

Concentrations of the immunoglobulin isotypes in the Caspian miniature horse: first report

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SUMMARY

The aim of the study is to investigate, for the first time, the concentrations of the various immunoglobulin (Ig) isotypes in healthy Caspian miniature horses, which is likely the ancestor of all modern breeds of hot-blooded horses and today reared in United States, England, New Zealand, Australia and Iran. For that, the specific Ig concentrations were measured using a single radio-immuno-diffusion method (VMRD kits) in 30 healthy Caspian horses after verifying absence of agammaglobulinemia by serum protein electrophoresis. After normalisation of the concentration distributions by a logarithmic transformation, the usual ranges of Ig isotype concentrations determined in the Caspian horse were 28.45 ± 13.20 g/L for IgG, 3.68 ± 0.32 g/L for IgG (T) (one of the IgG subclasses routinely measured for practical purposes), 0.66 ± 0.29 g/L for IgM and 0.92 ± 0.70 for Ig A. Compared with literature data, the IgG concentrations were higher than in other horse breeds or in ponies whereas the IgA and IgM concentrations were markedly depressed. These results underline the necessity to determine usual values specifically in the Caspian miniature horses and can be directly useful for veterinarian clinicians.

Keywords: Caspian miniature horse, serum, immunoglobulin isotypes, usual values.

RÉSUMÉ

Isotypes des immunoglobulines chez le cheval Caspien miniature : premier rapport

L'objectif de cette étude a été de déterminer, pour la première fois, les concentrations usuelles des différents isotypes d'immunoglobulines (Ig) chez le cheval miniature Caspien, qui est probablement l'ancêtre des races modernes actuelles et qui, actuellement, est élevé aux USA, en Angleterre, en Nouvelle Zélande, en Australie et en Iran. Pour cela, les concentrations spécifiques en Ig ont été mesurées chez 30 chevaux Caspiens sains par immuno-diffusion radiale simple (kits VMRD) après avoir vérifié au préalable l'absence d'agammaglobulinémie par électrophorèse des protéines sériques. Après normalisation des distributions des concentrations par une transformation logarithmique, les valeurs usuelles déterminées dans cette espèce ont été de 28.45 ± 13.20 g/L pour les IgG, 3.68 ± 0.32 g/L pour les IgG (T) (une des sous-classes des IgG mesurée régulièrement en pratique), 0.66 ± 0.29 g/L pour les IgM et 0.92 ± 0.70 pour les Ig A. Par comparaison aux données de la littérature chez les autres chevaux et les poneys, les concentrations en IgG ont été plus élevées et les concentrations en IgA et IgM plus faibles chez le cheval Caspien que dans les autres cas. Ces résultats soulignent la nécessité d'établir des valeurs usuelles spécifiques chez le cheval Caspien et peuvent être directement utilisées par les cliniciens.

Mots clés : Cheval Caspien miniature, sérum, immunoglobulines, isotypes, valeurs usuelles.

Introduction

Acquired humoral immune system, especially antibodies, has an important defensive role against foreign pathogens. Humoral immune deficiencies have been reported in horses, foals, cattle, dogs and other domestic animals [18]. For example, severe combined immune deficiency (SCID) in Arabian and Appaloosa foals, primary agammaglobulinemia in thoroughbred, standard bred and quarters horses, and also selective IgM deficiency in Arabian and quarter horses were noted [19, 21]. Also investigators reported selective IgG2 deficiency in 1-2% of red Danish cattle [19], and selective IgM and IgA deficiency respectively in Doberman pinscher and German shepherd dogs [19, 21].

Caspian miniature horse is likely one of the direct ancestors of the oriental breeds and all light horse breeds. It was believed that this tiny horse was pony, but researches cleared that Caspian horse is an ancient breed of miniature horse [7]. The Caspian horse is probably the ancestor of all modern breeds of hot-blooded horses [3, 7]. Its original habitat is Alborz Mountain and Caspian Sea in Iran [5].

The normal value of immunoglobulin (Ig) isotypes is essential in determining congenital or acquired immunodeficiency in veterinary medicine field. Because of few studies focusing on the equine humoral response, the field veterinarian has little information about parameters for examination healthy status of the immune system [2]. There are some reports about Ig reference values in horse and pony but according to recent information, normal concentrations of Ig isotypes in miniature Caspian horses have not been reported. Because of several differences of various haematological and biochemistry parameters between horses and Caspian miniature horses [1, 13], the concentrations and the distribution of immunoglobulin isotypes would be different. In this study, an attempt is made to establish normal values of Ig isotypes in this tiny horse for use in the field.

Materials and Methods

ANIMALS

The current research has been done on 30 healthy miniature Caspian horses, 4 to 20 years old (mean age: 8.5 years), from

both genders at the Khojir animal research station (Tehran National Zoo Park, Tehran, Iran). All horses were inspected by a veterinarian and were free from internal or external parasites. None of them exhibited any clinical sign of immunodeficiency disorder.

Blood samples (10 mL) were collected by the right jugular venipuncture into vacuumed sterile tubes without and with 10% disodium EDTA as anticoagulant (0.1 mL for 5 mL of blood). After clotting of the whole blood samples (30 minutes at room temperature) and centrifugation (1 000g, 15 minutes, at room temperature), sera were carefully harvested and stored at -20°C until analysis. Sera were used for the determination of Ig isotypes and serum protein electrophoresis. The EDTA blood samples were used for the routine haematological analysis (CBC count).

HAEMATOLOGICAL AND BIOCHEMICAL ANALYSES

Blood cell counts were determined using a manual method. Total RBC (red blood cells) counts and leukocytes were determined using a Neubauer haemocytometer. The identification of each leukocyte type was performed manually by microscopic examination of 100 leukocytes in Giemsa-stained blood smears and then absolute counts were calculated. PCV was measured using microhaematocrit capillary tubes (Hematokrit; Kapillaren, Hirschmann Laborgerate, Eberstadt, Germany) and a microcentrifuge (Hawksley and Sons Ltd, W Sussex, UK). The haemoglobin concentration was measured by the cyanmethaemoglobin method [9].

Serum protein electrophoresis was performed using an automated electrophoresis system on cellulose acetate strips (Sartorius GMBH, 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 minutes, destained for one minute and cleared for one minute respectively. Ponceau-S (GMBH, LRE Medizintechnik) and phosphoric acid were used as staining and destaining solutions, respectively. Finally after drying strips, the strips were scanned in a densitometer (software PhotoEP V7.51XP; Bender & Hobein GmbH, Munchen, Germany) and electrophoretic curves were obtained. Total protein concentrations of the samples were measured by the biuret method. Using total protein concentration, absolute (g/L) and relative concentration of each fraction were calculated. For calculating of absolute concentration, the percentage of each fraction in a serum sample was multiplied by the total protein concentration [4].

The Ig isotypes concentrations (IgG, IgM and IgA) were quantified by means of single radio-immuno-diffusion

method (SRID) (VMRD, Pullman, WA). The SRID kits have not been used for the separate determination of the different IgG subclasses except for the Ig G(T).

STATISTICAL ANALYSIS

The normal distributions of the Ig isotypes were evaluated by means of Kolmogorov–Smirnov test and SPSS software (SPSS 14.0.2, SPSS Inc., Chicago IL, USA) with significance of $P < 0.05$. For all the Ig isotypes except IgG (T) (one of the IgG subclasses routinely measured for practical purposes [2, 10, 11, 17]), the normality of the Ig concentration distribution was not confirmed but after logarithmic transformation, the distributions of the Ig isotypes were considered as normal according to the normality test.

Results

As shown in Table I, the total serum protein concentrations varied from 74.00 g/L to 97.00 g/L in the investigated Caspian horses and the γ -globulin proportions were comprised between 22.38% and 62.88%, leading to total immunoglobulin concentrations ranged between 15.00 g/L to 61.00 g/L. None animal has presented an agammaglobulinemia.

The concentrations of the different Ig isotypes in Caspian healthy horses were summarized in the Table II. The main Ig isotype was the IgG which roughly represented 94.5% of the immunoglobulins, while the proportions of IgM and of IgA were approximately 2.2% and 3.1% respectively. In addition, 50% and 25% of the Caspian investigated horses exhibited Ig G concentrations higher than 24.50 g/L and than 44.00 g/L respectively. Whereas the distribution of the IgG isotype was markedly large, those of the IgA and particularly IgM isotypes were narrower with 50% of values around the median value which maximally differed from each other by 0.94 g/L and 0.43 g/L respectively.

Discussion

Results were compared with serum Ig isotype concentrations found in other horse breeds (Table III) from a large population of adult horses or ponies or/and with a similar method (VMRD kits) for a more accurate comparison.

In the present study, the most exciting finding was higher concentration of IgG in Caspian miniature horse than values obtained from the majority of other studies [6, 8, 10-12, 17] (Table III). Among the different studies, only CAMARGO *et al.* [2] have confirmed the normality of the distributions of

	Proteinemia (g/L)	γ -globulin (%)	γ -globulin (g/L)
X \pm SD	84.00 \pm 9.55	40.20 \pm 10.1	34.00 \pm 9.82
Extreme values	74.00 – 97.00	22.38 – 62.88	15.00 – 61.00

TABLE I: Total serum protein and γ -globulin concentrations determined throughout electrophoresis in 30 healthy Caspian miniature horses (n = 30). Results are expressed as means \pm standard deviations (X \pm SD).

	Ig G	Ig G (T)	Ig M	Ig A
Normality after logarithmic transformation	< 0.05	< 0.05 ¹	< 0.10	< 0.10
X ± SD (g/L)	28.45 ± 13.20	3.68 ± 0.32	0.66 ± 0.29	0.92 ± 0.70
Descriptive analysis	Minimum	8.10	3.12	0.25
	25% percentile	17.00	3.50	0.50
	Median	24.50	3.53	0.54
	75% percentile	44.00	4.00	0.97
	Maximum	56.00	4.51	1.41

Ig G (T): one of the IgG subclasses routinely measured for practical purposes [2, 10, 11, 17]; ¹the distribution was normal without mathematical transformation.

TABLE I: Total serum protein and γ -globulin concentrations determined throughout electrophoresis in 30 healthy Caspian miniature horses (n = 30). Results are expressed as means ± standard deviations (X ± SD).

Equine population	Ig G	Ig G (T)	Ig M	Ig A	Ref.	
Caspian horses (n = 30), 4 – 20 years old	28.45 ± 13.20	3.68 ± 0.32	0.66 ± 0.29	0.92 ± 0.70		
Various breeds (n = 47), 6 – 23 years old	27.04 ± 14.24 ¹	4.19 ± 2.20	0.70 ± 0.30 ¹	1.96 ± 0.73 ¹	[2]	
Various breeds (n = 8), mean age: 13.7 year	19.13 ± 7.54	ND	1.03 ± 0.05	2.14 ± 1.00	[6]	
Various breeds (n = 60),	above 20 years old	15.21 ± 2.96	6.89 ± 3.37	1.91 ± 0.08	2.99 ± 1.34	[10]
	5-12 years old	14.47 ± 2.63	7.46 ± 3.95	0.75 ± 0.08	2.61 ± 1.49	
Various breeds (n = 39), adult	23.20 ± 8.60 ²	4.00 ± 2.50 ²	ND	0.40 ± 0.30 ²	[17]	
Standardbred (n = 36), adult	24.63 ± 13.37	ND	1.36 ± 2.18	3.05 ± 2.37	[8]	
Thoroughbred (n = 66), 1-14 days old	13.35 ± 6.52	ND	ND	ND	[12]	
Shetland ponies (n = 20), adult	13.34 ± 3.50	8.21 ± 3.01	1.20 ± 0.31	1.53 ± 0.86	[11]	

Ig G (T): one of the IgG subclasses routinely measured for practical purposes [2, 10, 11, 17]; ¹n = 33 for IgG, n = 47 for Ig M and n = 19 for IgA; ²the method used was ELISA whereas in all the other studies, commercial RID kits (VMRD) were used; Ref.: reference; ND: not determined.

TABLE III: Comparisons of serum immunoglobulin concentrations (g/L) in horses, ponies and Caspian miniature horses. Results are expressed as means ± standard deviations.

the different Ig concentrations obtained with VMRD kits. In comparison with this recent study [2], IgG and IgM concentrations were similar whereas the IgA concentrations were markedly higher in the CAMARGO's study. A considerable similarity between the 2 studies was high IgG concentrations. Twenty five percent of investigated horses had IgG concentrations above 40.00 g/L [2]. By contrast, the IgG concentrations reported in adult Shetland ponies [11] and in immature (1-14 days old) Thoroughbred horses [12] were notably low (13.34 ± 3.50 g/L and 13.35 ± 6.52 g/L, respectively) compared to results of the present study. Moreover, MC FARLANE *et al.* [10] have also reported low Ig G concentrations in old horses (older than 20 year old) from various breeds (15.21 ± 2.96 g/L) and also in 5-12 years old horses (14.47 ± 2.63 g/L). However, in 3 other studies [6, 8, 17], the Ig G concentrations measured by radio-immuno-diffusion were intermediate, around 20 g/L, in adult horses from various breeds.

On the other hand, MC FARLANE *et al.* [10] and MC GUIRE and CRAWFORD [11] determined higher concentrations of IgG(T) than in the present study (6.89 ± 3.37 g/L in old horses and 7.46 ± 3.95 g/L in adults for [10], 8.21 ± 3.01 g/L in Shetland ponies for [11] vs. 3.68 ± 0.32 g/L in the present study). Unfortunately because of little information, we don't examine causes of differences between various reports for IgG and IgG (T).

The Ig M concentrations determined in the present study (0.66 ± 0.29 g/L) were markedly lower than values observed in Shetland ponies (1.20 ± 0.31 g/L) [12], in old horses (1.91 ± 0.08 g/L) [10], in adult Standardbred horses (1.36 ± 2.18 g/L) [8] and in adult horses from various breeds (1.03 ± 0.05 g/L) by FLAMINIO *et al.* [6] at a lesser extend. Although the Ig concentrations were determined with VMRD kits in both genders and various breeds (Thoroughbred, Standardbred, Warmblood, and Quarter Horse) in this later study, the num-

ber of investigated horses was very small ($n = 8$) and prevented accurate comparisons. By contrast, CAMARGO *et al.* [2] and MC FARLANE *et al.* [10] have found similar values of IgM concentrations in adult (6-23 years old) and in old (> 20 year old) horses respectively.

As far as the IgA concentrations were concerned, all previously reported values [2, 6, 8, 10, 11] were markedly higher than those recorded in the present study (0.92 ± 0.70 g/L) except in the experiment of SHEORAN *et al.* [17] which reported considerably weak values (0.40 ± 0.30 g/L). Nevertheless, it would be noted that these authors have used an inhibition ELISA method instead of radio-immuno-diffusion methods.

The literature does not suffice for examining possible reasons for discrepancy in Ig isotype concentrations between the different animals and between the equine species. Some technical reasons have to be considered: some variations in the serum IgM concentrations can result from the inability to measure total IgM (i.e. free IgM and IgM bound to antigen or to C3b) or serum IgM translocated to mucosal surfaces [15]. Some efforts have also been made to establish reference values of Ig concentrations by VAERMAN *et al.* [20], PAHUD and MACH [14], and ROUSE and INGRAM [16], but the methods used have become not applicable now and for this reason, we did not include their data in the general comparison.

In conclusion, the profile of the Ig isotypes in the Caspian miniature horse differs from those of other horses or ponies: higher IgG concentrations were coupled to very lower IgA and IgM concentrations. Although the present study was the first report of normal Ig concentrations using SRID method (VMRD kits) in the Caspian horse, the results will be directly useful for clinicians to diagnose some clinically immune disorders. Furthermore, this study opens the way up to the exploration of humoral immunity in this particular horse specie.

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