

RESEARCH ARTICLE

Capillary hemoglobin electrophoresis of healthy and anemic dogs: Quantification, validation, and reference intervals of hemoglobin fractions

Ioannis L. Oikonomidis^{1*}, Theodora K. Tsouloufi¹, Mathios E. Mylonakis², Maria Kritsepi-Konstantinou¹

1 Diagnostic Laboratory, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece, **2** Clinic of Companion Animal Medicine, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece

* ioannis.oikonomidis@ed.ac.uk



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Abstract

Despite the advances in canine medicine and the rapid gaining of attention of canine models in biomedical field and particularly in hemoglobin genes research, the studies on canine hemoglobin composition are sparse with ambiguous findings. Our aim was: i) to investigate the electrophoretic pattern of canine hemoglobin and the possible effects of age, sex, and anemia using a capillary electrophoresis assay, and ii) to validate this assay and calculate reference intervals (RIs) for canine hemoglobin fractions. Blood samples were collected from 53 healthy and 42 dogs with regenerative and non-regenerative anemias. The Sebia Capillarys 2 flex-piercing was used for hemoglobin analysis and it was validated using canine blood samples. R statistical language was employed for the statistical analyses. A major hemoglobin fraction (named HbA₀) and a minor one (named HbA₂) were identified in 100% and 47.4% of samples, respectively. The within-run and between-run CV was 0.1% for HbA₀, and 9.1% and 11.2% for HbA₂, respectively. The extremely narrow range of HbA₀ and HbA₂ values hampered a linearity study using canine blood samples. The RIs for HbA₀ and HbA₂ were 98.9–100% and 0–1.1%, respectively. HbA₀ and HbA₂ values were not significantly correlated with age ($P = 0.866$) or reticulocyte count ($P = 0.731$). No differences were observed in the median HbA₀ and HbA₂ between the two sexes ($P = 0.887$), and healthy and anemic dogs ($P = 0.805$). In conclusion, the capillary electrophoresis revealed a major hemoglobin fraction and an inconsistently present minor fraction. No effect of age, sex, anemia, or regenerative status of anemia was detected. The assay used was validated and RIs were generated, so as to be suitable for use in future investigations.

Introduction

Hemoglobin is the oxygen-carrying moiety of erythrocytes. Structurally, it is a globular polypeptide tetramer, which consists of two pairs of unlike globin chains that form a shell around a

central cavity. The latter contains four oxygen-binding heme groups, each of which is covalently linked to a globin chain.

In healthy humans, hemoglobin consists of: i) a major fraction, HbA₀ ($\alpha_2\beta_2$), which comprises approximately 95% of the total hemoglobin; ii) a minor fraction, HbA₂ ($\alpha_2\delta_2$), which is normally less than 3.5% of total hemoglobin and iii) the fetal hemoglobin, HbF ($\alpha_2\gamma_2$) [1]. In human medicine, more than 700 hemoglobinopathies have been described to date with most of them being clinically benign [2]. The term hemoglobinopathy is broadly used to describe both quantitative (thalassemias) and qualitative (true hemoglobinopathies) hemoglobin disorders [3]. However, in a strict sense, hemoglobinopathies and thalassemias are two genetically distinct groups of diseases, although the clinical manifestations may overlap [1]. Specifically, thalassemias are characterized by a reduced production of the normal globin chain and may result from gene deletion or mutations that affect the transcription or stability of mRNAs [1]. On the other hand, the vast majority of hemoglobinopathies, including the clinically important ones, result from single nucleotide substitutions that are translated to single amino acid substitutions, primarily in the non- α chain, causing alterations in the secondary and tertiary structures of hemoglobin tetramer [1, 4].

High pressure liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) are the most widely used methods for human hemoglobin analysis and for the initial diagnosis of hemoglobinopathies, both of which have superior analytic and diagnostic performance when compared to other available methods, such as gel electrophoresis and mass spectroscopy [5]. CZE allows the successful separation of the normal human hemoglobin fractions, but it can also detect abnormal hemoglobin variants with altered charge resulting either from mutations that directly influence the charge of the molecule or indirectly from mutations that alter the higher-order structure [4]. In particular, Sebia Capillarys 2-flex piercing (Sebia, Norcross, USA), the updated model of Sebia Capillarys, has been successfully validated for human hemoglobin analysis and diagnosis of hemoglobinopathies [6]. Additionally, the same analyzer has been recently successfully validated for the measurement of the major fraction of glycated hemoglobin (HbA_{1c}) in dogs [7].

The α and β globin gene clusters have been only recently identified in dogs. Five genes in each cluster were found; α -globin gene cluster is composed of three embryonic-like genes (HBZ1, HBZ2, HBM) and two adult-like genes (HBA1 and HBA2), while the β -globin gene cluster comprises two embryonic/fetal-like genes (HBE and HBH) and three adult-like genes (HBD1, HBD2, HBB) [8]. Interestingly, the major canine hemoglobin in adult dogs contains HBD, while the minor one contains HBB; this finding is in contrast to humans and appears to be preserved in other carnivores as well [8]. Our knowledge about the electrophoretic pattern and composition of canine hemoglobin is currently limited and disparate. Additionally, the investigation of possible canine hemoglobin disorders in the clinical setting is hampered by the absence of a validated and readily available method for canine hemoglobin electrophoretic analysis. Currently, there is a dearth of published studies on hemoglobin composition in dogs and they have been conducted almost half a century ago and are not based on validated methods [9, 10], although dogs are rapidly gaining attention as potential models in various biomedical areas, while they are considered the ideal model particularly for the study of hemoglobin genes [8]. According to the above cited studies, no HbF is recognized in dogs, while a minor hemoglobin fraction may be detected [9, 10]. However, no further information is provided about the prevalence, quantification, and electrophoretic characteristics of the minor hemoglobin fraction. Only recently the minor hemoglobin fraction was quantified using acetate cellulose electrophoresis [11]. Surprisingly, the authors of this study also reported the presence of HbF in adult dogs, raising questions about our prior knowledge, but also about the utility of different assays for canine hemoglobin analysis [11].

In the aforementioned context, the objectives of this study were: i) to investigate the electrophoretic patterns of canine hemoglobin using a new automated capillary electrophoresis assay; ii) to study the effect of age, sex, and anemia unrelated to hemoglobin disorders on the electrophoretic pattern of canine hemoglobin; and iii) to validate the herein used assay for canine hemoglobin analysis and calculate appropriate reference intervals, so as to be suitable for use in future studies or in the clinical setting.

Materials and methods

The blood samples used in this study were aliquots of specimens collected (owners' consent provided) for diagnostic purposes, routine health check, or pre-operatively from healthy dogs referred to the Companion Animal Clinic, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece. The reference individuals were selected by a direct a priori method, based on the following inclusion criteria: age >6 months, up-to-date vaccination and deworming status, no history of illness or medication in the preceding month, and unremarkable physical examination and complete blood count. The distribution of canine breeds that were used in this study are presented in Table 1. Blood samplings were performed at admission by jugular venipuncture and the samples were collected into K3-ethylene diamine tetra-acetic acid (EDTA) coated 2mL tubes (Deltalab, Barcelona, Spain). Anemia was defined as red blood cell count $<5.36 \times 10^9/L$, or hemoglobin concentration $<122 \text{ g/L}$, or hematocrit $<0.372 \text{ L/L}$ [12]. The anemia was classified as regenerative when the absolute reticulocyte count was $>60,000/\mu\text{L}$ [13]. The diagnoses for all anemic dogs included in the present study can be found in Table 2. Grossly hemolyzed (in vitro hemolysis) and lipemic samples were excluded from the study. A complete blood count was performed on the Advia 120 hematology analyzer (Siemens Healthcare Diagnostics, Deerfield, USA) within 2 h of sampling.

Table 1. Distribution of canine breeds used in this study.

Canine breed	Number	Percentage
Beagle	2	2.1
Boxer	1	1.1
Cocker Spaniel	4	4.2
Collie	2	2.1
Doberman	1	1.1
French bulldog	2	2.1
Greek pointer	3	3.2
Greek shepherd	1	1.1
German shepherd	5	5.3
Jack Russell Terrier	1	1.1
Labrador	6	6.3
Maltese	5	5.3
Mixed-breed	44	46.3
Pekingese	1	1.1
Pincher	3	3.2
Poodle	3	3.2
Rottweiler	2	2.1
Setter	2	2.1
Shar Pei	2	2.1
Yorkshire Terrier	5	5.3
Total	95	100

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Table 2. Frequencies of diagnoses of the anemic canine population.

Diagnosis	Number	Percentage
Babesiosis	1	2.4
Bee sting envenomation	1	2.4
Blood loss	5	11.9
Chronic kidney disease	5	11.9
Chronic lymphocytic leukemia	1	2.4
Cutaneous sarcoma, cutaneous mast cell tumor	1	2.4
Diabetes mellitus	2	4.8
Immune-mediated hemolytic anemia	3	7.1
Immune-mediated thrombocytopenia	2	4.8
Leishmaniosis	4	9.5
Liver disease	5	11.9
Lymphoma	2	4.8
Periodontal disease	2	4.8
Pyometra	1	2.4
Septic peritonitis	2	4.8
Splenic hemangiosarcoma	2	4.8
Unknown	3	7.1
Total	42	100

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Hemoglobin electrophoresis was carried out within 4 h of sampling. Routine maintenance, assay and internal quality control procedures were conducted as defined in the analyser manuals. A normal electrophoretogram from a human patient was used for comparison. The automated analyser, Sebia Capillarys 2 flex-piercing, and the dedicated kit (Sebia, Norcross, USA) were used for the detection and quantification of different canine hemoglobin fractions as a percentage of total hemoglobin. The principle of the Capillarys 2 flex-piercing assay is CZE, in which charged molecules are discriminated by their electrophoretic mobility in an alkaline buffer (pH 9.4). The analyser is equipped with eight silica capillaries, which enable the simultaneous analysis of eight whole blood samples. In brief, the EDTA-treated whole blood sample is diluted (1:6) with a ready-made hemolysing solution and the resulting solution is then hydrodynamically injected at the anodic end of the capillary. A constant, high voltage is applied for 8 min, which allows the migration and separation of the hemoglobin variants. These are then directly detected by spectrophotometry (415 nm) and the electrophoretograms are automatically generated. The total output time is approximately 20 min for the first run and 12 min for every other run.

The validation of the analyzer was initially designed to include linearity, repeatability, and reproducibility. The repeatability or within-run precision was evaluated using blood samples from three dogs. Each sample was measured eight times in succession and the coefficient of variation (CV) was calculated. Blood samples from the same three dogs were used for the evaluation of reproducibility or between-run precision. Six aliquots were made from each sample and were measured over a period of 3 days; then, the CV was calculated.

The distribution of data was assessed using the Shapiro–Wilk test. The 95% reference intervals (RIs) were calculated using the non-parametric method, while the 90% confidence intervals (CIs) for the lower and upper reference limits were calculated by the bootstrap method. Cook's method was employed for the detection of outliers. For the determination of reference intervals, the R package referenceIntervals was used. The exact Wilcoxon and Kruskal–Wallis rank-sum tests were employed for median comparison between two or three different groups,

respectively. Spearman's rank correlation coefficients were used for correlation analyses. All the statistical analyses were conducted using the statistical language R (R Foundation for Statistical Computing, Vienna, Austria). Level of significance was set at 0.05 ($P < 0.05$).

Results

In total, 95 dogs were sampled. The reference population comprised 53 dogs (27 males and 26 females) with mean (\pm SD) age of 6.0 ± 3.8 years, hemoglobin concentration of 155 ± 16 g/L, and reticulocyte count of $31.1 \pm 15.5 \times 10^6$ /L. The anemic population comprised 42 dogs (19 males and 23 females) with mean (\pm SD) age of 6.6 ± 4.1 years, hemoglobin concentration of 75 ± 27 g/L, and reticulocyte count of $179.5 \pm 158.4 \times 10^6$ /L. The anemia was classified as non-regenerative in 16/42 (38.1%) dogs and regenerative in 26/42 (61.9%) dogs.

The inspection of the electrophoretograms revealed one major and one minor hemoglobin fraction. The major canine hemoglobin fraction migrated slower towards the anode than the respective human HbA₀ (Fig 1) and it was consistently present in all examined samples (95/95, 100%). The minor fraction migrated slightly slower towards the anode compared to human HbA₂ (Fig 1) and it was evident in 26/53 (49.1%) reference individuals and in 19/42 (45.2%) anemic dogs. For the purposes of this study, we refer to the major canine hemoglobin fraction as HbA₀ and to the minor one as HbA₂.

The total within-run and between-run CV for HbA₀ was 0.1%, while for HbA₂ was 9.1% and 11.2%, respectively. Specificity (dilutional linearity study) using canine blood samples could not be performed due to the extremely narrow range of HbA₀ and HbA₂ percentages in our canine population. No outliers were detected in the reference population using Cook's method. The 95% RI for HbA₀ was 98.9–100% with the CIs for the lower and upper reference limits being 98.8–99.0% and 100%, respectively. The 95% RI for HbA₂ was 0–1.1% with the CIs for the lower and upper reference limits being 0 and 1.0–1.2%, respectively.

The data distribution of HbA₀, HbA₂ and reticulocyte count was non-Gaussian ($P < 0.05$) using the Shapiro-Wilk test. HbA₀ and HbA₂ values were not significantly correlated with age ($\rho = 0.024$ and $\rho = -0.024$, respectively, $P = 0.866$). No statistically significant difference ($P = 0.887$) was observed in the median HbA₀ and HbA₂ between male and female dogs. The median (range) HbA₀ and HbA₂ was 100% (98.9–100%) and 0% (0–1.1%), respectively, in both sexes. No statistically significant difference ($P = 0.805$) was detected in the median (range) HbA₀ and HbA₂ between the reference population [100% (98.9–100%) and 0% (0–1.1%), respectively] and dogs with non-regenerative [100% (98.9–100%) and 0% (0–1.1%), respectively] or regenerative anemia [100% (99.0–100%) and 0% (0–1.0%), respectively] (Fig 2). Finally, HbA₀ and HbA₂ were not significantly correlated with reticulocyte count ($\rho = -0.036$ and $\rho = 0.036$, respectively, $P = 0.731$).

Discussion

In this study, the electrophoretic pattern of canine hemoglobin was investigated using a new automated capillary electrophoresis assay, addressing a significant knowledge gap in hemoglobin research. This is the first study to date in which an automated assay has been validated for canine hemoglobin analysis and appropriate reference intervals have been calculated for adult dogs. The effect of age and sex on canine hemoglobin electrophoretic pattern was also evaluated. Finally, we investigated if anemias that were not attributed to a hemoglobin disorder, could affect the hemoglobin electrophoretic pattern.

The inspection of the electrophoretograms revealed two hemoglobin fractions: one major fraction that was constantly present in all of the enrolled dogs and one minor fraction that was detected in approximately half of the dogs. The major canine hemoglobin fraction was found

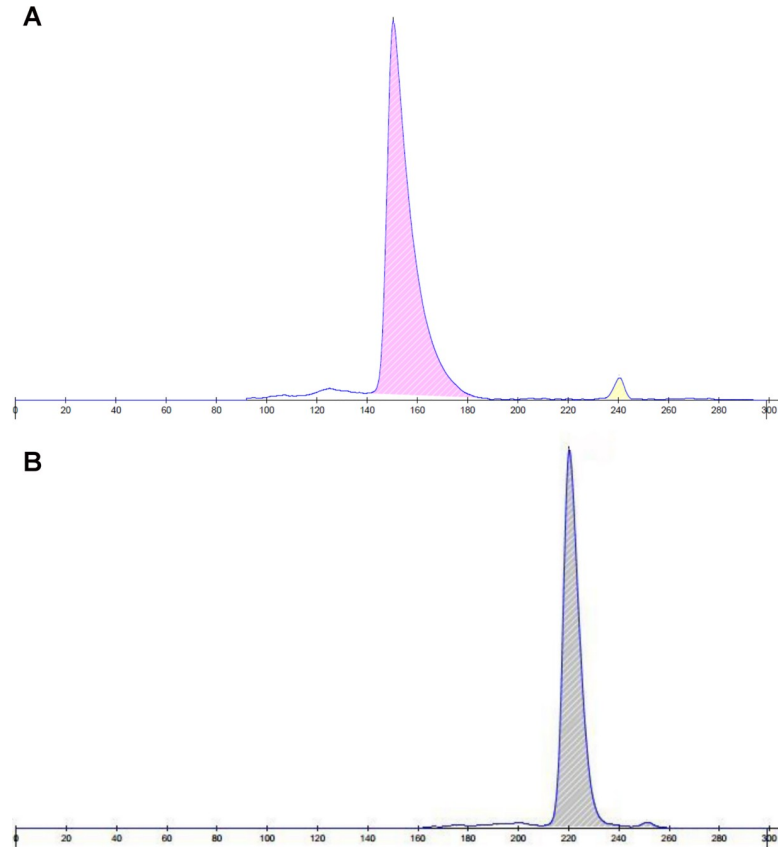


Fig 1. Two representative hemoglobin electrophoretograms from a healthy human (A) and a healthy dog (B). The major (HbA₀) and the adult minor (HbA₂) hemoglobin fractions are depicted in both electrophoretograms. The major canine hemoglobin fraction migrates slower towards the anode than the respective human one. The minor fraction migrates slightly slower towards the anode compared to human HbA₂ and it is inconsistently present in dogs.

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to migrate slower towards the anode compared to human HbA₀, while the minor canine hemoglobin fraction migrated slightly slower than human HbA₂. As previously mentioned, in

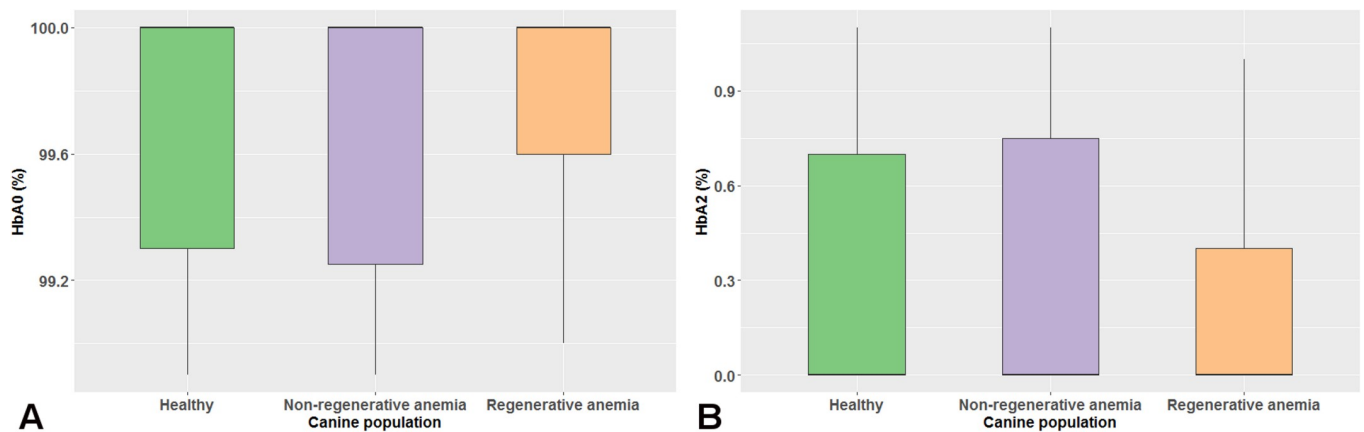


Fig 2. Boxplots of the major (A) and minor (B) hemoglobin fraction values of the reference populations and dogs with non-regenerative or regenerative anemia are depicted. The colored boxes represent the main body of data; they are bisected by a line, which stands for the median value. No statistically significant difference ($P = 0.805$) was detected in the median values of both canine hemoglobin fractions between the three groups.

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contrast to human, major hemoglobin of adult dogs and other carnivores contains HBD, while the minor one contains HBB [8]. In an attempt to correlate the detected hemoglobin fractions with the reported canine globin sequences [8], we could propose that HbA₀ is likely composed of HBA1 and/or HBA2 (which differ by one amino acid, namely Ala/Thr, in position 131) combined with HBD1 and/or HBD2 (which are identical), while HbA₂ is likely formed by the combination of HbA1 and/or HBA2 with HBB. A third hemoglobin fraction consistent with HbF was not detected in any of the dogs included in this study. Our findings are in agreement with previous studies using gel electrophoresis or peptide mapping techniques, which reported the absence of HbF in both embryonic and adult canine samples and the presence of one or two hemoglobin fractions in adult dogs [9, 10]. However, in the aforementioned studies, no further information was provided about the electrophoretic features, the prevalence, and the quantification of the different hemoglobin fractions. The characterization of HbA₂ was only recently done in canine samples [11]. In this study, the prevalence of HbA₂ in healthy dogs was higher, yet similar to ours (64.1% versus 49.1%, respectively). However, the range of HbA₂ value was wider and roughly three times the one reported in our study. The absence of detectable HbA₂ in almost half of the dogs included in our study and in the study of Atyabi et al. [11] may indicate the absence of this fraction or it may be attributed to the presence of HbA₂ in such a low amount that is undetectable by both assays. However, Atyabi et al. surprisingly reported the presence of HbF in 50.0% of their samples [11], as opposed to current and previously published studies [9, 10], which reported the absence of canine HbF in dogs. The source of the observed discrepancy between the study of Atyabi et al. [11] and the rest of the published studies, including the present one, cannot be easily explained. Given that the population used in the aforementioned study was exclusively composed of adult dogs, other preanalytic (handling and storage of blood samples) and analytic factors (inherent limitations of the used method) may have contributed to the observed differences. In the study of Atyabi et al., there is no available information about the duration and conditions under which the blood samples were stored before the analysis [11]. Therefore, we cannot exclude the possibility the fraction called HbF to be simply a hemoglobin derivative due to prolonged storage. In fact, in our previous study, we demonstrated that a new hemoglobin fraction, possibly corresponding to methemoglobin, is detected after prolonged storage of blood samples at -20°C [7]. Furthermore, the observed discrepancies between the results of our study and those of Atyabi et al. may also be explained by the different assays used for hemoglobin analysis. In our study, a new automated capillary electrophoresis assay was employed and subsequently validated for use in dogs, while in the aforementioned study cellulose acetate electrophoresis was used without being validated by the authors.

HPLC and CZE are the most widely used methods for human hemoglobin analysis and for the initial diagnosis of hemoglobinopathies [5]. These two methods have comparable results and share some major advantages, such as the accuracy, rapidness, and high throughput; however, each of them has its disadvantages, primarily referring to inability for identification of some human-specific hemoglobin variants [5]. However, a major advantage of CZE over HPLC, which is potentially applicable to different species, is the substantially better visualization of the results; indeed, post-translational modification and degradation peaks are often present in HPLC chromatograms, potentially making the interpretation problematic [5]. Gel electrophoresis and mass spectroscopy can likewise be used for the hemoglobin analysis and diagnosis of hemoglobinopathies; notwithstanding, a major disadvantage is recognized in both of them. Gel electrophoresis is characterized by an inherent lower accuracy and sensitivity [5], while mass spectroscopy is unable to detect intact globin chains with a slightly different mass, reportedly less than 6 Da [14].

The capillary electrophoresis assay used in this study has been recently successfully validated for the measurement of canine HbA_{1c} [7]. However, to our knowledge, this is the first time that this assay is utilized for canine hemoglobin electrophoresis and thus, a study of the analytic performance of this assay is valuable. The repeatability and reproducibility of this assay for HbA₀ measurement, using canine blood samples, was excellent and in agreement with studies in human medicine [6]. However, the within-run and between-run CV for HbA₂ measurement was considerably higher than the one reported for human HbA₂ [6]. The higher imprecision in canine HbA₂ measurement can be attributed, at least partially, to the extremely low values of the HbA₂ in dogs, which are not normally seen in humans; however, the performance is likely acceptable for use, although this cannot be clearly stated given the absence of specific performance goals in dogs. It should be noted that none of the previously used assays for canine hemoglobin electrophoresis was validated for use in dogs. Additionally, in our study, appropriate RIs were calculated for adult dogs with the range for both hemoglobin fractions being narrower compared to human ones. Finally, the age and sex do not appear to have an effect on canine hemoglobin electrophoretic pattern, in accordance to human studies reporting only a minimal, effect of age and sex, and the study by Atyabi et al. which found no difference between male and female dogs [11, 15].

Given that anemia (of variable severity) is the usual clinical manifestation of hemoglobinopathies in humans [3], we also decided to investigate whether anemias that were not related to hemoglobin disorders, might have an effect on the electrophoretic pattern of canine hemoglobin. In addition, we also included both regenerative and non-regenerative anemias of various etiology in order to investigate if the regenerative status (as assessed by the presence of reticulocytes in peripheral blood) might have an effect on canine hemoglobin electrophoretic patterns. No quantitative or qualitative hemoglobin abnormalities were detected in the electrophoretic pattern of anemic dogs when compared to our reference population, and no significant correlation was detected between the reticulocyte count and HbA₀ or HbA₂. In spite of the small sample size of anemic dogs, this finding indicates that an anemia not attributable to a hemoglobin disorder and the regenerative status do not interfere with the capillary electrophoresis assay used in our study.

Conclusions

The canine hemoglobin consists of a major fraction and a minor one, inconsistently present in very low proportions. A new automated capillary electrophoresis assay was validated for the separation of canine hemoglobin fractions and appropriate RIs were generated. Our study indicates no effect of age, sex or regenerative status of anemia on hemoglobin electrophoretic pattern among adult dogs, while no quantitative or qualitative hemoglobin abnormalities were detected in the anemic dogs without evidence for a hemoglobin disorder. The capillary electrophoresis assay used in this study is the only validated assay that can be used in future research studies on canine hemoglobin or in clinical cases suspected of having a hemoglobin disorder.

Supporting information

S1 File. Raw data used in this study.
(XLSX)

Author Contributions

Conceptualization: Ioannis L. Oikonomidis, Maria Kritsepi-Konstantinou.

Data curation: Ioannis L. Oikonomidis.

Formal analysis: Ioannis L. Oikonomidis.

Funding acquisition: Ioannis L. Oikonomidis, Theodora K. Tsouloufi, Mathios E. Mylonakis, Maria Kritsepi-Konstantinou.

Investigation: Ioannis L. Oikonomidis, Theodora K. Tsouloufi, Mathios E. Mylonakis, Maria Kritsepi-Konstantinou.

Methodology: Ioannis L. Oikonomidis, Theodora K. Tsouloufi.

Project administration: Ioannis L. Oikonomidis, Theodora K. Tsouloufi, Maria Kritsepi-Konstantinou.

Resources: Ioannis L. Oikonomidis, Theodora K. Tsouloufi, Mathios E. Mylonakis, Maria Kritsepi-Konstantinou.

Software: Ioannis L. Oikonomidis.

Supervision: Ioannis L. Oikonomidis, Mathios E. Mylonakis, Maria Kritsepi-Konstantinou.

Validation: Ioannis L. Oikonomidis, Theodora K. Tsouloufi.

Visualization: Ioannis L. Oikonomidis, Theodora K. Tsouloufi.

Writing – original draft: Ioannis L. Oikonomidis.

Writing – review & editing: Ioannis L. Oikonomidis, Theodora K. Tsouloufi, Mathios E. Mylonakis, Maria Kritsepi-Konstantinou.

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