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ORIGINAL ARTICLE

Analysis of serum proteins electrophoresis in clinically healthy miniature Caspian horse

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Abstract The Iranian miniature Caspian horse is the most ancient domestic breed of horse; it is the likely ancestor of all modern breeds of hot-blooded horses. Blood serum protein analysis has not been sufficiently studied in miniature Caspian horses. Since serum electrophoresis may be an important preclinical tool for diagnosis of equine diseases, evaluation of serum protein fractions in miniature Caspian horses using acetate cellulose strips was undertaken. In addition, serum protein concentrations were also examined. Blood samples were collected from 30 clinically healthy horses with and without anticoagulant. Using cellulose acetate electrophoresis (CAE), serum samples were fractionated and absolute and relative concentrations from each sample calculated. From 30 samples, 26 samples (86.6%) had five protein fractions and four samples (13.4%) had four fractions. Samples with five fractions were a common pattern (χ^2, $P<0.01$). Albumin and other protein fraction concentrations were distributed normally ($P>0.05$). Absolute values of albumin, $\alpha 1$, $\alpha 2$, β, and γ globulin were 2.9±0.24, 0.97±0.19, 0.96±0.24, 1.2±0.36, and 1.3±0.29 g/dl, respectively. Mean albumin to globulin ratio was 0.67. In conclusion, analysis of serum proteins in the miniature Caspian horse using CAE showed five protein fractions including albumin, $\alpha 1$, $\alpha 2$, β, and γ globulin.

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Introduction

The miniature Caspian horse is the most ancient domestic breed of horse in the world. It was previously believed that this tiny horse was a kind of pony, but detailed scientific research verified that this animal was a small horse and not a pony. It is likely to be a direct ancestor of the oriental breeds and subsequently of all light horse breeds and is probably the ancestor of all modern breeds of hot-blooded horses (Hendricks 1995). They are very rare, and the breed was rescued from extinction by Louise Firouze, an American living in Iran, in 1965 (Seifi et al. 2002).

Equine diseases that cause quantitative and qualitative changes in serum proteins include liver disease, congenital or acquired immunodeficiency states, protein-losing enteropathies, protein-losing nephropathies, local or systemic infections, neoplasia, and parasitism. Blood serum electrophoresis is a useful technique for diagnosing these diseases (Riond 2008).

Based on the dividing method of protein fractions, interpretation of serum electrographs are variable (Bierer 1969; Coffmen 1968; Green et al. 1982; Kirk et al. 1975; Osbaldiston 1972; Mattheeuws et al. 1966). Standardization of serum protein electrophoresis (SPE) is vital to obtain precise and reproducible results (Keay and Doxey 1982; Jeppson et al. 1979). Investigators have determined various numbers of protein bands in normal horse blood serum (Bierer 1969; Coffmen 1968; Green et al. 1982; Kirk et al. 1975; Osbaldiston 1972; Mattheeuws et al. 1966; Riond et al. 2008). Recently, Riond et al. (2008) standardized protein fractions in some horse breeds (Thoroughbred and

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Belgian Shire horses). To our knowledge, neither the electrophoretic techniques nor the classification of serum protein fractions has been standardized in miniature Caspian horses.

The present study was therefore conducted to examine the serum protein fractions in the Caspian horse using cellulose acetate electrophoresis (CAE) with particular focus on interindividual variations.

Materials and methods

The study was performed on 30 clinically healthy miniature Caspian horses at Khojir animal research station (Tehran National Zoo Park, Tehran, Iran). These animals were between 4 and 20 years old (mean=8.5 years), and both males and females were sampled. All horses were inspected by the station veterinarian and declared free from internal and external parasites.

Blood samples were collected from the jugular vein using a disposable syringe and an 18 G needle. Approximately 10 ml of blood was taken from each horse into glass tubes containing anticoagulant (0.1 ml of 10% disodium ethylenediaminetetraacetic acid solution for 5 ml of blood) and tubes without anticoagulant. After clotting the blood samples for 30 min, serum was harvested by centrifugation at 3,500×*g* for 7 min and stored at -20°C until analysis. In addition, a CBC count and chemistry profiles were done for each sample. None of the horses were clinically sick.

Total protein concentrations of the samples were measured by the biuret method. SPE and densitometry were performed using an automated electrophoresis system on cellulose acetate strips (Sartorius, Munich, Germany) according to the procedure described by the manufacturer. Briefly, after electrophoresis of the strips (buffer solution: tris barbiturate, pH 8.6, ionic strength 0.1) for 45 min at 13 mA and 220 V, samples were stained for 10 min, destained for 1 min, and cleared for 1 min. Ponceau-S (LRE Medizintechnick) and phosphoric acid were used as staining and destaining solutions, respectively. Finally, after drying, the strips were scanned in a densitometer (software PhotoEP V7.51XP; Bender & Hobein, Munich, Germany) and electrophoretic curves were obtained. Using total protein concentration, absolute (g/dl) and relative concentrations of each fraction were calculated. For calculating the absolute concentration, the percentage of each fraction in a serum sample was multiplied by total protein concentration (Eckersall 2008). Normality of the protein fraction distributions were evaluated using Shapiro–Wilk test at a significance of <0.05 using SPSS software (SPSS 14.0.2, SPSS, Chicago, IL, USA). Chi-square (χ^2) test was used for comparison of the fraction resolution pattern.

Results

From 30 samples, 26 samples (86.6%) had five protein fractions and four samples (13.4%) had four fractions (Fig. 1). The χ^2 test demonstrated that patterns with five fractions were a common pattern ($P<0.01$). Total serum protein and albumin concentrations within the samples with five fractions were 7.3±0.54 and 2.9±0.24 mg/dl, respectively (Table 1). Using the Shapiro–Wilk test, the distribution of the albumin and other protein fractions was identified as normal. But distribution of the β globulin was normal after data transformation logarithmically ($P=0.05$). Absolute and relative values of each protein fraction are presented in Table 1.

Discussion

Electrophoresis is a selective method for evaluating serum protein fractions. If results obtained by SPE are properly interpreted, it may be a useful diagnostic method for clinicians (Riond 2008). The history of the usage of the cellulose acetate membrane for SPE goes back to the mid-20th century (Kohn 1957), and although agarose gel electrophoresis has become more popular in diagnostic laboratories, CAE is widely used in human and veterinary clinical biochemistry laboratories (Manera and Britti 2008).

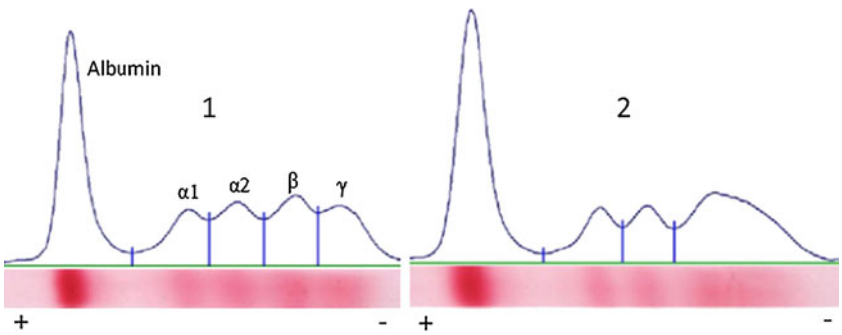

Fig. 1 SPE using cellulose acetate in Caspian miniature horses. *1* Strips with five fractions (26 samples), *2* Strips with four fractions (four samples)

Table 1 Serum protein fractions in Caspian miniature horses determined by CAE

Protein fraction		Mean±SD	Range	Normality test (P=0.05)[a]
Albumin	AV (g/dl)	2.9±0.24	2.4–3.3	0.54
	RV (%)	39.4±3.6	34.2–46.9	
α1	AV (g/dl)	0.97±0.19	0.7–1.4	0.15
	RV (%)	13.3±2.4	9.8–19.3	
α2	AV (g/dl)	0.96±0.24	0.6–1.4	0.14
	RV (%)	12.3±3.5	9.2–19.2	
B	AV (g/dl)	1.2±0.36	0.6–1.9	0.05
	RV (%)	17.4±4.6	8–28.4	
Γ	AV (g/dl)	1.3±0.29	0.9–2.1	0.18
	RV (%)	17.4±4.6	10.8–25.1	
A/G ratio		0.66±0.11	0.5–.88	

Data were collected from 26 samples (86.6%) had five protein fraction as common pattern (χ^2, P<0.01)

AV Absolute value, *RV* relative value

[a] Shapiro–Wilk test (P<0.05)

There are many advantages when using CAE including little absorption of albumin during electrophoresis, achieving sharper fractions, and ease of staining and scanning the strips (Osbaldiston 1972).

There are several techniques for determining dividing lines between fractions, but based on studies by Riond et al. (2008) and Osbaldiston (1972), relative migration routes of proteins are common. In this method, the distance of albumin from the point of application is assumed as 100, and distances of the other fractions are calculated from this point.

Various studies have presented different numbers of protein peaks in the serum of horses (Table 2). Separation technique and quantification procedures are the main reasons for reported differences. Specially the "shoulder" on the alpha side of the albumin peak leads to these differences (Green et al. 1982; Kirk et al. 1975; Riond et al. 2008). Some authors named this alpha-1 (Kirk 1975) or alpha-1a (Bierer 1969) globulin while others included it with albumin (Coffman 1968; Green et al. 1982; Irfan 1967; Osbaldiston 1972). However, in our study, the shoulder was not observed on any of the studied strips.

Because patterns with five fractions were seen as a common pattern (χ^2, P<0.01), we propose the following five albumin fractions: α1, α2, β, and γ globulin for miniature Caspian horse serum. Other studies using the same method (see Table 2) have suggested various protein fractions. These studies mostly showed five fractions for horse serum electrophoresis (Coffman 1968; Green et al. 1982; Kao et al. 1954; Osbaldiston 1972). By means of zone electrophoresis, and Deutsch and Goodloe (1945) and Kao (1954) fractionated horse serum into five fraction including similar bands to the present study. In addition, SPE on agarose gel, as a supporting matrix, provided various protein bands (Kristensen and Firth 1977; Riond 2008; data not shown).

Although, the serum protein concentration (7.3±0.54 g/dl) for Caspian horse determined in this study was similar to that presented by others (see Table 2), both Massip and Fumiere (1974) and Mattheeuws (1966) showed lower values (6.1±0.07 and 6.74±0.28 g/dl, respectively). Our absolute value (2.9±0.24 g/dl) for albumin was also similar to other studies except for data presented by Coffman (3.3 g/dl). Massip and Fumiere (1974), Coffman (1968), and Mattheeuws (1966) reported higher relative values for albumin. Six studies recognized α1 fraction in their protein electrophoresis strips (Table 1). In all studies except by Osbalsdiston (1972), α1 absolute concentration was significantly lower than the present study (0.97±0.19 g/dl). Compared to our study, Massip and Fumiere (1974) and Mattheeuws et al. (1966)) obtained a lower relative value for α1 globulin. Absolute (0.96±0.24 g/dl) and relative values (12.3±3.5%) of the α2 globulin was similar to other studies findings. Using separation of horse serum CAE, Kao (1954) indicated a single β globulin fraction. Moreover, Green (1982) presented two β fractions, but calculated one β fraction concentration. Our absolute value for β globulin (1.2±0.36 g/dl) was lower than the value presented by Green et al. (1982). All authors are, however, in agreement on a single γ fraction. γ Globulin concentration in the Caspian horse was slightly lower than values obtained by Kirk (1975), Green (1982), and Osbalsdiston (1972). To our knowledge, none of these studies examined normality of their results except for Riond (2008). Data analyzed by Rinond (2008) was not normally distributed in the studied horses (data not shown).

Rinond (2008) analyzed SPE from clinically healthy horses [warmbloods (including Thoroughbreds) and draught horses (Belgian, Shire horses)]. Using agarose gel electrophoresis, they identified six protein fractions. In addition, they calculated absolute and relative protein fractions. Our total serum protein concentration (7.4 g/dl) was higher than theirs. Although γ globulin levels in both studies were close, our absolute values for the other protein fractions (α1, α2) were higher. But albumin concentration and percentage in the present study was lower than their findings.

Table 2 Serum protein fractions values (g/dl or%) in normal horse obtained by different studies and in Caspian miniature horse

	Our study[a]		Kao	Bierer	Osbalsdiston	Kirk	Green[b]	Coffman		Massip and Fumiere		Mattheeuws	
	AV	RV	RV	RV	AV	AV	AV	AV	RV	AV	RV	AV	RV
TP	7.3±0.54				7.3	7.53±0.16	–	7.0 (6.0–7.7)		6.1±0.07		6.74±0.28	
Albumin	2.9±0.24	39.4±3.6	39.6	43.0 (37.3–50.3)	3.0 (2.8–3.3)	2.7±0.06	3.13 (2.60–3.84)	3.3 (2.9–3.8)	47 (39–53)	2.76±0.08	45.4±1.47	3.14±0.24	48.5
α1	0.97±0.19	13.3±2.4	16.9	–	1.0 (0.66–1.15)	0.3±0.02	0.42 (0.36–0.47)	–	–	0.25±0.02	4.06±0.33	0.19±-0.02	2.9
α1a	–	–	–	2.22 (1.43–3.79)	–	–	–	–	–	–	–	–	–
α2b	–	–	–	3.12 (1.91–4.89)	–	–	–	–	–	–	–	–	–
α2	0.96±0.24	12.3±3.5	15.0	–	–	–	1.05 (0.76–1.37)	1.0 (0.7–1.3)	14 (10–18)	–	–	0.65±0.13	10.1
α2a	–	–	–	12.8 (8.3-17.6)	–	0.21±0.02	–	–	–			–	–
α2b	–	–	–	5.61 (2.37-12.1)	–	0.82±0.08	–	–	–			–	–
β	1.2±0.36	17.4±4.6	13.6	–	–	–	1.93 (1.46–3.19)	–	–	–	–	–	–
β1	–	–	–	11.9 (8.2–18.2)	1.0 (0.7–1.44)	1.27±0.11	–	0.8 (0.6–1.2)	13 (8–17)	0.81±0.04	13.2±0.53	0.92±0.30	14.2
β2	–	–	–	–	0.8 (0.55–1.16)	0.82±0.06	–	0.6 (0.4–1.2)	9 (6–19)	0.47±0.03	7.7±0.43	0.57±0.11	8.8
β2a	–	–	–	3.54 (1.78–4.78)	–	–	–	–	–	–	–	–	–
β2b	–	–	–	3.69 (1.42–6.48)	–	–	–	–	–	–	–	–	–
γ	1.3±0.29	17.4±4.6	14.9	–	1.5 (1.04–1.81)	1.41±0.06	1.43 (1.05–1.72)	1.2 (0.9–1.5)	17 (14–21)	0.82±0.05	13.5±0.73	1.00±0.14	15.5
γ1	–	–	–	12.1 (8.6–25.4)	–	–	–	–	–	–	–	–	–
γ2	–	–	–	2.18 (0.48–7.17)	–	–	–	–	–	–	–	–	–
A/G ratio	0.66±0.11	–	0.67	0.77 (0.62–1.01)	–	–	0.66 (0.46–0.92)	–		–		0.96	
No. of bands	5		5	10	5	7	5	5		7		6	

Values are expressed as mean±SD (values from Kirk et al. and Massip and Fumiere are based on mean±SE). Numbers in *parenthesis* are data range

AV Absolute value (g/dl), *RV* relative value (%), *TP* total protein

[a] Data were obtained from 26 samples (86.6%) had five protein fractions as common pattern

[e] Data were obtained from ponies>2 years

Albumin/globulin (A/G) ratio is used for the interpretation of total protein value (Evans and Duncan 2003). Although the SPE profile and absolute values of the individual fractions are valuable parameters, it is best to accompany these values with A/G ratio (Eckersall 2008). A/G ratio in the present study (0.66±0.11) was similar to other studies (Table 2), but values from Riond (2008) and Mattheeuws (1966) were higher than our findings.

All diseases affecting serum proteins and changes in their concentrations have been assessed by advanced methods such as proteomics. But these methods are not routinely applied in veterinary laboratories (Eckersall 2008). Therefore, the results obtained from SPE may be useful for diagnosing some diseases affecting serum proteins quality and quantity in Caspian horse and other equids.

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