Haemoglobin typing and its variations in Iranian domestic dogs

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Abstract The study of canine haemoglobin (Hb) components can help to forecast Hb changes during many pathological and physiological processes such as responsive anaemia. The aims of our study were to show canine Hb electrophoretic pattern on cellulose acetate and identify Hb types similar to the human Hb pattern. Blood samples from 78 different canine breeds were randomly collected in tubes containing anticoagulant EDTA. Animals were brought to the Small Animal Teaching Centre for a check-up and vaccination. All blood samples underwent electrophoresis on cellulose acetate paper strips to determine Hb types. Haematocrit and Hb were measured simultaneously. The Hb electrophoresis results showed that Hb A₁ was assigned to most of Hb components on cellulose acetate paper. Also, in some blood samples, Hb A₂ was detected at the cathode end of cellulose acetate paper similar to human Hb A₂, by densitometer. Small amounts of Hb F were detected in some dogs which was not significant. According to our study, Hb A₁ composes most of the total Hb percent in canine blood. Two types of Hb, A₂ and F, were detected in a few animals, but the majority did not possess Hb F. It seems that Hb F is not significant in these animals. We concluded that one or two Hb types could be seen in dogs. There is no difference in electrophoretic pattern between male and female dogs.

Keywords Electrophoresis · Haemoglobin A₁ · Haemoglobin A₂ · Dog · Sex · Breeds

Introduction

Red blood cells (RBCs) play the main role in oxygen transportation, which take oxygen from the lungs and deliver it to numerous tissues during circulation. The major constituent of RBCs is haemoglobin (Hb); it has the capacity to bind oxygen and later releases carbon dioxide. In man at rest, about 250 ml of oxygen is used whilst 200 ml of carbon dioxide is produced per minute and therefore highlights the importance of the following topic since Hb transportation capacity is 100 times more than plasma transportation capacity alone. This molecule is spherical with a molecular weight approximately 64,500 D (Telen 2009). Hb is a tetrameric protein consisting of four polypeptide globin chains, each of which contains a heme prosthetic group within a hydrophobic pocket. In adults, this molecule consists of two identical alpha and two non-alpha chains that are generally classified as beta chains (Harvey 1997).

In humans, different kinds of Hb such as A₁, A₂, F, Gower I, Gower II and Portland are found depending on age. The globin structure of A₁, A₂ and F has molecular structure of $\alpha_2\beta_2$, $\alpha_2\delta_2$, $\alpha_2\gamma_2$, respectively. Hb Gower I, Gower II and Portland which dominate during the first 3 months of fetal development are known as embryonic Hb, while fetal Hb predominates over the production of embryonic Hb during the early infancy period (Telen 2009). Most animals also synthesize embryonic and fetal Hb at the embryonic and fetal stages, respectively. In animals, the embryonic globin chain is designated as Hb ϵ , like humans. Beta globin chain synthesis is predominant around mid-gestation. At birth, a mixture of adult and fetal Hb is seen. Soon after birth, the level of Hb F decreases, but its synthesis ability in response to anaemia still remains (Kaneko 2000).

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Normal and different variants of Hbs are identified by standard laboratory techniques. Cellulose acetate electrophoresis can detect most variants of Hb at alkaline pH as well as isoelectric focusing (IEF) method. Confirmation is done by HPLC, citrate agar and IEF (Telen 2009).

Chickens, albino rats, guinea pigs, rabbits, horses, pigs, dogs and cats all lack fetal Hb. Embryonal Hb is replaced by adult Hb during the fetal period. In dogs, fetal Hb cannot be distinguished from adult Hb by electrophoretic or peptide mapping. It has been demonstrated that there are one or two types of adult Hb in dogs. Postulated genetic control for dogs includes designated $\alpha_2\epsilon_2$ as embryonic Hb and $\alpha_2\beta_2$ for adult Hb. In felines, there are three different types of embryonic Hb components during 21 days post conception, and 20–30 days post conception, adult Hb has already emerged (Kitchen and Brett 2006).

The acetate cellulose electrophoresis technique is widely used for detection of serum protein and its quantitation. The biggest advantage of Hb electrophoresis on acetate cellulose is simplicity. All Hb types as A₁, A₂, F as well as abnormal Hb C and S are indentified and quantitated (Rosenbaum 1966). Rose et al. demonstrated that impregnation of cellulose acetate with citrate agar would provide Hb S and C separation and haemoglobinopathies associated with them. This method is also an excellent established method for separation of Hb A and F (Schneider et al. 1974).

Process simplicity and the absence of adsorption yield a clean white background between fractions. Therefore, there is no adsorptive loss of protein during migration (Bartlett 1963). The existence of large pores in the cellulose acetate membrane provides minimal sieving effect on proteins. Therefore, electrophoretic separation is entirely dependent on charged density of the molecules (Westermeyer and Gronau 2005). Hb molecules have negative net charge in alkaline pH 8.6 and move towards the anode in cellulose acetate electrophoresis. The electrophoretic mobility of Hb A is greater than that of Hb F and A₂, but Hb A₂ is not far from the cathode, and Hb F is located between them (Elghetany 2007). The aims of this study were to assess canine Hb components by cellulose acetate electrophoresis and to detect different types of canine Hb.

Materials and methods

Blood samples were collected randomly from 78 different canine breeds (41 male and 37 female) which were presented for check-up and vaccination to the Small Animal Teaching Hospital in the College of Veterinary Medicine, University of Tehran, during the 6-month period from November 2009.

The dogs had no suspected clinical symptoms and were from different breeds such as bulldog, Chihuahua, Doberman,

German shepherd, Great Dane, mixed breed, Pekingese, Poodles, Shih Tzu and Terrier (data shown in Table 1). All blood samples were electrophorized at room temperature (25°C). Haematocrit (Hct) and Hb levels were determined prior to electrophoresis by a haematologic analyzer (Hema-Screen 18, HOSPITEX DIAGNOSTICS, Sesto Fiorentino, Italy). Other information and data such as sex, breed and age were also recorded. To produce the Hb haemolysate, whole blood samples were collected in microcentrifuge tubes containing the anticoagulant EDTA, centrifuged for 10 min at 2.3 g (5,000/rpm) to provide RBC pellet and washed three times with saline. After centrifugation, the washed RBCs were suspended in equal volume of saline. In the following process, 100 μ L of haemolysate reagent (Cellogel Electrophoresis Co., Milan, Italy) was added to 20 μ L RBC pellet and shaken vigorously for 5–7 min to lyse all cells to produce a clear red-coloured solution. Then the lysate was centrifuged to obtain the supernatant which was discarded. The Cellogel/Myl cellulose acetate paper (Cellogel Electrophoresis Co., Milan, Italy) strips were soaked in Tris glycine buffer solution (Cellogel Electrophoresis Co., Milan, Italy) for 15 min as per manufacturer's instruction. In the next step, 10 μ L of the centrifuged pellet was loaded onto the cellulose acetate paper using an applicator, and electrophoresis was for between 30 and 40 min at 250 V and 7 mA. After electrophoresis, the paper was stained with Amido black (Cellogel Electrophoresis Co., Milan, Italy) for 7 min and then washed roughly three times for 5 min for each turn in destaining solution (Cellogel Electrophoresis Co., Milan, Italy). The strips were cleared in clearing solution (Cellogel Electrophoresis Co., Milan, Italy) for 5 min and dried in an oven at 70°C for 20 min. The strips were analysed by densitometer and using PhotoEP V7.51XP software (Bender & Hobein GmbH, Munchen, Germany). In addition, several human male blood samples were used to compare Hb running pattern on cellulose acetate paper.

Results

In the present study, Hb electrophoresis was done on cellulose acetate paper. There were no differences between female and male Hb electrophoresis pattern on macroscopic view (Fig. 1), and no difference was found according to age. The greatest Hb components were at the anode end in the same line approximately, but in some cases at cathode side, low values of Hb A₂ were also observed by densitometer analysis (Fig. 3, c and d). The human Hb moved faster than canine species (Fig. 2). According to this study, distribution of the levels of Hb was normal, and no statistically significant difference was observed between males and females (*t* test, *P*=0.939) (Table 2). Hct distribution was normal and similar to Hb. Hct distribution for all samples was

Table 1 Distribution of descriptive characteristics of the 78 dogs that underwent blood sampling for cellulose acetate electrophoresis at the Veterinary School, Small Animal Teaching Hospital, University of Tehran

Breed	Frequency (%)	Sex		Age median (months)
		Male	Female	
1 Terrier	39 (50)	17	22	17
2 German shepherd	13 (16.7)	9	4	15
3 Mixed breed	10 (12.8)	6	4	13.5
4 Pekingese	4 (5.1)	2	2	9.5
5 Shih Tzu	4 (5.1)	2	2	10.25
6 Doberman	3 (3.8)	2	1	9
7 Great Dane	2 (2.6)	1	1	8.75
8 Chihuahua	1 (1.3)	1	0	24
9 Poodle	1 (1.3)	1	0	24
10 Bulldog	1 (1.3)	0	1	4
Total	78 (100)	41	37	15

41.6±0.11% with no correlation or with increasing age ($r=0.03$). Also, Hb showed normal distribution (12.78 ± 3.21 g/dL). No significant difference was obtained between males and females (t test, $P=0.020$). Hb levels increased slightly with increasing age ($r=0.26$). In one sample (a 12-month female Terrier), Hb A₁ reached a minimum level of 96.2% while in others, 28 out of 78 dogs (~36%), it was composed of 100% total Hb and showed a slight increase with age ($r=0.26$) whilst more than 75% of samples possess 98% or more (Fig. 3). On the other hand, 28 out of 78 blood samples showed no Hb A₂, and 69.2% had lower than 1%. In one case, Hb A₂ was lower than 2.9%. In general, Hb A₂ decreased slightly with age ($r=-0.27$). Thirty-nine cases out of 78 showed Hb F, but only in one case did it exceed 2.3%. Hb F in 91% of all cases was below 1% of the total Hb and resembles what is seen in humans. Hb F in the blood of all animals was slightly decreased with age ($r=-0.18$).

Fig. 1 Electrophoretic pattern of canine Hb on cellulose acetate. *A* Male Great Dane, 1 year old; *B* female mixed breed, 7 years old; *C* Male mixed breed, 18 months old; *D* male Terrier, 2 years old; *E* female German shepherd, 3.5 years old; *F* female Terrier, 2 years old. Note the Hbs approximately located in the same line

Hb A₁, A₂ and F were not significantly different between sexes using the Mann–Whitney test (data not shown). Male and female did not show an age difference with other Hb types. Age distribution in male and female dogs was approximately the same. There were no statistically significant differences between male (41) and female (37) dogs among all the measured parameters.

Discussion

The present study showed canine Hb electrophoretic pattern on cellulose acetate paper strips. The electrophoresis analysis was performed using densitometer software where we assumed that the Hb components A₁ and A₂ were the same as human Hb. The results indicate that Hb A₁ is the major Hb component, but Hb A₂ was detected at the

Fig. 2 Electrophoretic pattern of human and canine Hb. *A* Male, human, 28 years old. The black arrow shows Hb type A₁. The arrowhead indicates HbA₂. *B* Male German shepherd, 5 years old. *C* Male Terrier, 1 year old. *D* Female Terrier, 5 years old. Human HbA₁ moved faster than canine Hbs

Table 2 Distribution of different statistical measurements of HCT and Hb (A1, A2 and F) in canine blood samples at the Small Animal Teaching Hospital, Veterinary School University of Tehran ($n=78$)Corresponding P values have been presented^a r Pearson correlation coefficient (between age and each parameter)

Parameter	Median	Range		Mean $\pm$ SD	Correlation with age	
		Min	Max		r^a	P value
Hct (%)	44	17	64	41.63 $\pm$ 0.11	0.03	0.789
Hb (g/dL)	12.5	5.3	20	12.78 $\pm$ 3.21	0.26	0.020
A1 (%)	99.5	96.2	100	0.99 $\pm$ 0.011	0.26	0.022
A2 (%)	0.4	0	2.9	0.0067 $\pm$ 0.0077	-0.27	0.016
F (%)	0.05	0	2.3	0.0031 $\pm$ 0.0047	-0.18	0.114

cathode side at minor levels in some dogs. Kitchen and Brett in 1974 claimed that canine adult Hb includes one or two types (Kitchen and Brett 2006). Electrophoretic pattern of 20 individuals of *Canis familiaris*, by Seal in 1969, showed only one Hb A band and did not report any fetal Hb (Brimhall et al. 1977). It is interesting to note in our results that the curve obtained from canine Hb A₁ looks sharper than that of human Hb A₁ (Fig. 2). This indicates that canine Hb A₁ has greater homogeneity than human A₁ Hb. Not only does the heterogeneity of Hb depend genetically on amino acid interchanges, but it could also originate from non-related genetic heterogeneity of Hb, for example alteration of protein structure, i.e. β chain N-acetylation in feline Hb B or glycosylation in diabetic dogs (Harvey 1997). Peptide sequencing of canine α and β chains has described two kinds of α chains which differ with threonine

and alanine at position 130 (Brimhall et al. 1977). In some samples, proteins like Hb F were seen between Hb A₁ and A₂, but it was not significant in the present study. Three canine Hb phenotypes were observed by immobile isoelectric focusing in polyacrylamide gel technique in Braend's study (Braend 1988). The sensitivity of our technique is less than that of the polyacrylamide gel technique. Cellulose acetate electrophoresis is used as a routine screening technique for haemoglobinopathies, but agarose is the medium of choice because it can separate normal and more abnormal Hb easily and is commercially supported (Higgins et al. 2006). Considering the common heme structure of human and domestic animal Hb, α - and non- α -chain amino acid sequences are responsible for electrophoretic differences in animals (Kaneko 2000). There are no reports of globin chain synthesis abnormalities in

Fig. 3 Hb line graph. **a** Male dog, Terrier, 18 months old. **b** Female dog, mixed breed, 7 years old. There is no Hb A₂ detectable by cellulose acetate electrophoresis in either male or female dogs. **c** Human, male, 28 years old. The major human Hb component is Hb A₁ which is located at the anodal end, but at the cathodal side, Hb A₂ was seen, also. **d** Male dog. There is a trace amount of Hb A₂ at the cathode side in some of the dogs, similar to humans. In our study, a number of dog Hb patterns were like the human Hb pattern. It seems that the curve obtained from canine Hb A₁ looks sharper than that of human Hb A₁. (Densitometer analysis is done by PhotoEP V7.51XP software)

animals as seen in humans (Kaneko 2000; Broukus and Andreasen 2003). Therefore, globin charge and molecular weight are determined on electrophoretic pattern. Under alkaline condition, globin chains become negative. Hb A₁ yields more net negative charge and moves faster towards the cathode end. Normal Hb A₂ is closer to the anode end as a lower negative charge. We did not see any differences between electrophoretic patterns among canine breeds and sexes. It should also be noted that faster mobility has been discovered in human Hb in comparison to canine Hb on gel electrophoresis. The canine Hb subunits α (15,217.31 molecular weight (MW), 141 amino acids, PI=7.98) (UniProt: HBA_CANFA; available at: <http://www.uniprot.org/uniprot/P60529>, last modified July 21, 1986) and β (15,996.37 MW, 146 amino acids, PI=7.96) (UniProt: HBB_CANFA; available at: <http://www.uniprot.org/uniprot/P60524>, accessed February 19, 2010) are not that different from human Hb α (15,126.36 MW, 142 amino acids, PI=8.73) (UniProt: HBA_HUMAN; available at: <http://www.uniprot.org/uniprot/P69905>, accessed February 19, 2010) and β (15,867.22 MW, 147 amino acids, PI=6.81) (UniProt: HBB_HUMAN; available at: <http://www.uniprot.org/uniprot/P68871>, accessed February 19, 2010). Additionally, the average isoelectric point of canine and human Hb is 7.97 and 7.77, respectively. Therefore, it seems logical that the less PI a protein has, the greater the negative charge it will have and the faster it will move under pH conditions. In addition, human Hb molecules weigh less than canine Hb, but as mentioned previously, because of the large pores in the cellulose acetate paper, the sieving effect is entirely dependent on net charge rather than molecular weight. In summary, we found that in canine species, Hb A is the most important Hb component although in some cases, Hb A₂ was also found at the cathode end.

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