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Genetic Polymorphism of Antithrombin III, Haptoglobin, and Haemopexin in Wild and Domestic European Rabbits

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Genetic polymorphism of European rabbit (Oryctolagus cuniculus) plasma proteins antithrombin III, haptoglobin, and haemopexin was investigated by means of isoelectric focusing in free and immobilized pH gradients followed by immunoblotting. The study of two wild and one domestic populations led to the recognition of six alleles of antithrombin III and haptoglobin, and five alleles of haemopexin.

KEY WORDS: European rabbit; *Oryctolagus cuniculus*; antithrombin III; haptoglobin; haemopexin; hybrid isoelectric focusing; genetic polymorphism.

INTRODUCTION

Several studies on the genetic polymorphism of domestic rabbit haptoglobin (HP) and haemopexin (HPX) have been performed in the past. Nevertheless, both the separation and detection methods used are far from being efficient for large population phenotyping and resolving power for the detection of cryptic variation. Genetic variation of HP was investigated by immunodiffusion with the detection of two alloantigens with no differences in electrophoretic mobility (see Altman and Katz, 1979), and several studies on the genetic polymorphism of HPX by conventional electrophoresis in starch and polyacrylamide gels led to the recognition of four electromorphs (Grunder, 1966, 1968; Hagen *et al.*, 1978). In the

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present work isoelectric focusing techniques in free and immobilized pH gradients rehydrated with carrier ampholytes followed by immunoblotting detection for the study of rabbit antithrombin III (ATIII), HP, and HPX genetic polymorphism are described. A survey of one domestic and two wild populations revealed a high level of genetic variation at the three loci. We detected six alleles of ATIII and HP, and five alleles of HPX.

MATERIAL AND METHODS

EDTA blood samples from domestic rabbits were obtained in slaughterhouses while samples from wild animals were obtained during the hunting season. Plasma was separated from red cells by centrifugation at 1500*g* for 5 min at 4°C and stored at −70°C until use. ATIII hybrid isoelectric focusing (HIEF) separation was achieved in immobilized pH gradients 4.55–5.05 in polyacrylamide gel ($T = 5\%$, $C = 3\%$, $247 \times 112 \times 0.5$ mm) constructed as indicated in the Application Note Nr 324 of LKB. Immobiline gels were dehydrated overnight at 45°C and rehydrated in a 20% (w/v) sucrose, 2.7% (v/v) 4.5–5.4 Pharmalyte solution for 2 h at room temperature. A mixture of 0.025 M glutamic acid and 0.025 M aspartic acid, and 0.1 M sodium hydroxide was used as anode and cathode solutions, respectively. Ten microliter of plasma diluted 1:3 (v/v) in distilled water was applied 1.5 cm from the cathode in a silicone strip. Focusing was performed at constant voltage setting limits at 1500 V, 4 mA, 12 W (30 min), 3000 V, (3 h), and 5000 V (2 h). For HP detection, 5 μL of plasma was incubated with 15 μL of neuraminidase (0.6 U/mL) for 1 h at 37°C and overnight at room temperature. Prior to IEF, 10 μL of dithiothreitol (DTT) (0.06 M) was added and incubated at 37°C for 1 h. A further 15 μL of IAC (0.12 M) was added and left for 30 min at 37°C. DTT and iodoacetamide (IAC) solutions were prepared in 6 M urea. Isoelectric focusing separation of HP was done in polyacrylamide gels ($T = 5\%$, $C = 3\%$, urea 48% (w/v); $230 \times 100 \times 0.3$ mm) with a pH gradient of 4–9 established with Pharmalyte 4–6.5 (1.50%, v/v), Ampholine 6–8 (4.00%, v/v), and Ampholine 7–9 (0.75%, v/v). The anode and cathode solutions were 0.04 M glutamic acid and 1 M sodium hydroxide, respectively. After prefocusing at constant power setting limits at 1500 V, 25 mA, 1 W (30 min), 2 W (15 min), and 3 W (15 min), 8 μL of plasma solution was applied 1 cm from the cathode in a silicone strip. Focusing was performed at constant power setting limits at 2500 V, 25 mA, 4 W (1 h), 5 W (1 h), and 6 W (1 h). IEF separation of HPX was done in the same conditions as HP in a pH gradient 5–8 established with Pharmalyte 5–6 and 5–8 in the same final concentration of 3.13% (v/v), and proline 3% (w/v). After focusing, proteins were transferred to a nitrocellulose membrane and patterns detected by immunoblotting with antihuman ATIII (ATAB 81932), antihuman HP (ATAB 81952), and antihuman HPX (ATAB 80288), and rabbit antigoat immunoglobulin peroxidase conjugate (ATAB 83806), as previously described by Branco *et al.* (1998).

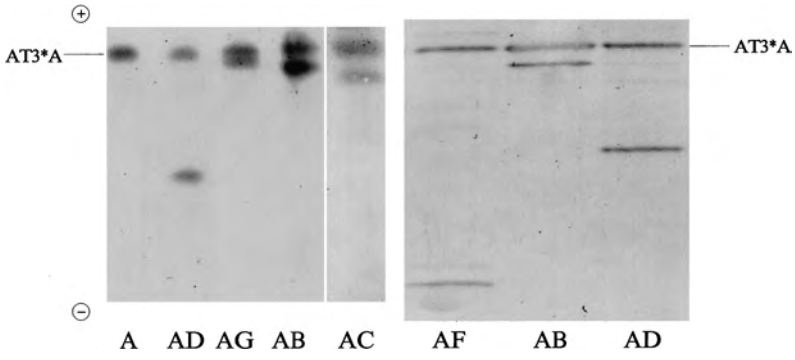

Fig. 1. ATIII phenotypes comprising all alleles detected in one domestic and two Iberian wild rabbit populations after hybrid isoelectric focusing separation and immunoblotting.

RESULTS AND DISCUSSION

Figure 1 depicts the typical pattern of rabbit ATIII obtained after HIEF separation. The eight ATIII phenotypes detected can be explained by a genetic model of six codominant alleles at an autosomal locus for a monomeric protein. Table I shows the estimated gene frequencies for the three populations studied. ATIII*A is fixed in the domestic stock and is most abundant in wild rabbits. ATIII*B and ATIII*C were only detected in the Navarra population while ATIII*D, ATIII*F, and ATIII*G were only found in the Doñana population.

In Fig. 2 the typical pattern of rabbit HP obtained after IEF separation is represented. The 13 phenotypes detected are in agreement with a genetic model of six codominant alleles at an autosomal locus for a monomeric protein.

According to Altman and Katz (1979) two HP alloantigenes were detected in the domestic rabbit but no electrophoretic separation of the native protein was obtained. In the present work, the use of denaturing agents during both sample treatment and isoelectric focusing allowed the identification of six alleles (Table II). Although the unavailability of the antibodies precludes cross identification with the electromorphs, the detection of two alleles in the domestic stock

Table I. Allele Frequencies for Antithrombin III in Three European Rabbit Populations

Population	*N*	Allele frequencies					
		ATIII*A	ATIII*B	ATIII*C	ATIII*D	ATIII*F	ATIII*G
Wild rabbit							
Doñana (Spain)	58	0.88			0.05	0.05	0.02
Navarra (Spain)	44	0.77	0.22	0.01			
Domestic rabbit	143	1.00					

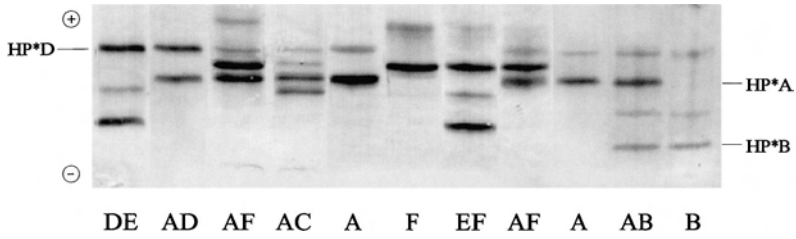

Fig. 2. HP phenotypes comprising all alleles detected in one domestic and two Iberian wild rabbit populations after isoelectric focusing separation and immunoblotting.

suggests that these are most probably the same. Allele HP*A is the most common in both domestic and wild populations, HP*B was detected in the domestic stock and in the Navarra population and HP*F is common to both wild populations. HP*C, HP*D, and HP*E are low frequency alleles only detected in the Doñana population.

Figure 3 represents the pattern of IEF separation of rabbit HPX. The 11 phenotypes detected can be explained by a genetic model of five codominant alleles at an autosomal locus for a monomeric protein. Table III describes the estimated allele frequencies for the three populations studied. Research of haemopexin genetic variation in domestic rabbit by means of conventional electrophoresis led to the detection of four alleles (Grunder, 1966, 1968; Hagen *et al.*, 1978). To identify the alleles described, reproduction of the electrophoretic techniques followed by peroxidase activity of the heme-HPX complex was attempted, but the poor quality of the electrophoretic patterns only allowed the discrimination of two electromorphs in domestic rabbit (results not shown), thereby precluding the identification of the alleles previously described. According to their electrophoretic mobility they were named HPX*F and HPX*S. IEF screening of domestic and wild rabbits revealed heterogeneity in both forms named HPX*F1, HPX*F3, HPX*S1, HPX*S3, and HPX*S4, in chronological order of appearance. HPX*F1 and HPX*S1 were detected in all populations, the former being the most abundant in all of them. HPX*F3 and HPX*S3 are shared by domestic rabbits and the populations of

Table II. Allele Frequencies for Haptoglobin in Three European Rabbit Populations

Population	*N*	Allele frequencies					
		HP*A	HP*B	HP*C	HP*D	HP*E	HP*F
Wild rabbit							
Doñana (Spain)	99	0.41		0.05	0.05	0.03	0.47
Navarra (Spain)	36	0.72	0.02				0.26
Domestic stock	96	0.80	0.20				

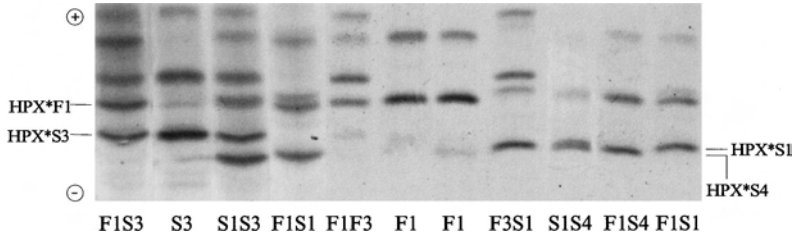

Fig. 3. HPX phenotypes comprising all alleles detected in one domestic and two Iberian wild rabbit populations after isoelectric focusing separation and immunoblotting.

Doñana and Navarra, respectively. HPX*S4 is a low frequency allele detected only in the Doñana population.

Preliminary analysis of wild rabbits revealed the existence of further genetic variants in both ATIII (ATIII*E) and HPX (HPX*F2, HPX*S2) (results not shown) proving that investigation of protein polymorphism by IEF separation methods is still a powerful tool to uncover extensive genetic variation within the rabbit species. Notably, the HPX locus has already proved to be highly polymorphic in other leporid species, like the Iberian hare and the European hare (Alves *et al.*, 2000).

Identification of the electromorphs previously described in domestic rabbits at the HP (Altman and Katz, 1979) and HPX (Grunder, 1966, 1968; Hagen *et al.*, 1978) loci using rudimentary methods or specific alloantigenes was not possible. By contrast, IEF in free and immobilized pH gradients followed by immunoblotting have highly standardized protocols easy to establish in any laboratory, which makes them of great interest for studies that depend on extensive population surveys.

The patterns of allelic distribution presented here show a high level of differentiation between southern and northern Iberian populations which is in agreement with the description of two evolutionary diverging lineages within the rabbit species (Branco *et al.*, 2000, 2002; Ferrand, 1995; Monnerot *et al.*, 1994; van der Loo *et al.*, 1991,1999). It further supports the idea that domestic breeds belong to the northern group from which they probably had a single origin (Ferrand, 1995).

Table III. Allele Frequencies for Haemopexin in Three European Rabbit Populations

Population	*N*	Allele frequencies				
		HPX*F1	HPX*F3	HPX*S1	HPX*S3	HPX*S4
Wild rabbit						
Doñana (Spain)	43	0.35	0.33	0.28		0.04
Navarra (Spain)	40	0.66		0.22	0.12	
Domestic stock	139	0.54	0.02	0.35	0.09	

HPX maps to linkage group I (Hagen *et al.*, 1978) together with the hemoglobin beta chain locus and HP maps to linkage group VI (Korstanje *et al.*, 2001). Linkage groups I and VI were, respectively, assigned to chromosome 1 (Xu and Hardison, 1989) and chromosome 5 (Korstanje, 2000). The present description of three highly polymorphic type I markers (O'Brien *et al.*, 1983) may be of interest for the ongoing research of the genetic map of the rabbit, but also for the characterization of wild and domestic populations as well as stock identifications. Microsatellites are known to be more polymorphic than biochemical markers and are now the most used genetic markers for population surveys. Nevertheless, in the rabbit it has also been shown that, because of their high mutation rate and homoplasy, microsatellites have a poor capacity to discriminate populations, especially in their natural range, the Iberian peninsula (Queney, *et al.*, 2001). Our knowledge of the different levels of rabbit genetic variation, demonstrates that the study of highly poymorphic protein markers is still one of the most valuable tools to address population genetic questions. Finally, their combination with studies on nuclear DNA sequencing will provide a significant contribution to evolutionary analyses.

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