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Validation of a method for measuring canine HbA1c

Title: Validation and Determination of a Reference Interval for Canine HbA1c Using an Immunoturbidimetric Assay

Short Title: Validation of a method for measuring canine HbA1c

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Abstract

Background: Hemoglobin A1c (HbA1c), provides a reliable measure of glycemic control over 2 to 3 months in human diabetes mellitus (DM). In dogs, formation of HbA1c has been demonstrated, but there are no validated commercial assays.

Objective: To validate a commercially available automated immunoturbidimetric assay for canine HbA1c and determine a reference interval in a hospital population of various ages and breeds.

Methods: The specificity of the assay was assessed by inducing glycosylation in vitro using isolated canine hemoglobin. Repeatability was assessed by measuring canine samples 5 times in succession, long term inter-assay imprecision by measuring supplied control materials, stability using samples stored at 4˚C over 5 days and -20˚C over 8 weeks, linearity by mixing samples of known HbA1c in differing proportions, and the effect of anticoagulants by taking paired samples. A reference interval was determined using EDTA-anticoagulated blood samples from 60 non-diabetic hospitalised animals of various ages and breeds. HbA1c was also measured in dogs with DM (n=10).

Results: HbA1c increased proportionally with glucose concentration in vitro. The mean repeatability was 4.1% (range 1.2% - 6.1%). Samples were stable for 5 days at 4˚C. The assay was linear within the assessed range. EDTA- and heparin-anticoagulated blood can be used interchangeably for HbA1c measurement. The reference interval for HbA1c was 9 – 18.5mmol/mol. There was no apparent effect of age or breed on HbA1c. HbA1c ranged from 14 - 48 mmol/mol in dogs with DM.

Conclusions: The assay provides a reliable method of canine HbA1c measurement with good analytical performance.
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Key words: analytical performance, diabetes mellitus, dogs, glycosylated hemoglobin, method validation

Abbreviations used

ANOVA – analysis of variance
CBC – complete blood count
CI – confidence interval
CV – coefficient of variance
DM – diabetes mellitus
EDTA – ethylenediaminetetraacetic acid
HbA1c - hemoglobin A1c
RCF – relative centrifugal force
SD – standard deviation

Introduction

Glycosylated hemoglobin A1c (HbA1c) is formed when glucose binds to the n-terminal valine of the β-subunit of hemoglobin A and makes up the largest fraction of the total glycosylated hemoglobin (HbA1)\(^1\). The total amount of HbA1c formed is dependent on erythrocyte lifespan, erythrocyte permeability to glucose and the average blood glucose concentration throughout that erythrocyte lifespan\(^1\). In people it is related to the average blood glucose concentration over the preceding 2-3
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months, weighted towards the most recent 2-4 weeks. Fructosamine – glycosylated plasma proteins - are currently a commonly used measure of longer term diabetic stability in the dog. Fructosamine are related to the average blood glucose concentration over the previous 1-2 weeks. However a number of factors other than plasma glucose concentration affect fructosamine concentration including hypoproteinemia, hyperlipidemia and azotemia. Compared to fructosamine measurements, HbA1c is less affected by pathological conditions other than DM.

In people HbA1c has shown itself to be a highly specific and reliable biomarker for the long term control of both type 1 and type 2 DM. HbA1c is also used prognostically with increasing values corresponding to increased risk of diabetic complications. In dogs, there have been no studies looking at the relationship between fructosamine or HbA1c and outcome in DM.

The measurement of HbA1c in dogs has been previously described. Several methods developed for human use have been evaluated for use in canine DM but none have been adopted into standard clinical practice. All published studies have however shown that canine diabetics have higher average ratios of glycosylated hemoglobin: total hemoglobin compared to non-diabetic controls.

The first aim of this study was to determine the effect of increasing glucose concentrations on the production of canine HbA1c as measured by an immunoturbidimetric assay, the Siemens DCA™ Vantage (Siemens Healthcare plc. Surrey, UK), in canine erythrocyte preparations. The second aim was to validate this method and to establish a reference interval from a hospital population. The final aim was to assess the effect of breed pre-disposition and age on canine HbA1c within the reference population.
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Materials and Methods

Animals and Samples

Specimens obtained were all taken from dogs referred to the Small Animal Hospital, University of Glasgow. Ethical approval for this study was obtained from the School of Veterinary Medicine Ethics and Welfare committee, University of Glasgow.

Hospital Population

Surplus EDTA-anticoagulated and heparin-anticoagulated blood from samples that had been taken for diagnostic purposes by jugular venipuncture were used for the method validation aspect of the study. Screening for eligibility into the reference interval occurred between June and August 2015 with 60 samples meeting all criteria selected. The inclusion criteria were a record of a CBC measured using a Cell-Dyn hematology analyzer (Abbott Laboratories, North Chicago, IL, USA) with WBC differential counts performed manually, and biochemistry analyses including a near normal plasma glucose concentration of 3-7mmol/L (54-126mg/dL) measured by the hexokinase G-6-PDH method in a fluoride oxalate-anticoagulated sample within 8 hours using an Olympus AU640 biochemistry analyzer (Olympus Corporation, Tokyo, Japan). As part of the biochemistry analyses cholesterol (by combined cholesterol esterase/oxidase method) and triglycerides (by combined lipolysis/ glycerol kinase/ glycerol phosphate oxidase method) were also measured on the Olympus AU640. Exclusion criteria were a diagnosis of DM or other endocrine disorders. Grossly hemolyzed and lipemic samples were excluded. Animals with a hemoglobin concentration <12 g/dL were excluded from the reference interval.
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population as anemia has been shown to have an effect on HbA1c in previous canine studies. Animals with diseases unrelated to carbohydrate metabolism were not excluded. Information was collected on the animal’s age; breed, sex, co-morbidities, current medication, blood glucose, and hemoglobin as well as any abnormalities found on hematology and biochemistry. To ensure a wide range of dogs were included in the reference interval population, dogs were prospectively recruited into 4 similarly sized sub-groups based on age and diabetic predisposition; young (\(<\ 5\) years old) non-predisposed, older (\(>5\) years old) non-predisposed, young predisposed, and older predisposed. The diabetic predisposition was based on the list as shown in the supplementary information.

**Diabetic population**

Surplus heparin-anticoagulated and EDTA-anticoagulated blood samples, taken for monitoring purposes were also available for 10 diabetic dogs receiving insulin therapy. Diagnosis had been confirmed before sample collection on the basis of consistent clinical signs of polyuria, polydipsia and weight loss, concurrent hyperglycemia and glucosuria. Information was collected on age, breed, sex, co-morbidities, medications, blood glucose, hemoglobin and any hematological and biochemical abnormalities as well as any serum fructosamine measurements performed using the Nitrotetrazolium blue reduction method on an Olympus AU640 biochemistry analyser with ABX Horiba reagent kit (Horiba UK Ltd – Medical, Northampton, UK) as part of routine monitoring.

**Analysis of HbA1c**

Samples were analysed on a DCA™ Vantage using HbA1c reagent cartridges (Siemens DCA™ Systems Hemoglobin A1c reagent kit) which contain all reagents in
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a single disposable cartridge that the sample is slotted into, effectively eliminating carry-over. The Siemens DCA™ Vantage uses murine anti-human HbA1c monoclonal antibodies specific to the last few amino acids on the N-terminal of the β-chain\(^{19}\). Canine and human hemoglobin A are only 80% homologous, but the first 5 residues of the N-terminal of the β-chain are identical\(^{16}\). HbA1c in the sample causes an inhibition of latex agglutination by binding competitively to the anti-HbA1c antibodies and decreasing absorbance at 531nm. Concurrently total hemoglobin is measured following the oxidation to methemoglobin, complexed with thiocyanate to form a colored compound which is measured spectrophotometrically at 531nm. HbA1c is expressed as a ratio of total hemoglobin in mmol/mol within 6 minutes. All reactions and calculations are carried out internally\(^{19}\).

The samples were analysed according to the manufacturers’ instructions using 1μL of EDTA-anticoagulated blood. Before each batch run and when changing cartridge batch numbers a quality control run with one normal (range 24.6 - 48.6mmol/mol) and one abnormal (range 70 - 116.4mmol/mol) sample was performed using commercial control reagents (Siemens DCA™ systems).

**Incubation of canine hemoglobin with glucose**

EDTA-anticoagulated blood from 5 non-diabetic dogs with no hematological abnormalities was pooled. This sample was washed 3 times with phosphate buffered saline by centrifuging at a relative centrifugal force (RCF) of 1439 using a Beckman Coulter Allegra X-12R (Beckman Coulter, High Wycombe, UK) for 10 minutes. The washed erythrocytes were hemolyzed using a modified osmotic shock procedure\(^{20}\), then centrifuged at an RCF of 2249 for 15 minutes to sediment the cell debris. The hemolysate was divided into 5 aliquots of 600μL. The hemolysates were incubated at
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glucose concentrations of 0, 50, 100, 200 and 400 mmol/L (equivalent to 900, 1800, 3600 and 7200 mg/dL), stored at room temperature, and measured at days 0, 7, 11, and 17 after preparation. A separate pool of intact erythrocytes was washed in the same manner and half the sample was hemolyzed. Both hemolysates and intact erythrocytes were divided into 500µL aliquots and incubated at 4°C in the same glucose concentrations as the room temperature hemolysates. Samples were measured at 0, 4, 7 and 14 days after preparation.

**Repeatability**

Samples were collected as described previously from both diabetic and non-diabetic animals to represent a large range of glucose concentrations. To evaluate the repeatability, the HbA1c of each sample (n=4) was measured 5 times in succession for each sample. To evaluate the inter-assay variability over the course of the study, the results from the commercially available quality control materials were used.

Storage stability was evaluated using 3 EDTA-anticoagulated samples kept at 4°C. Samples were measured at baseline and then for 4 consecutive days, with the measurements at each time point compared to baseline. Stability of canine HbA1c at -20°C was assessed by freezing 5 samples, measuring these after 4 and 8 weeks and comparing to baseline.

**Linearity**

Samples from 2 dogs were used. To achieve a range, one set of samples was from the reference interval and the other from the diabetic population. HbA1c was measured in the EDTA-anticoagulated samples and then mixed in varying proportions (25:75, 50:50 and 75:25) to create 3 new intermediate expected values,
which were then compared to the measured HbA1c\textsuperscript{21}. This was repeated with the heparin-anticoagulated samples to assess both linearity of the assay and the effect of different anti-coagulants to the measured HbA1c.

**Interference**

An initial investigation into the effect of lipemia on the assay was performed. EDTA-anticoagulated samples were obtained as previously described. Samples from 7 dogs with known total lipid concentrations, triglycerides from 0.68 – 25.58 mmol/L and cholesterol from 4.9 – 11.7 mmol/L were used. 4 of the samples came from diabetic dogs, 2 from non-diabetics who had been excluded from the reference interval due to gross hyperlipemia, and one control from the reference interval population. After assessing the baseline HbA1c, the samples were centrifuged (Beckman Coulter Allegra X-12R) at 2073 RCF at 6°C for 12 minutes. The plasma was completely removed and an equal volume of 0.9%NaCl added. The packed red cells were re-suspended by mixing well and the samples were measured again.

To assess the effect of complete hemolysis on HbA1c measurements, the starting time point results from the haemolyzed preparations used in the 4°C glucose incubation experiments (described earlier) were compared to the results from the intact erythrocyte preparations (which were from the same pooled sample but had not undergone haemolysis).

**Statistical methods**

Statistical analysis was carried out in SPSS v22 (IBM) or Microsoft Excel 2010 for Windows with Reference Value Advisor Add-In \textsuperscript{22}. Statistical significance was set at $p=0.05$ for all analyses.
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For the glucose incubation regression lines were fitted. The lines of measured HbA1c over time were fitted for each glucose concentration and compared against the control (0 mmol/L glucose). The change in HbA1c from baseline was compared to glucose concentration for each time point using a linear regression and Pearson correlation.

To evaluate linearity a least squares regression analysis was performed on expected against observed measurements. The difference between EDTA and heparin anticoagulant was evaluated by calculating the mean difference and performing a least squares regression analysis.

The reference interval for the hospital population was determined using the non-parametric method with bootstrapping to arrive at the 90% confidence interval for the whole population. Both Tukey and Dixon-Reed tests were used to identify potential outliers. The reference interval comprises the central 95%, with 90% confidence intervals around the lower and upper limits.

All factors assessed – HbA1c age, breed-predisposition, glucose, hemoglobin, sex – were summarised descriptively. Linear regression and correlations were performed to look at the relationship between HbA1c and glucose concentration, age and hemoglobin concentration. A regression model of HbA1c was fitted with age, haemoglobin concentration, sex, breed-predisposition and glucose concentration as co-factors.

A Kruskal-Wallis ANOVA was used to assess the overall difference in HbA1c between the pre-specified reference interval groups. Subgroup analysis was performed within the reference population by comparing the median HbA1c in each
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subgroup to that of the median HbA1c of the other subgroups combined using a
Mann-Whitney U test.

For the diabetic population all factors assessed were summarised descriptively and
compared to the reference interval population. Normally distributed factors were
compared using an independent sample t-test and non-normally distributed factors
were compared using a Mann-Whitney U test.

Results

HbA1c synthesis in vitro at room temperature using canine hemolysates increases
with time and glucose concentration (Figure 1). Using a least squares regression to
fit lines for each concentration over time, the concentrations were found to be
significantly different from each other (p<0.001). Additionally at each time point, the
change in HbA1c from baseline against glucose concentration gave a linear
relationship; day 7, Pearson correlation 0.968, R² linear 0.937 (p=0.007); day 11, R²
0.941 (p=0.006); day 17, R² 0.941(p=0.006). The increase in HbA1c over time was
initially linear for all glucose concentrations, but between days 11 and 17 the rate of
change started to decrease. Further points were not measured. The aliquots kept at
4°C did not show any significant deviations from baseline after 14 days.

The mean co-efficient of variation using canine samples measured 5 times in
succession was 4.08% (range 1.16% - 6.10%) (Table 1) with a maximum difference
of 2 mmol/mol between the highest and lowest measurement within any sample.
One measurement of 57mmol/mol was excluded from the analysis of the canine
diabetic sample due to operator error.
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The inter-assay imprecision over the course of the study was 2.97% with the normal concentration quality control material (stabilised human hemoglobin) supplied by the manufacturer. The inter-assay imprecision with the high HbA1c commercial control had a larger CV of 8.81%. Bias was seen with an overestimation of the mean relative to the stated value (mean 93.5mmol/mol) (Table 1).

There was a low variation due to storage over 5 days using the canine samples with a mean CV of 2.12% (Table 1). The maximum absolute change from baseline at any time point was 1 mmol/mol with a mean change from baseline after 5 days of -0.3mmol/mol. After freezing for 4 weeks there was low variation compared to baseline with a maximum absolute change of 1 mmol/mol. After 8 weeks 4 samples showed a small change from baseline with the fifth showing a decrease of 7 mmol/mol (-14%) from baseline.

In the study of linearity, regression analysis of the heparin-anticoagulated and EDTA-anticoagulated blood samples gave a linear relationship for both (Figure 2). R² linear for EDTA was 0.982 and for heparin 0.957. For EDTA samples the y intercept was -2.23mmol/mol (95% CI -4.539 to 0.077mmol/mol) and the slope was 1.031 (95% CI 0.951 to 1.111). For the heparin samples the y intercept was -1.846mmol/mol (95% CI -5.5 to 1.8 mmol/mol) and the slope 1.046 (95% CI 0.918 – 1.174). For both lines the y intercept was not significantly different from 0, the slope was not different from 1 and the lines were not significantly different from each other (p=0.92). The mean difference between the heparin and EDTA samples was -0.8mmol/mol.

Regarding interference from hyperlipemia, the initial HbA1c measured ranged from 11 to 34mmol/mol with a trend for increasing interference from lipids as the
triglyceride concentration increases (Table 2). The largest difference was 6mmol/mol (60% change from baseline for sample 5 and a 20.7% change for sample 7).

The HbA1c values obtained from the hemolyzed pooled sample were higher, mean 22mmol/mol, than the intact erythrocytes, mean 17.75mmol/mol, with no overlap between the two samples.

The reference population comprised of 9 predisposed breeds and 18 non-predisposed breeds. The most common predisposed breeds were Border collies (n=9), Bichon Frises (n=3), Cavalier King Charles Spaniels (n=3) and Yorkshire terriers (n=2). The most common non-predisposed breeds were Labradors (n=6), Crossbreeds (n=5), German Shepherd dogs (n=5), Golden Retrievers (n=4) and Cocker Spaniels (n=4). The reference interval was calculated from 60 animals. No outliers were excluded. The distribution of the reference values was not normal, Shapiro-Wilk p=0.03, showing evidence of a binomial distribution (Figure 3). Using the non-parametric method (n>40) a reference interval for HbA1c of 9 – 18.5 mmol/mol was obtained with a lower limit 90% CI of 9 - 10.5mmol/mol and an upper limit 90% CI of 18 - 19mmol/mol. The mean value obtained was 14.3mmol/mol HbA1c (SD 2.5) with a total range of 9 - 19 mmol/mol observed for the reference population (Table 3).

There was a weak positive correlation between HbA1c and plasma glucose concentration within the reference population, Spearman’s σ 0.332, $r^2=0.089$. The correlations between age and HbA1c and between hemoglobin and HbA1c were not significant within the reference population. Within the regression model the overall adjusted $r^2$ was low (0.146) and the only significant factor was glucose concentration (p=0.02) with an increase in HbA1c of 1.259 mmol/mol for every 1 mmol/L increase.
in plasma glucose concentration. Both being neutered and being of a non-
predisposed breed decreased HbA1c in the model (estimate -0.8448, p=0.19 and -
1.002, p=0.12 respectively) but neither was statistically significant. Age, hemoglobin
concentration and male vs female were all non-significant factors (p=0.09, p=0.23,
p=0.68).

Differences in age, sex and breed predisposition were observed between the groups
(Table 3), but only the old non-predisposed group had a higher HbA1c (mean
15.28mmol/mol) when compared to the rest of the reference interval (mean
13.88mmol/mol) p=0.045. There were no significant differences in HbA1c across the
sub-groups using a Kruskal-Wallis ANOVA. There were also no significant
differences when partitioning by age (p=0.442) or breed predisposition
(p=0.213)(Figure 3).

The DM group (n =10) was significantly older (p=0.005) and had a higher proportion
of dogs of predisposed breeds(7/10) (p=0.047) compared to the reference interval
population. As expected, the DM group had significantly higher glucose
concentrations, mean 19.56mmolL vs. 4.9mmolL (p<0.001) (Table 3). The HbA1c,
mean 36.5mmol/mol vs. 14.3mmol/mol (p<0.001), was also significantly higher
(Figure 4). There were no differences in hemoglobin (p=0.454) and sex (p=0.442)
between DM group and the reference population. Within the DM group there was no
significant correlation between HbA1c and glucose concentration p=0.618 (n=9) or
fructosamine p=0.827 (n=6).

Discussion

Using pooled erythrocytes from non-diabetic dogs it was demonstrated that
incubation with glucose increased the proportion of HbA1c measured in vitro with
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increasing glucose concentration. From the known kinetics of the reaction\(^1\) and work using human erythrocyte preparations\(^{23}\), this increase was expected and thus suggests that the DCA™ Vantage is measuring the stable canine HbA1c fraction and that canine HbA1c can be used to quantify the long term glycemic control of a canine patient. Using human erythrocytes it was previously shown that HbA1c increases with increasing glucose concentrations at 4°C\(^{23}\). However this was not replicated in our experiments, with values not deviating significantly from baseline after 14 days. The previous study used agar gel electrophoresis for detecting HbA1c, which does not differentiate between stable and labile fractions of HbA1c. However, the reaction of hemoglobin with glucose to form labile HbA1c occurs 11.9 times faster than to stable HbA1c\(^2\) and so increases were seen more quickly than in our study. The only previous study using canine erythrocyte preparations also looked at increases in labile HbA1c, incubating their preparations with a high glucose concentration for just 6 hours and increasing glucose concentrations were not evaluated\(^{18}\).

Intact erythrocytes were not used for the room temperature incubation as they are prone to extensive and variable hemolysis\(^{24}\). Using hemolysates controlled for this variable, but made the model less representative of in vivo conditions. We were however able to show that canine HbA1c increases in a dose dependent manner with increasing glucose concentration at room temperature. Despite supraphysiological glucose concentrations the reaction was slow. This is consistent with HbA1c as a measure of average glucose concentration over 2-3 months\(^2\). The results from this in vitro incubation are also consistent with previous findings that persistent hyperglycemia over 2 weeks is needed in order to see increases in HbA1c in vivo\(^{13}\).
Important components of a method validation study are imprecision and accuracy\textsuperscript{25}. Imprecision in the DCA™ Vantage was low with a CV of 2.97% for the normal commercial quality control. This is comparable to CVs obtained by several independent studies validating the DCA™ Vantage for use in humans where < 3.1% was achieved\textsuperscript{26, 27}. Slightly higher CVs were obtained during assessment of inter-assay variation using canine samples, mainly due to the low samples showing proportionally much more variation with an overall range of 1mmol/mol compared to samples with a higher value. The overall imprecision is likely to be acceptable for veterinary use\textsuperscript{28}. A much higher inter-assay variation (8.81%) was obtained from the high control. It is known that the DCA™ Vantage exhibits increased variance at readings above 64mmol/mol\textsuperscript{26}. However as only a small number of diabetic animals were included in the study (with a maximum HbA1c of 48mmol/mol) it cannot be said whether this will impact veterinary use. Bias in the DCA™ Vantage could not be assessed for canine HbA1c measurements due to the absence of a canine reference method and the inaccessibility of previously validated methods. Comparison using high performance liquid chromatography set up for human HbA1c has been unsuccessful in previous canine studies\textsuperscript{3, 11, 18}. With the human reference material the abnormal control showed considerable bias compared to the reported mean of the material, but this was marginal in the normal control. The effect of storage at 4°C was minimal with a mean change from baseline of -0.33mmol/mol after 5 days. Different samples were used for the repeatability and storage assessments so variation cannot be compared directly, but the total variance from the storage assessment (CV 2.12%) was comparable to the inter-assay variation. The linearity study provided an indirect assessment of bias. The regression analysis confirmed that the line obtained experimentally was not significantly different from
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...and thus there was no internal bias against expected values. The results obtained here are consistent with human data from independent studies\textsuperscript{19, 26}. The high HbA1c used in assessing linearity was significantly lower than the highest reading obtained from any animal in this study (40mmol/mol vs. 48mmol/mol), so there is potential for unknown bias in the upper expected working ranges of the assay and means that we have not fully established the reportable range of this assay. However as validation of this assay for human samples has established a reportable range of $< 143$mmol/mol\textsuperscript{19} it is unlikely that any samples will exceed the reportable range when using the DCA™ Vantage for veterinary purposes. No significant difference was found between measurements obtained from blood anticoagulated with EDTA or heparin before analysis. The mean difference of $-0.8$mmol/mol and lack of significant differences between the regression lines, suggests that these anticoagulants can be used interchangeably in the measurement of canine HbA1c.

In these preliminary investigations, hemolysis and hyperlipemia appear to interfere with the HbA1c measurement obtained by the DCA™ Vantage. The preliminary investigations were designed to test whether there was interference when using patient samples with the concentration range of interferent expected with clinical use, to determine where further investigation is warranted\textsuperscript{29}. The manufacturers report that, for human samples, there is a bias of $-1.81\%$ at triglyceride concentrations of 15.2mmol/L for HbA1c measurements in the 42-48mmol/mol range, but no data on the magnitude of interference beyond this is available. As dogs may have triglyceride concentrations in excess of 15.2mmol/L\textsuperscript{30}, it was important to determine whether there may be an effect of hyperlipidemia on the HbA1c as measured by the device. Samples with a total lipid concentration of 25mmol/L and above showed increases
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from baseline in measured HbA1c concentration after lipid removal that were larger than the inherent imprecision within the method. It gives an early indication that measured HbA1c may be artificially reduced in samples with gross hyperlipidemia and this should be investigated further using established guidelines\textsuperscript{21}. Similarly, hemolyzing a pooled sample significantly increased the HbA1c reading obtained compared to baseline. This was surprising as the DCA™ Vantage hemolyzes the sample internally before analysis. So far only 100% hemolysis has been looked at and further investigation will be necessary to determine the maximum acceptable sample hemolysis. The other main interferent which is often looked at in validation studies is bilirubin, however unfortunately no sufficiently icteric samples were obtained during the course of the study.

The reference interval was 9 – 18.5mmol/mol for canine HbA1c in a hospital population. With an assay specific for canine HbA1c we would expect a lower reference interval compared to the human reference interval due to the decreased glucose permeability of canine erythrocytes compared to human erythrocytes\textsuperscript{1}. The human reference interval is 20 – 42mmol/mol. As there is no canine reference method, our results cannot be readily compared to previous studies. When comparing our results to those obtained with previously evaluated methods for canine HbA1c still available for human use\textsuperscript{16,17}, our reference interval was significantly lower. As a point of note, one of the previous studies\textsuperscript{35} determined health based on the absence of clinical signs and did not specifically exclude anemic dogs. Anemic dogs were excluded in our study as there is evidence of an effect on canine HbA1c independent of glucose concentration\textsuperscript{13,16,31}.

The hospital population may not mirror the true healthy population, despite evidence that most pathological processes do not affect HbA1c\textsuperscript{13}. A previous study did not
show any differences between the reference interval obtained from a hospital sample compared to healthy animals, but their healthy population consisted of 18 juvenile colony beagles. The ethical issues involved with collecting samples from healthy animals for research purposes preclude the use of healthy reference animals (regulated under the Animals Scientific Procedures Act 1986). The reference interval is however relevant to dogs presented to a small animal hospital. Using this reference interval in other situations may not be appropriate. Sample size is another potential limitation in this study in that it reduced the power of the study to detect small differences between sub-groups. Despite a previous study with a larger sample size observing normality, the distribution of reference values of HbA1c in our study was not normal (Figure 3). The reason for this distribution is not clear. The study was large enough, however, to establish a reference interval in line with American Society of Veterinary Clinical Pathology recommendations with good confidence in the range as shown by the tight 90% confidence intervals.

Information was specifically collected with a view to partitioning the reference population into subgroups. When performing subgroup analysis, the HbA1c was found to be significantly higher in the old non-predisposed group when compared to the rest of the reference interval, but a Kruskal-Wallis ANOVA identified no significant differences between the groups. There was no difference when partitioning solely by age (Figure 3), which is consistent with previous studies. To our knowledge breed predisposition has not been previously examined in conjunction with HbA1c. There was no significant effect of breed predisposition on HbA1c. However it is acknowledged that, as we only looked at predisposed compared to non-predisposed breeds, our categorization may have reduced the ability to detect a true difference in HbA1c in specific breeds or in the small sub-
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group of non-predisposed breeds that might be considered ‘protected’ against diabetes mellitus. Predisposed breeds are not likely to have a higher HbA1c as the clinical course of canine DM is comparable to human type 1 disease. However as little is known about breed differences in erythrocyte permeability, but at least one breed associated difference has been identified, it follows that some breeds may have higher (or lower) HbA1c concentrations.

In order for canine HbA1c to be adopted as an effective biomarker for long term diabetic control, an independent objective assessment of diabetic control needs to be developed. It will then be possible to assess the relative values of HbA1c and fructosamine as markers of long term diabetic control.

Acknowledgements

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References


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Table 1: Co-efficients of variance and measured HbA1c (mmol/mol) for control materials and canine samples and variation during storage over 5 days

<table>
<thead>
<tr>
<th>Sample type (n)</th>
<th>HbA1c range</th>
<th>HbA1c mean</th>
<th>CV</th>
<th>Reported mean</th>
<th>Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>canine 1</td>
<td>8 - 9</td>
<td>8.2</td>
<td>6.10%</td>
<td>-</td>
<td>-</td>
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<tr>
<td>canine 2</td>
<td>13 - 15</td>
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<td>5.75%</td>
<td>-</td>
<td>-</td>
</tr>
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<td>canine 3</td>
<td>17 - 18</td>
<td>17.4</td>
<td>3.32%</td>
<td>-</td>
<td>-</td>
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<td>canine 4</td>
<td>43 - 44</td>
<td>43.3</td>
<td>1.16%</td>
<td>-</td>
<td>-</td>
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<td>mean canine samples</td>
<td></td>
<td></td>
<td></td>
<td>4.08%</td>
<td></td>
</tr>
<tr>
<td>Inter-assay¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal control (n=9)</td>
<td>36 - 39</td>
<td>36.9</td>
<td>2.97%</td>
<td>36.6</td>
<td>-0.49%</td>
</tr>
<tr>
<td>abnormal control (n=9)</td>
<td>90 - 116</td>
<td>104.7</td>
<td>8.81%</td>
<td>93.5</td>
<td>10.29%</td>
</tr>
<tr>
<td>Storage²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>canine 5</td>
<td>17 - 18</td>
<td>17.6</td>
<td>3.11%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>canine 6</td>
<td>39 - 40</td>
<td>39.4</td>
<td>1.39%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>canine 7</td>
<td>48 - 50</td>
<td>48.4</td>
<td>1.85%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mean canine samples</td>
<td></td>
<td></td>
<td></td>
<td>2.12%</td>
<td></td>
</tr>
</tbody>
</table>

¹Long term inter-assay variation using commercially available control materials (stabilised human hemoglobin). ²Storage over 5 days at 4°C. ³The mean HbA1c of the commercially available material as reported by the manufacturer. ⁴The bias in the mean of the measured HbA1c against the reported mean value.
Validation of a method for measuring canine HbA1c

Table 2: Interference in the HbA1c assay using patient samples with increasing hyperlipidemia

<table>
<thead>
<tr>
<th>n</th>
<th>Diabetic</th>
<th>total lipids</th>
<th>TG (RI &lt;0.6)</th>
<th>cholesterol (RI &lt;7)</th>
<th>HbA1c pre</th>
<th>HbA1c post</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>5.62</td>
<td>0.68</td>
<td>4.94</td>
<td>15</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>15.35</td>
<td>7.05</td>
<td>8.30</td>
<td>34</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>19.55</td>
<td>9.26</td>
<td>10.29</td>
<td>13</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>21.59</td>
<td>11.64</td>
<td>9.95</td>
<td>13</td>
<td>14</td>
<td>+1</td>
</tr>
<tr>
<td>5</td>
<td>Yes</td>
<td>25.28</td>
<td>14.88</td>
<td>10.40</td>
<td>16</td>
<td>16</td>
<td>+6</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>31.7</td>
<td>4.7</td>
<td>27.00</td>
<td>11</td>
<td>14</td>
<td>+3</td>
</tr>
<tr>
<td>7</td>
<td>Yes</td>
<td>36.65</td>
<td>25.58</td>
<td>11.07</td>
<td>29</td>
<td>35</td>
<td>+6</td>
</tr>
</tbody>
</table>

TG: Triglycerides, RI: Reference Interval. ¹Sample number. ²Total measured lipids is defined as the sum of triglycerides and cholesterol. ³HbA1c as measured at baseline. ⁴HbA1c after removal of interferent containing plasma and replacement with an equal volume of 0.9% NaCl. ⁵Difference between post and pre HbA1c.

Table 3: Characteristics for the reference interval subgroups and combined reference interval
Validation of a method for measuring canine HbA1c

<table>
<thead>
<tr>
<th>Subgroups of the reference population</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>young non-pre disposed</td>
<td>Reference population</td>
</tr>
<tr>
<td>old non-pre disposed</td>
<td>n=20</td>
</tr>
<tr>
<td>young pre disposed</td>
<td>n=18</td>
</tr>
<tr>
<td>old pre disposed</td>
<td>n=9</td>
</tr>
<tr>
<td>Category</td>
<td>n=13</td>
</tr>
<tr>
<td>Total</td>
<td>n=60</td>
</tr>
<tr>
<td>Age Mean (SD)</td>
<td>3.25 (1.29)</td>
</tr>
<tr>
<td>Median</td>
<td>4</td>
</tr>
<tr>
<td>Range</td>
<td>1 - 5</td>
</tr>
</tbody>
</table>

Sex

- male entire n(%) | 5 (25%) | 8 (44.4%) | 4 (44.4%) | 1 (7.7%) | 18 (30%) | 1(10%) |
- male neut n(%) | 1 (5%) | 4 (22.2%) | 1 (11.1%) | 4 (30.8%) | 10 (16.7%) | 5 (50%) |
- female entire n(%) | 5 (25%) | 2 (11.1%) | 0 (0%) | 3 (23.1%) | 10 (16.7%) | 1 (10%) |
- female neut n(%) | 9 (45%) | 4 (22.2%) | 4 (44.4%) | 5 (38.5%) | 22 (36.7%) | 3 (30%) |

Breed predisposition

- Yes n(%) | 22 (36.7%) | 7 (70%) |
- no n(%) | 38 (63.3%) | 3 (30%) |

Blood glucose (mmol/L)

- Mean (SD) | 4.83 (0.469) | 4.96 (0.544) | 5.18 (0.954) | 4.94 (0.549) | 4.9 (0.596) | 19.56 (10.6) |
- Median | 4.9 | 5 | 5.3 | 4.9 | 4.9 | 19.3 |
- Range | 3.8 - 5.9 | 3.7 - 5.8 | 3.6 - 6.8 | 4.1 - 5.9 | 3.6 - 6.8 | 3.9 - 35.1 |

Hemoglobin (g/dL)

- Mean (SD) | 15.98 (2.32) | 15.51 (1.80) | 15.77 (1.71) | 15.78 (3.25) | 15.8 (2.29) | 15.09 (1.43) |
- Median | 15.8 | 15.15 | 16.1 | 15.5 | 15.7 | 15.1 |
- Range | 13 - 20.3 | 12.6 - 19.2 | 12.6 - 17.9 | 11 - 23.2 | 11 - 23.2 | 13.4 - 17.5 |

HbA1c (mmol/mol)

- Mean (SD) | 14.1 (2.5) | 15.28 (1.97) | 13.89 (2.32) | 13.62 (3.20) | 14.3 (2.5) | 36.5 (9.94) |
- Median | 13.5 | 15.5 | 13 | 13 | 14 | 39 |
Validation of a method for measuring canine HbA1c

Figure Legends

Figure 1: Change in HbA1c in hemolysates incubated with increasing concentrations of glucose at room temperature. ♦ 400mM glucose, ▼ 200mM glucose, ▲ 100mM glucose, ■ 50mM glucose, ● 0mM glucose.

Figure 2: Scatter plot of expected HbA1c values against observed HbA1c measurements in mmol/mol using 2 baseline samples and 3 intermediate mixes (25:75, 50:50, 75:25) using EDTA (squares) and heparin-anticoagulated blood (triangles) blood. Straight lines are regression lines; dotted line is y=x.

Figure 3: Histogram of all reference values (n=60) partitioned by age (left panel) and breed disposition (right panel).

Figure 4: Boxplot of HbA1c values of the reference group compared to the diabetic group. The box represents the interquartile range and is bisected by a line representing the median. The lines represent the main body of data with open circles representing outlying points.