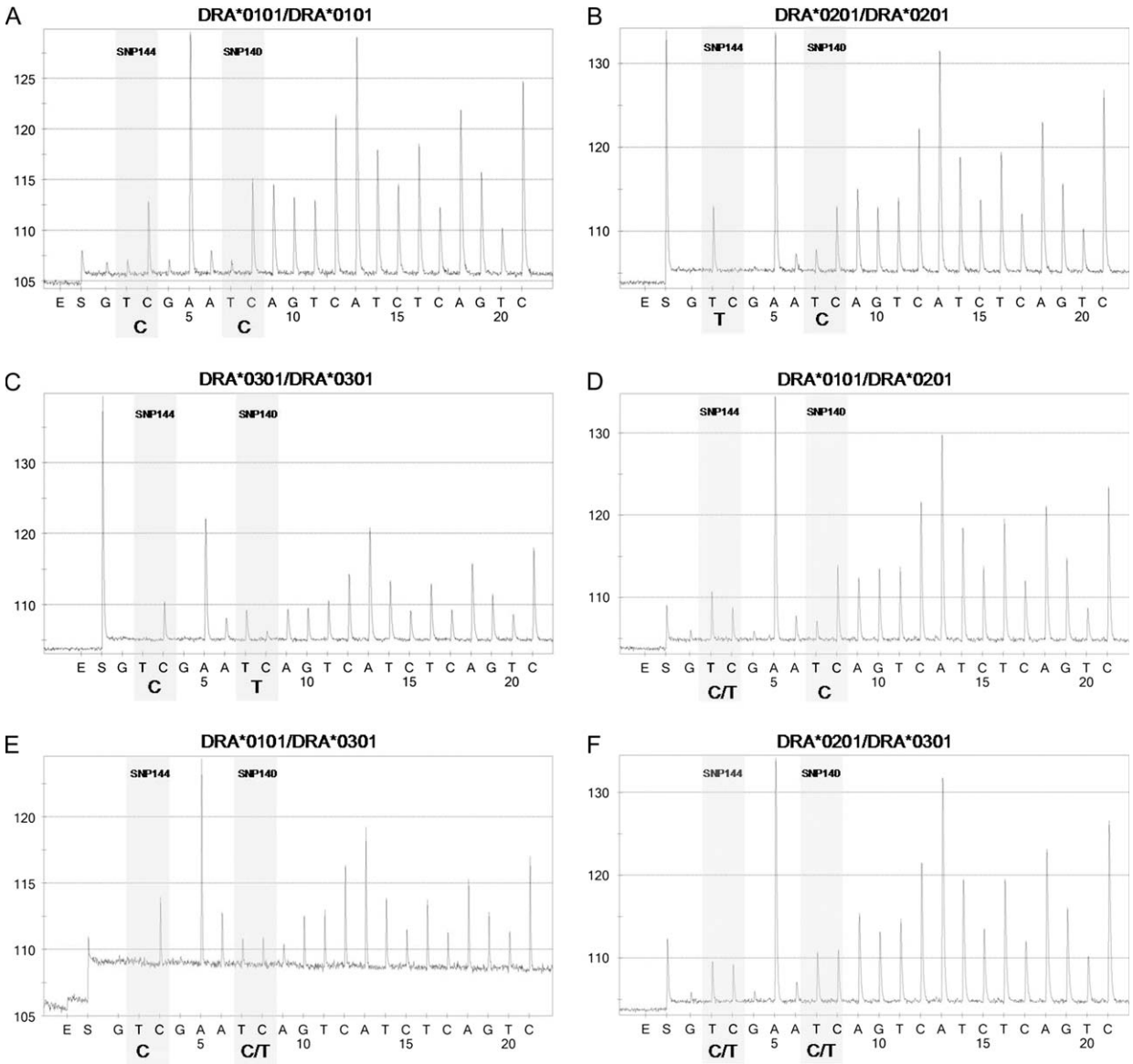

Figure 1 Pyrograms of the raw data obtained from solid-phase pyrosequencing on six horses representing homozygous and heterozygous genotypes of *ELA-DRA* exon 2 alleles. Nucleotide addition, according to the order of nucleotides, is indicated below the pyrogram and the corresponding *DRA* genotype is indicated above the pyrogram. (A–C) homozygous; (D–F) heterozygous genotypes.

typing (10, 11, 34). By the proposed sequence-based methodology, it was possible to confirm the genetic variability of *DRA* gene and preliminarily determine the allele distribution in different breeds from Argentinean and Spanish horse populations. The *DRA*0101* allele was the most common among all breeds, while *DRA*0301* was not detected in Arabs, Quarter Horses and Spanish Breeds (Table 1), but the absence of this allele may be because of the low number of individuals tested. However, allele frequencies were estimated for comparison purposes to the same and other breeds screened by PCR-SSCP and RSCA (10, 11, 34). Table 1 showed the comparison of allelic frequencies and expected heterozygosities (He) in all domestic horse breeds reported up to date. Frequency differences among the same breed could be influenced by the origin and number of horses of the different equine populations examined.

Multiple *SNPs* have been identified to have a major impact on susceptibility/resistance to autoimmune and infectious diseases, consequently it is necessary to have rapid and efficient SNP evaluation techniques to analyse candidate genes such as *ELA-DRA* and genes that influence immune response. As a first approach to screen polymorphism simultaneously in a large number of individuals, SSCP typing is a very useful tool but is very dependent on temperature and ion concentration, so electrophoresis reproducibility is a relevant point if the objective of the investigation is to associate genotypes to resistance/susceptibility to a particular disease.

The close proximity of two or three variable positions of interest allowed *ELA-DRA* base substitutions to be analysed all in one reaction. Additionally, several approaches would be used to analyse more SNPs at the same time within the same PCR amplicon. Furthermore, pyrosequencing technique generates 30 to 40 base sequences with each primer, and the throughput is 96 samples in approximately 20 min (33, 35). The potentiality of this methodology increases because haplotype analysis is an area of intense research for complex genetic phenotypes, so the knowledge of haplotype for several SNPs in one gene is likely to provide more information about genotype–phenotype links than the underlying SNPs (36).

In conclusion, the pyrosequencing technology is an efficient and faster new technology to SNP evaluation than RFLP or SSCP analysis. SNP analysis in large population studies is highly improved because of the reduction in the amount of reagents used, the automation in outcome acquisition and result interpretation. This could aid in the rapid and efficient analysis of SNPs in many genes that encode or are related to disease susceptibility.

Table 1 *ELA-DRA* allele frequencies in domestic horse breeds. The allele frequency data of other breeds were retrieved from the literature

	DRA allelic frequencies					
Breed (*n*)	0101	0201	0301	JBH11[a]	He	References
Thoroughbred (8)	0.57	0.21	0.21	—	0.63	This study
Thoroughbred (265)	0.62	0.15	0.21	0.02	ND	(11)
Thoroughbred (20)	0.40	0.25	0.35	—	0.60	(34)
Criollo Argentino (8)	0.62	0.19	0.19	—	0.58	This study
Silla Argentino (39)	0.37	0.41	0.22	—	0.65	This study
Argentine Peruvian Paso (10)	0.65	0.30	0.05	—	0.51	This study
Arabian (10)	0.85	0.15	—	—	0.27	This study
Arabian (20)	0.63	0.22	0.15	—	0.50	(34)
Quarter Horse (3)	0.50	0.50	—	—	0.60	This study
Spanish Pure Breed (8)	0.75	0.19	0.63	—	0.43	This study
Spanish Native (11)	0.64	0.37	—	—	0.49	This study
Irish Draught (51)	0.70	0.14	0.14	0.02	ND	(11)
Warmblood (26)	0.69	0.12	0.17	0.02	ND	(11)
Brasilian Criollo (12)	0.96	—	0.04	—	0.08	(10)
Garrano (32)	0.91	0.08	0.02	—	0.19	(10)
Lusitano (31)	0.58	0.38	0.03	—	0.53	(10)
Lusitano (17)	0.06	0.94	—	—	0.00	(34)
Mangalarga (25)	0.82	0.06	0.12	—	0.28	(10)
Paso Fino (10)	0.80	0.15	0.05	—	0.40	(10)
Sorraia (52)	0.98	0.02	—	—	0.04	(10)
Sorraia (17)	0.59	0.29	0.12	—	0.47	(34)
Sulphur Mustang (10)	0.95	—	0.05	—	0.10	(10)
Lipizzaner (24)	0.73	0.17	0.10	—	0.25	(34)
Polish primitive (6)	0.83	0.17	—	—	0.33	(34)
Exmoor (38)	0.79	0.20	0.01	—	0.37	(34)

He, expected heterozygozity; ND, not determined.

[a] Allele not detected with the methodologies employed in these studies.

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