Detection of the Protease Inhibitor (Pi) Systems of the Light Breed Horses by Isoelectric Focusing

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Abstract Detection of the equine protease inhibitor (Pi) systems was carried out by isoelectric focusing (IEF) with a high capacity for resolution and repeatability. The Pi systems of the light breed horses were classified and their usefulness as marker genes for parentage testing was discussed. 1. To increase resolution of Pi electrophoretic patterns, serum samples were treated with a reducing agent and a separating agent was added to the polyacrylamide gels. Consequently, Pi bands could be detected more clearly because of increased separation between Pi and Pa regions, and made it possible to remove any extra proteins. New attached bands in Pi systems could then be detected in the regions which has been called Xc/Xd/Xh. 2. It became apparent that the Pi types in the light breed horses are controlled by eleven codominant autosomal alleles; Pi^F , Pi^G , Pi^I , Pi^L , Pi^{L1} , Pi^M , Pi^N , Pi^{S1} , Pi^{S2} , Pi^U and Pi^Z . The Pi^L allele was found to have the highest frequency of 44.7% in the light breed horses. 3. As the theoretical paternity exclusion probability of Pi system was 0.532, it was recognized that the system could be very useful as the marker gene for parentage testing in the light breed horses.

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The equine prealbumin system (Pre) was first reported by GAHNE (1966)¹⁾. Subsequently, the electrophoretic methods used for determination of Pre types were improved, and Pre types were studied in many kinds of horses. It became apparent that Pre system is controlled by a minimum of 15 codominant autosomal alleles; Pre^{D1} , Pre^{D2} , Pre^F , Pre^G , Pre^I , Pre^L , Pre^{L1} , Pre^M , Pre^N , Pre^S , Pre^T , Pre^U , Pre^W , Pre^Z , and $Pre^{Y2,3,4)}$.

On the basis of studies by EK (1979)⁵⁾, MATTHEWS (1979)⁶⁾ and JUNEJA *et al.* (1979)⁷⁾, it was found that Pre protein corresponds to human Pi protein, with regard to its antitrypsin activity, electrophoretic mobility and molecular weight. As the α -protease inhibitor activities of Pre bands described above were also confirmed, it was suggested that Pre system should be renamed as Pi system. Since the system has subsequently been used as Pi system, it should be referred to as such in the present paper.

JUNEJA *et al.*⁷⁾ have also analyzed equine Pi types by means of two-dimensional gel electrophoresis, and two groups of α_1 -globulin designated Pi 1 and Pi 2, for which complete genetic linkage was observed, were found to be protease inhibitors. It was shown that the Pi protein has a high heterogeneity.

At present, there is a problem in that the results obtained for the Pi system in each laboratory which participated in the horse blood typing comparison test did not correspond as compared with those of other systems. This might be due to differences in the electrophoresis technics and multiplicity of Pi protein zones. However, as SCOTT (1976)⁸⁾ mentioned, since *Pi* locus had a high polymorphism, it was thought that Pi system could be the most useful one as a marker gene for the identification and parentage test.

This report presents the results of examining the detection of equine Pi types by isoelectric focusing (IEF) with a high capacity for resolution and repeatability. At the same time, the Pi types of the light breed horses were classified and their usefulness as the marker gene for parentage testing was also studied.

Materials and Methods

Sera used: Serum samples were obtained from 2501 horses consisting of Thoroughbreds (1514), Anglo-Arabs (979) and Arabs (8). The serum samples were treated with 100 mg/2.56 ml dithiothreitol in distilled water and 4.6 mg/2 ml iodoacetamide in distilled water in a 2:1:2 ratio overnight at 4°C.

Making of gels: Gels were made according to the method reported by YOKOHAMA *et al.*⁹⁾, except that 0.4 ml of Pharmalyte (Pharmacia) 4-6.5 and 0.3 ml of SERVLYT (SERVA) AG 4-5 were used as the carrier ampholyte, with 117.3 mg of ACES (Sigma) added the stock solution per a gel plate.

Electrophoresis: Contacts of gel with anode and cathode were made using strips of TOYO No. 526 filter paper soaked in 0.04 M DL-glutamic acid and 0.2 M L-histidine, respectively. Running was carried out initially at 450 V (2.5 W) and finally at 1200 V for 4.5 hours. The sample papers (TOYO No. 526 filter paper measuring 4 mm × 5 mm) were removed after 15 minutes of running.

Fixing and staining: Gels were fixed and stained according to the method reported by YOKOHAMA *et al.*⁹⁾.

Genetical analysis: 707 pairs of family data were used studying the inheritance of Pi types.

Exclusion probabilities: The theoretical paternity exclusion probabilities were calculated by the method of RENDEL and WINER *et al.*^{10,11)}, and then the practical exclusion probabilities were studied by using 340 pairs of parentage cases of two sires offered for the parentage test.

Results and Discussion

Fig. 1 shows the electrophoretic patterns of Pi phenotypes which were detected by the IEF method. As can be seen, the patterns on the gel were separated into three regions, Pi (Pre), Xc/Xd/Xh and Pa (Xk) regions, which were the same as those of acidic prealbumin types reported by BRAEND^{12,13)}. Compared with the equine Pi types determined by IEF which were first reported by MATTHEWS (1979)¹⁴⁾, the IEF method reported here differed in several ways. Namely, before running, serum

Horse Pi System by IEF

Fig. 1. Electrophoretic patterns of the equine Pi and Pa systems by IEF.

Fig. 2. Diagram to illustrate Pi and Pa phenotypes

samples were treated with a reducing agent, and then a separating agent was added to the gel solution. Consequently, extra proteins on Pi zones were removed and the separation between Pi and Pa regions was increased, making it possible to distinguish Pi bands more clearly.

As shown in the photograph in Fig. 1, 12 basic bands (F, G, I, L, L₁, M, N, S₁, S₂, U, Z₂ and Z) of Pi could be classified by the IEF method reported here, although the No. 11 individual with Z₂ type was taken from the samples of the 1983 horse blood typing comparison test. As individuals Nos. 6, 13 and 14 were shown to be M-(F), IL-(F) and L-(F) types, respectively, which were contained F band of Es system was detected within Pi zones.

Fig. 2 shows a diagram of each Pi type. As can be seen, the bands in each Pi type were shown by assigning arabic figures to them in order of decreasing electrophoretic mobility. The patterns of L and L₁ types resembled each other, except for the fact that L₁ type did not have Nos. 3 and 4 bands shown by L type, and that No. 4 band absent from L type was observed in L₁ type. In I, L, L₁ and M types, new attached bands which were named Nos. 5, 6, 5 and 4, respectively, were de-

tected in Xc/Xd/Xd region, and these attached bands were available for use as markers for classifying Pi types.

The segregation ratios of Pi types except for the Z₂ type were studied using the results of 707 pairs of matings. Consequently, it was reconfirmed that Pi types were controlled by a pair of codominant autosomal alleles at a single locus.

Table 1. Gene frequencies of Pi system in the light breed horses

Alleles	Breeds			Average
	Thoroughbreds	Anglo-Arabs	Arabs	
<i>Pi^F</i>	0.036	0.092	0.188	0.059
<i>Pi^G</i>	0.007	0.058	0.	0.027
<i>Pi^I</i>	0.065	0.079	0.188	0.071
<i>Pi^L</i>	0.466	0.420	0.250	0.447
<i>Pi^{L1}</i>	0.011	0.009	0.	0.010
<i>Pi^M</i>	0.	0.024	0.	0.009
<i>Pi^N</i>	0.196	0.098	0.	0.157
<i>Pi^{S1}</i>	0.034	0.055	0.313	0.043
<i>Pi^{S2}</i>	0.054	0.059	0.063	0.056
<i>Pi^U</i>	0.127	0.106	0.	0.118
<i>Pi^Z</i>	0.003	0.001	0.	0.002
No. of animals	1514	979	8	2501

Table 2. Estimation of the average probability of negating one of the two possible sires in parentage cases

Loci	No. of alleles	Breeds		Average
		Thoroughbreds	Anglo-Arabs	
Red cell loci alone*		0.454	0.458	0.460
<i>Tf</i>	5	0.421	0.430	0.429
<i>Alb</i>	2	0.143	0.172	0.160
<i>Es</i>	3	0.118	0.128	0.126
<i>Pi</i> (S. G. E.)	4	0.120	0.196	0.160
<i>6-PGD</i>	2	0.183	0.185	0.183
<i>Hb-a</i> (IEF)	2	0.112	0.126	0.117
12 loci combined		0.847	0.872	0.862
<i>Pi</i> (IEF)	11	0.522	0.581	0.532
<i>Pa</i> (IEF)	2	0.	0.026	0.016
13 loci combined which added <i>Pi</i> and <i>Pa</i> (IEF) instead of <i>Pi</i> (S. G. E.)		0.917	0.935	0.925

*: The case that genotype of sire is not clear, and 6 loci are A, C, D, P, Q and U systems.

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Table 3. Number and percentage of negating one of two possible sires when Pi system (IEF) was adopted for parentage test

Items studied	Case that genotype of dam was clear		Case that genotype of dam was not clear		Average
	Thoroughbreds	Anglo-Arabs	Thoroughbreds	Anglo-Arabs	
No. of parentage cases	231	36	52	21	340
Parentage cases solved by Pi system (S. G. E.)	(11) 0.048	(7) 0.194	(3) 0.058	(2) 0.095	(23) 0.068
Parentage cases solved by Pi system (IEF)	(100) 0.433	(21) 0.583	(18) 0.346	(5) 0.238	(144) 0.424

(): No. of animals, S. G. E.: Starch gel electrophoresis, IEF: Isoelectric focusing.

If it is accepted that Pi system consists of eleven codominant autosomal alleles, then 66 different genotypes should theoretically be possible. However, only 56 genotypes were observed in this study using 2501 light breed horses.

Table 1 shows the results of gene frequencies of *Pi* alleles in the light breed horses. Differences in the frequencies were not extreme between Thoroughbreds and Anglo-Arabs. However, whereas *Pi^M* allele was not recognized in the former, its frequency was 2.4% in the latter, while *Pi^N* allele was about 10% higher than that of the latter. Also *Pi^M* allele was found to correspond with *Pre^s* allele which was first classified by GAHNE¹⁾.

Table 2 shows the results obtained for the theoretical paternity exclusion probabilities of Pi and Pa systems along with 12 loci. It can be seen that the exclusion probabilities of Pi types in Thoroughbreds and Anglo-Arabs were 52.2 and 58.1%, respectively. Their values were the highest as compared with other loci. The exclusion probabilities of the 13 loci including Pa system that can be detected along with Pi types were 93% in the light breed horses used. This figure could be expected to increased by about 6% when the 13 loci have been adapted for the blood typing.

Table 3 shows the results obtained when the practical exclusion probabilities of Pi system were studied by using the parentage cases of the two sires which were offered for the parentage test. In Thoroughbreds, eleven (4.8%) of 231 parentage cases could be solved using *Pre* (Pi) types by means of starch gel electrophoresis, whereas by using Pi types detected by IEF, 100 (43.3%) of these cases could be solved. In Anglo-Arabs, using the former types, seven (19.4%) of 36 parentage cases could be solved, whereas 21 cases (58.3%) could be solved using the latter types. Accordingly, it was recognized that Pi types characterised by means of IEF were considerably useful as marker genes in the parentage test. But, if we introduce this Pi system to routine works, we should have to check for by means of two-dimensional electrophoresis some Pi types whether they are heterozygotes or not.

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等電点電気泳動法による軽種馬の Pi system

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再現性および分析能が高い等電点電気泳動法 (IEF) による Protease inhibitor (Pi) 型の検出法の検討を行うと同時に、軽種馬における Pi 型の分類およびその標識遺伝子としての有効性を調べた結果、次の如く成績が得られた。

1. Pi 型の泳動像をより鮮明にするため還元剤による血清処理および Separator をゲルに加えたところ、Pi 域と Pa 域間が拡大でき、さらに余分な蛋白の除去も可能となったため、各 Pi 型バンドがより鮮明に検出できた。また従来から Xc/Xd/Xh と呼ばれている域に Pi 型

の新しいバンドが検出できた。

2. IEF 法により検出された軽種馬の Pi 型は Pi^F , Pi^G , Pi^H , Pi^L , Pi^{Li} , Pi^M , Pi^N , Pi^{S1} , Pi^{S2} , Pi^U および Pi^Z の 11 種類の対立遺伝子から成ることが分かった。また、 Pi^L の頻度が 44.7% と最も高い値であった。

3. 軽種馬の Pi 型における理論的な父権否定の確率は 0.532 で、親子判定上きわめて有効な標識遺伝子であることが認められた。

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