Full title

Effects of storage time and temperature on thromboelastographic analysis in dogs and horses

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Short title: Sample stability for canine and equine TEG

Abstract

Background: Accessibility of thromboelastography (TEG) to general practitioners is limited by short sample storage times (30 minutes) and storage temperature (20-23°C).

Objectives: To evaluate the stability of canine and equine citrated blood samples when stored for extended periods of time, both at room temperature (RT) (20-23°C) and refrigeration temperature (FT) (2-7.5°C).

Methods: Citrated whole blood samples from healthy dogs and horses (n=10 for each) were stored for 30 minutes (baseline) at RT before TEG analysis. Baseline values for TEG variables R, K, α, MA, LY30 and LY60 were compared to those from samples stored for 2 hours, 8 hours, and 22.5 hours, both at RT and FT. Results were compared using ANOVA ($p < 0.05$). Total allowable analytical error (TE_a) based on biological variation data was used to evaluate stability.

Results: In dogs, statistically significant differences included shorter R, longer K, decreased MA, and increased LY60 at various time points and storage temperatures from 2 hours onwards. Only samples stored for 2 hours at FT showed acceptable stability compared to TE_a.

In horses, statistically significant differences included shorter R and K, and decreased α, LY30 and LY60 at various time points and storage temperatures from 2 hours onwards. Samples were not stable at any time, regardless of temperature, compared to TE_a.

Conclusions: In this study, canine samples could be stored for up to 2 hours at FT without affecting TEG results; equine samples should be stored for 30 minutes at RT.

Key words

Hemostasis, preanalytical, stability, TEG, viscoelastic testing

1 Introduction

Thromboelastography (TEG) is an analytical method used for assessment of hemostasis that was first introduced in the 1940s by Hartett.[1] TEG delivers an analysis of hemostasis that is more extensive (includes cellular and soluble components) than routine coagulation assays, providing a continuous detection and recording of viscoelastic changes in whole blood during clot initiation, amplification, propagation and lysis.[2,3]

TEG was originally designed to be a bed-side, point-of-care analysis of non-anticoagulated (“native”) whole blood. Native whole blood had to be placed in the analyser within four minutes after venipuncture (before significant coagulation started in the specimen), which made it impossible to perform the test when the analyser was not in close proximity to the patient.[4] The subsequent use of citrated whole blood has prolonged the amount of time a sample can rest before analysis. Currently, the recommendation for viscoelastic testing in veterinary species is that citrated samples should be stored at room temperature for 30 minutes between collection and analysis.[5]

The four standard variables generated from a thromboelastograph include R (reaction time; representing time between clot initiation and the production of the initial insoluble fibrin strands (“clot”) and is proportional to the coagulation protein concentration), K (clot formation time; representing the time between initial clot formation and a

pre-determined strength of clot formation), α (clot angle; representing the acceleration of fibrin formation and cross-linking) and MA (maximum amplitude; representing maximum clot strength and is proportional to platelet count, platelet function and fibrinogen level).[3,6-11] These variables are collectively used to determine whether a patient is normocoagulable, hypocoagulable (longer R, longer K [or absent K if MA never reaches 20 mm], decreased α and decreased MA) or hypercoagulable (shorter R, shorter K, increased α and increased MA).[12] Values for the variables LY30/60 (clot lysis; representing the percentage of clot lysis detected 30/60 minutes after MA was reached) can also be generated, but are not in common clinical use.[6]

The limitation on storage time means that TEG analysis is usually performed at referral institutions and is not widely available to general practitioners. The limitation of room temperature storage further decreases availability to general practitioners, especially those working under field conditions. Existing TEG storage studies in veterinary medicine comprise of varying combinations of species, subject health status, activators and storage time. In terms of kaolin-activated TEG, studies have assessed the effect of storage on TEG variables in cows, horses and Asian elephants.[13-15] These studies have presented conflicting results and conclusions. There are no published kaolin-activated TEG storage studies for dogs, to our knowledge.

Unlike the approach commonly used in the veterinary literature of only testing for statistically significant differences between results from differing storage times and conditions, attempts have been made in human laboratory medicine to ascertain if there is a clinically relevant difference ("unstable") or if there is no clinically relevant difference ("stable") with regards to sample stability. Documented approaches include using Shewart's X-charts, the significant change limit method, reference change values and total allowable analytical error (TE_a)[16-20] A recent review found that there was no consensus on the experimental design or the criteria used to assess stability in storage studies.[21] The authors of this review subsequently published a guideline protocol for stability storage studies in 2019, which recommends that stability be assessed by comparison of individual biases at different time points to a maximum permissible instability (MPI).[22] The MPI is a quality performance specification equivalent to TE_a that should be established by an individual laboratory according to the hierarchical model defined by the European Federation of Laboratory Medicine.[23] Most veterinary clinical pathologists are familiar with the concept and use of TE_a, and so this recommended approach was used in our study.

The aims of this study were to determine whether TEG results in healthy dogs and horses are affected by storage times exceeding 30 minutes and by storage temperature. We hypothesized that storage time and temperature will have significant effects on TEG variables.

2 Methods & materials

This study was approved by the Research Ethics Committee of the Faculty of Veterinary Science (Project REC073-18) and the Animal Ethics Committee (Project V081-18) of the University of Pretoria, South Africa. Written consent was obtained for use of all animals. All data collection and analyses were performed at the Onderstepoort Veterinary Academic Hospital at the Faculty of Veterinary Science, University of Pretoria, South Africa.

2.1 Study subjects

Ten healthy dogs and ten healthy horses were selected as study subjects. All ten horses were Nooitgedacht mares, part of the Onderstepoort Teaching Animal Unit between 8 and 17 years old and weighed more than 300 kg. All ten dogs were donors for the Onderstepoort Animal Blood Bank. The dogs ranged in age from 1-7 years and in weight from 21-60 kg and included seven German Shepherd Dogs, one Border collie, one Doberman and one mixed breed.
All subjects were deemed healthy based on history, physical examination and complete blood count results.

2.2 Blood collection

Horses were gently restrained in a crush with a halter and lead rein. Dogs were gently restrained in lateral recumbency by trained veterinary personnel. No chemical restraint was used. For each subject (dog or horse), blood was collected by the same operator from the jugular vein, through careful venipuncture, using a 21G needle and vacutainer shoulder. Blood was collected in a specific sequence, through vacuum assistance only, into a serum vacutainer tube (discard tube), four 3.2% sodium citrate vacutainer tubes and an EDTA vacutainer tube (BD, Johannesburg, South Africa). The 3.2% sodium citrate tubes were filled to ensure a 1:9 (citrate:blood) dilution, and gently inverted at least three times. The EDTA tube was used to run a CBC on an ADVIA 2120i (Siemens Healthcare, Erlangen, Germany) within an hour of sampling.

2.3 TEG analysis

Of the four citrate tubes, two tubes were stored at room temperature (RT) (20-23°C) for 30 minutes, 2 hours (hrs), 8 hrs and 22.5 hrs prior to TEG analysis. The remaining two citrate tubes were immediately stored in the fridge (FT) (2-7.5°C) for 2 hrs, 8 hrs and 22.5 hrs prior to TEG analysis. For each subject, analyses at the set time points for both room temperature and fridge storage were performed simultaneously. The first two animals (horses) to be sampled had a final storage time of 24 hours. This caused a logistical difficulty during the period of data collection, and the final storage time was consequently changed to 22.5 hrs.

TEG analysis was performed on TEG 5000 Thrombelastograph Hemostasis System® (Haemonetics Corporation, Braintree, MA, USA) analyzers as follows: a sodium citrate tube was gently inverted at least five times to ensure mixing and then 1 mL of citrated whole blood was added to a kaolin-coated vial. The vial was gently inverted five times after which 340 µL of kaolin-activated blood was added to a pre-warmed sample cup containing 20 µL of 0.2 M $CaCl_2$. The assay was then started. An electronic test and assay of two levels of quality control material (Haemonetics TEG 5000 Level I and Level II controls, Haemonetics Corporation, Braintree, MA, USA) were performed daily, and analyses were only performed if the quality control passed the manufacturer's performance goals. Samples stored in the fridge were not allowed to return to room temperature before analysis. R, K, α, MA, LY30 and LY60 variables were recorded. The TEG analysis was performed by two operators: AVL (horses) and EHH (dogs).

2.4 Statistical significance evaluations

Descriptive statistics were performed, and data was evaluated for normality using the Shapiro-Wilk test. Results from the 30 minutes baseline were compared to the differing storage times and temperatures and evaluated using Friedman's ANOVA with a post-hoc Conover pairwise comparison of variables for non-parametric data and a repeated measures ANOVA with a post-hoc Bonferroni correction for parametric data. Significance level was set at $p < 0.05$. Statistical analysis was performed using MedCalc Statistical Software version 18.11 (MedCalc Software bvba, Ostend, Belgium).

2.5 Evaluation of the percentage of results within total allowable analytical error limits

Acceptable stability (absence of clinically relevant differences) was further determined using the TE_a, as recommended in a recently published human laboratory medicine guideline.[22] To our knowledge, no recommendations are currently available for quality specification goals for TEG testing in any species, and so TE_a was derived from biological variation data, as recommended in various resources.[23-25]

For dogs, TE_a for R, α and MA was calculated using CV_i published in a TEG biological variation study using canine citrated plasma with tissue-factor activation.[26] Data for K was not available. For horses, TE_a for R, α and MA was calculated from intra-individual variation (CV_i) derived for equine citrated whole blood with kaolin activation.[27] An estimation of CV_i for K using kaolin-activated TEG was not available, and the CV_i for tissue-factor activation was used instead. The following calculations were used:[26]

$$TE_a = (1.65 \times CV_{max}) + Bias_{max}$$

where

$$CV_{max} = 0.5 \times CV_i i$$

and

$$Bias_{max} = 0.25 \times \sqrt[2]{CV_a^2 + CV_i^2}$$

The imprecision derived from results of internal quality control measurements was used for CV_a.

The bias for each measurement for each TEG variable for each storage condition was calculated as:

$$Bias(\%) = \frac{(Result\ for\ storage\ condition - Result\ for\ 30\ minutes)}{Result\ for\ 30\ minutes} \times 100$$

This was done for both dogs and horses. Then, the number of times the bias fell inside of the respective TE_a range around the result for the 30 minutes measurement was counted in order to determine the % of values within total allowable analytical error limits (WTAAEL):[17,19]

$$\%WTAAEL = \frac{\#\ bias\ values\ within\ TEa\ limits}{total\ \#\ of\ bias\ values} \times 100$$

Biological variation data was not available for LY30 or LY60 in either species, and these variables were not evaluated using this method.

3 Results

All ten dogs had a normal hematocrit (0.475 ± 0.043 L/L) and platelet count (243 ± 26x10^9/L) based on automated counting and blood smear evaluation. For the ten horses, hematocrit was 0.351 ± 0.061 L/L and automated platelet count was 141 ± 72 x10^9/L. Horses with low automated platelet counts had platelet aggregation on blood smears and platelet numbers were thus deemed to be acceptable. For the dogs, 30 minutes baseline TEG results fell within the laboratory's internally generated population-based reference intervals (Table 1) except for one specimen with a LY30 of 2.4%; this was considered acceptable as all other variables including LY60 were within normal limits. However, for the Nooitgedacht horses, 30 minutes baseline TEG results were often outside of these reference intervals (Table 1). Only Thoroughbred horses were used to generate these reference intervals and a significant breed difference was suspected. Generation of breed-specific reference intervals for Nooitgedacht horses was not within the scope of this study. As no horses showed any evidence of disease or hemostatic defects, these results were accepted as normal for this breed. Estimates of analytical imprecision derived from results of internal quality control measurements were 7.4% for R, 0% for K, 0.8% for α and 5.2% for MA.

Table 1: Reference intervals used by the Clinical Pathology Laboratory at the Faculty of Veterinary Science, University of Pretoria for kaolin-activated thromboelastographic (TEG) analysis of dogs and horses derived from citrated whole blood samples stored for 30 minutes.

	Dogs	Horses
R (min)	2-7	7-17
K (min)	1-4	2-6
α (°)	49-74	34-59
MA (mm)	49-74	48-61
LY30 (%)	0-2	0-1
LY60 (%)	0-8	0-6

Results for each storage condition (mean or median and range) are shown in Table 2. For some measurements, the sample size was less than ten, due to technical problems with the TEG analyzers. Graphic comparisons of results for dogs and horses are provided in Figures 1 and 2.

Table 2: Mean or *median* and (range) for kaolin-activated thromboelastography (TEG) variables in healthy dogs and horses derived from citrated whole blood samples stored for different lengths of time and at different temperatures (room temperature (RT) or refrigerator temperature (FT)).

	30 min	2 hr RT	2 hr FT	8 hr RT	8 hr FT	22.5 hr RT	22.5 hr FT
				Dogs			
R (min)	4.9 (3.1-6.2)	*4.1* (2.3-4.7)*	5.6 (4.5-6.6)	3.1 (1.7-4.3)*	4.2 (3.2-5.7)	3.6 (2.7-4.8)* **	3.2 (2.6-3.9)*
K (min)	*1.7* (1.1-1.8)	1.5 (1.2-2.1)	*1.8* (1.4-2.4)*	1.5 (1.2-1.9)	2.0 (1.3-3.3)	1.6 (1.1-2.8) **	2.3 (1.3-4.3)*
α (°)	66.8 (62.5-73.2)	66.9 (57.4-71.4)	64.4 (58.5-68.3)	69.9 (65.4-73.6)	64.8 (56.7-70.4)	67.7 (55.2-74.6) **	62.8 (53.3-71.5)
MA (mm)	60.1 (54.3-69.7)	60.1 (50.1-65)	*58.1* (56.3-65.8)	60.0 (51.2-68.8)	54.5 (45-65.8)	59.1 (41.4-68.7) **	53.8 (43-62.2)*
LY30 (%)	*0* (0-2.4)	*1.4* (0-17.7)	*0.1* (0-11.5)	*0.05* (0-11.2)	*0* (0-9.2) **	*0* (0-3.2) **	*0.05* (0-27.5)
LY60 (%)	*0.9* (0-7.7) **	*6.1* (1.3-27)*	*2.8* (0-36.3)*	*1.7* (0-29.3)	*2.5* (0-21.5) **	3.7 (0-10.9) ***	*6.9* (0.3-55.6)*

Horses							
R (min)	10 (7.8-11.8)	8.0 (4.9-10.2)*	8.7 (4.5-11.5)	7.3 (4.0-10.0)*	8.1 (5.0-12.3)	7.3 (4.8-10.6)*	5.7 (4.2-7.6)*
K (min)	2.3 (1.8-2.8)	1.9 (1.3-2.8)*	2.5 (1.9-3.6)	1.9 (1.4-2.4)*	2.2 (2.0-3.0)	1.8 (1.3-2.9)*	2.6 (1.8-3.3)
α (°)	60.1 (54.7-67.5)	63.4 (52.8-70.1)	58.9 (47.5-66.1)	61.2 (40.5-71.2)	*60.7* (50.2-62.9)	62.5 (46.6-71.8)	53.8 (43.7-66.4)*
MA (mm)	62.8 (48.4-76.3)	63.3 (45.0-74.2	62.5 (34.0-81.1)	64.2 (44.1-75.3)	63.8 (47.4-72.9)	63.0 (46.6-75.3)	58.5 (48.1-73.2)
LY30 (%)	0.9 (0.0-2.4)	*0.4* (0.0-7.1)	*0.2* (0.0-2.4)	*0.3* (0.0-3.5)	0.0 (0.0)*	*0.2* (0.0-2.9)	*0.0* (0.0-0.3)*
LY60 (%)	4.7 (1.6-7.7)	5.1 (1.7-12.4)	3.6 (1.0-8.5)*	4.3 (1.0-8.5)	*1.4* (0.0-2.0)*	3.7 (0.4-7.6)*	*0.4* (0.0-2.7)*

* indicates results significantly different to 30 min values (ANOVA, $p < 0.05$)

Unless otherwise stated, n=10

** n=9

*** n=8

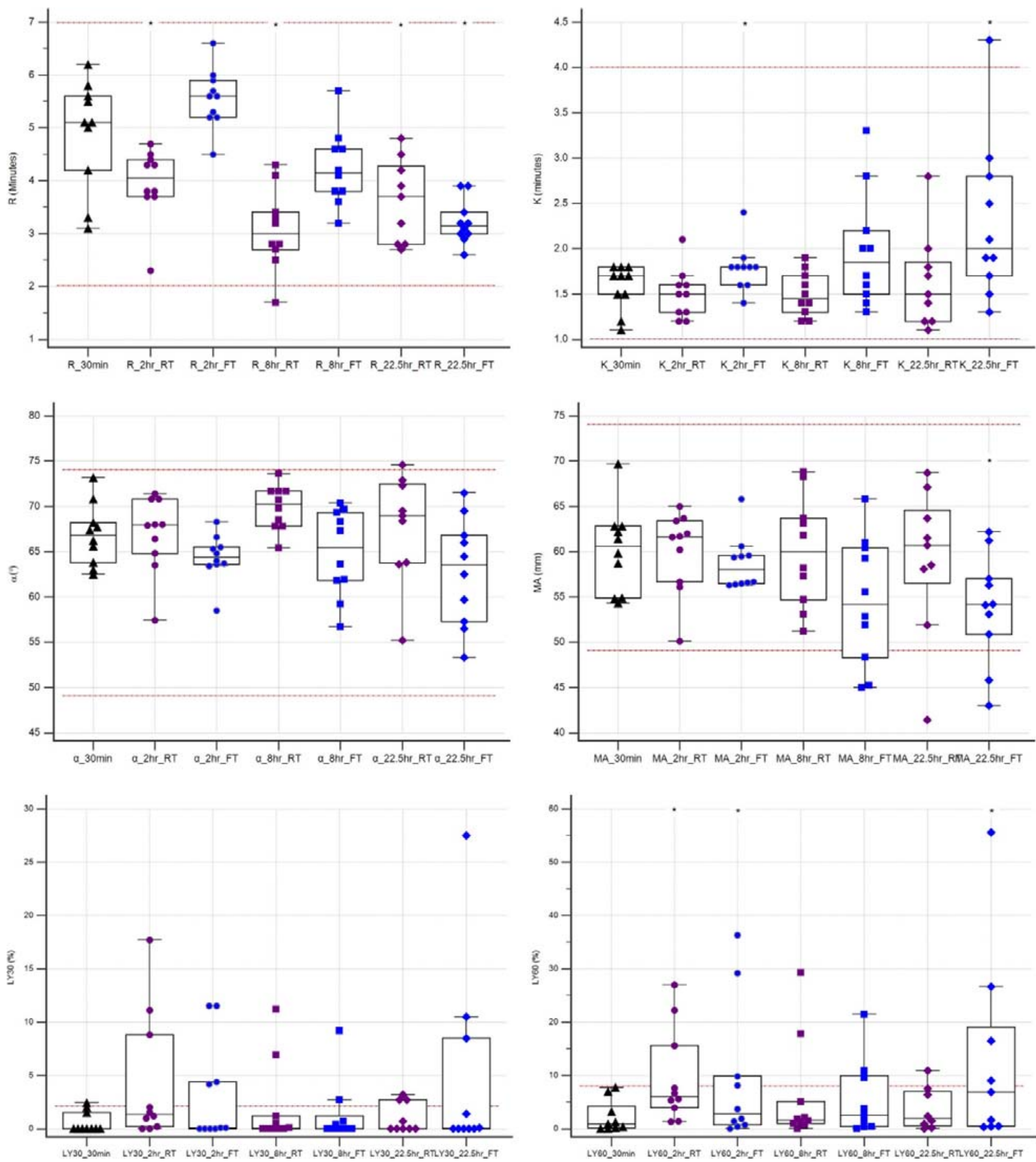

Figure 1: Box-and-whisker plots comparing kaolin-activated thromboelastography (TEG) variables in healthy dogs derived from citrated whole blood samples stored under various conditions. The dotted red lines indicate the reference intervals; * indicates a statistically significant difference ($p < 0.05$) from the 30 minute group. RT room temperature; FT refrigerator temperature

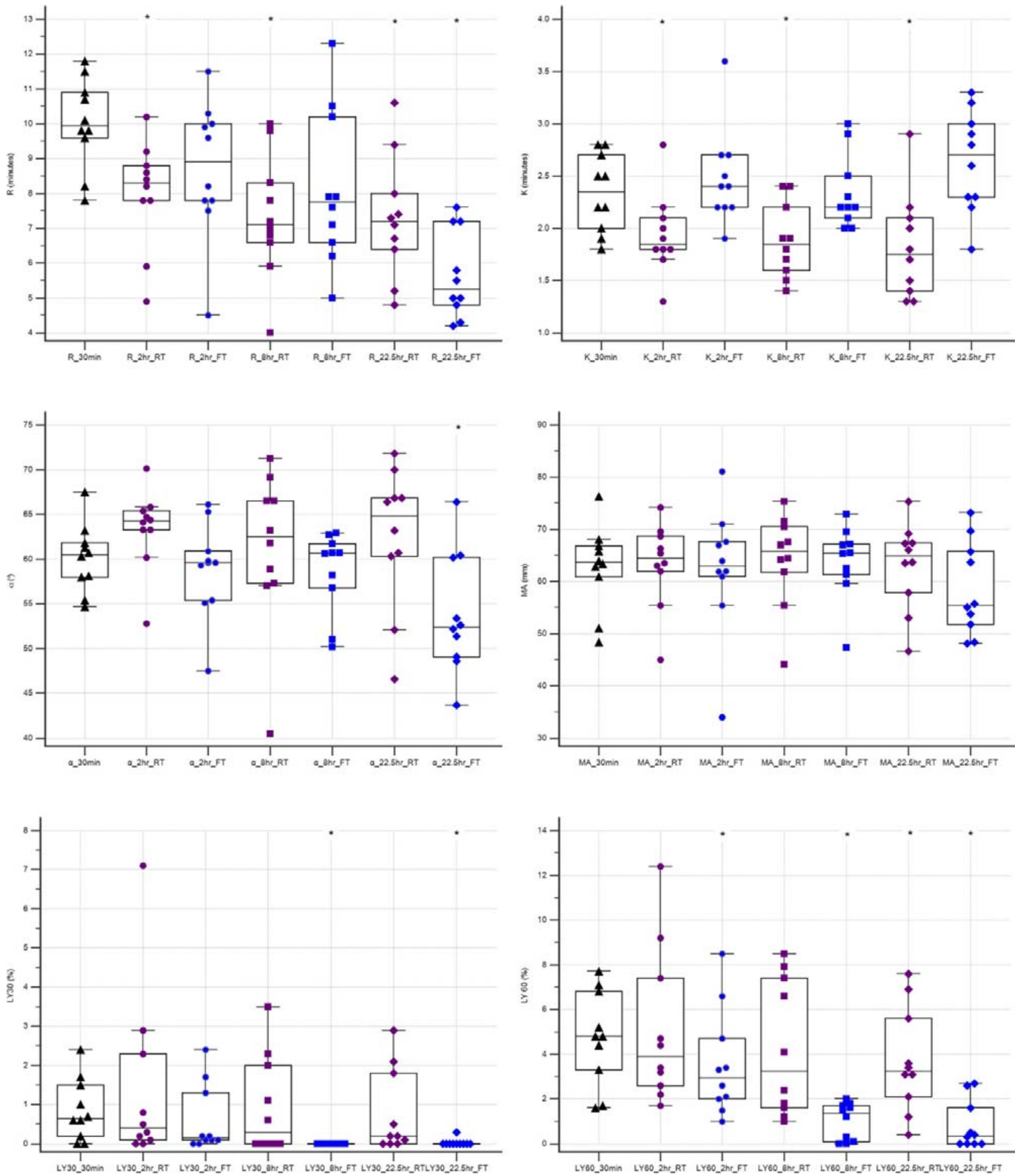

Figure 2: Box-and-whisker plots comparing kaolin-activated thromboelastography (TEG) variables in healthy horses derived from citrated whole blood samples stored under various conditions. * indicates a statistically significant difference ($p < 0.05$) from the 30 minute group. RT room temperature; FT refrigerator temperature

3.1 Dogs

The following statistically significant differences were found: shorter R from 30 minutes to 2 hrs RT, 8 hrs RT, 22.5 hrs RT and 22.5 hrs FT; increased K from 30 minutes to 2 hrs FT and 22.5 hrs FT; decreased MA from 30 minutes to 22.5 hrs FT; increased LY60 from 30 minutes to 2 hrs RT, 2 hrs FT and 22.5 hrs FT. α and LY30 values showed no statistically significant differences when stored up to 22.5 hrs RT and FT. No statistically significant differences were found for R, K, α and MA values at 8 hrs FT.

For dogs, calculated TE_a was R 22.8%, α 20.0% and MA 14.4% (CV_i R 20.9%, α 18.6%, MA 13.2%). The number and percentages of acceptable results, as well as the direction of the bias for unacceptable results are shown in Table 3. The storage condition with the highest percentage of results WTAAEL was 2 hr FT for dogs (90% overall). Unacceptable bias was often found for R and was mostly negative, apart from only 3/10 positive unacceptable bias results for 2 hr FT. Most results for α were WTAAEL. The percentage of unacceptable bias results for MA ranged from 0% (2 hr FT) to 40% (8 hr FT) and was variable in direction but mostly negative.

Total allowable analytical error could not be calculated for the TEG variable K; mean bias for K at different storage conditions was: -4.0% at 2hr RT, 16.4% at 2hr FT, -3.7% at 8hr RT, 30.3% at 8hr FT, 4.7% at 22.5 hr RT and 51.8% at 22.5 hr FT. Bias could not be calculated for LY30 and LY60 as several results for 30 min RT were zero but the magnitude of changes can be seen in Figure 2.

Table 3: The number and percentage of kaolin-activated thromboelastography results from healthy dogs and horses with values within total allowable analytical error limits (WTAAEL) for different storage times and temperatures (room temperature (RT) or refrigerator temperature (FT)). Results from 30 minutes RT specimens were used as the baseline. The direction of the bias (positive, +ve; negative, -ve) for unacceptable results is presented in parentheses.

	2 hr RT	2 hr FT	8 hr RT	8 hr FT	22.5 hr RT	22.5 hr FT
Dogs						
R (min)	4/10 40% (1 +ve, 5 -ve bias)	7/10 70% (3 +ve bias)	1/10 10% (1 +ve, 8 -ve bias)	4/10 40% (2 +ve, 4 -ve bias)	5/9 56% (4 -ve bias)	3/10 30% (7 -ve bias)
α (°)	9/10 90% (1 -ve bias)	10/10 100%	10/10 100%	10/10 100%	9/9 100%	9/10 100% (1 -ve bias)
MA (mm)	8/10 80% (1 +ve, 1 -ve bias)	10/10 100	7/10 70% (1 +ve, 2 -ve bias)	6/10 60% (4 -ve bias)	6/9 67% (1 +ve, 2 -ve bias)	7/10 70% (3 -ve bias)
Total results WTAAEL	21/30 70%	27/30 90%	18/30 60%	20/30 67%	20/27 74%	19/30 63%
Horses						
R (min)	8/10 80% (2 -ve bias)	8/10 80% (2 -ve bias)	5/10 50% (5 -ve bias)	6/10 60% (4 -ve bias)	4/10 40% (6 -ve bias)	0/10 0% (10 -ve bias)

K (min)	5/10 50% (5 -ve bias)	7/10 70% (3 +ve bias)	4/10 50% (6 -ve bias)	8/10 80% (1 +ve, 1 -ve bias)	2/10 20% (8 -ve bias)	7/10 70% (3 +ve bias)
α (°)	3/10 30% (7 +ve bias)	5/10 50% (2 +ve, 3 -ve bias)	3/10 30% (5 +ve, 2 -ve bias)	6/10 60% (1 +ve, 3 -ve bias)	3/10 30% (6 +ve, 1 -ve bias)	3/10 30% (7 -ve bias)
MA (mm)	7/10 70% (2 +ve, 1 -ve bias)	4/10 40% (4 +ve, 2 -ve bias)	4/10 40% (5 +ve, 1 -ve bias)	5/10 50% (4 +ve, 1 -ve bias)	6/10 60% (2 +ve, 2 -ve bias)	2/10 20% (2 +ve, 6 -ve bias)
Total results WTAAEL	23/40 58%	24/40 60%	16/40 40%	25/40 63%	15/40 38%	12/40 30%

3.2 Horses

The following statistically significant differences were found: shorter R from 30 minutes to 2 hrs RT, 8 hrs RT, 22.5 hrs RT and 22.5 hrs FT; decreased K from 30 minutes to 2 hrs RT, 8 hrs RT and 22.5 hrs RT; decreased α from 30 minutes to 22.5 hrs FT; decreased LY30 from 30 minutes to 8 hrs FT and 22.5 hrs FT; decreased LY60 from 30 minutes to 2 hrs FT, 8 hrs FT, 22.5 hrs RT and 22.5 hrs FT. MA values showed no statistically relevant differences when stored up to 22.5 hrs RT and FT. No statistically significant differences were found for R, K, α and MA values at 2 hrs FT and 8 hrs FT.

For horses, calculated TE_a was R 23.4%, K 19.8%, α 6.4% and MA 3.5% (CV_i R 22.3%, K 18.2%, α 5.9%, MA 2.5%). The number and percentages of acceptable results are shown in Table 3. Storage for 2hr FT and 8 hr FT had the highest percentage of results WTAAEL (60% and 63%). For R, the percentage of unacceptable bias results increased with storage time, and these biases were always negative. The percentage of results WTAAEL for K was highest for FT storage; bias was variable in direction. The percentage of unacceptable bias results for α and MA was generally high

for all storage conditions, and the direction of bias was very variable. Bias for LY30 could not be calculated due to the presence of zero results for 30 min RT, and absolute bias for LY60 varied from zero to 475% (see Figure 3).

4 Discussion

In our study, for dogs, the four most commonly utilized TEG variables (R, K, α, MA) showed no statistically significant differences when samples were stored for up to 8 hrs at FT, apart from K which showed a small increase at 2 hrs FT. However, when estimates of bias were compared to TE_a, samples may be stable for a maximum of 2 hrs at FT, and not under any other storage conditions . For horses, R, K, α and MA variables showed no statistically significantly differences when samples were stored for up to 8 hrs at FT. However, when estimates of bias were compared to TE_a, samples did not exhibit stability under any storage conditions.

In our study, with both dogs and horses, there were often contradictory findings regarding statistically significant versus clinically relevant differences (as determined by comparison to TE_a), with the latter approach being much stricter. For the purpose of this study, where the ultimate goal is to facilitate a wider availability of TEG to veterinary general practitioners, consideration of whether storage-induced changes will affect clinical interpretation, is foremost. In our attempt to evaluate clinically relevant changes, we used an approach recently recommended for storage stability studies in human laboratory medicine.[22] We did however encounter some limitations when trying to follow this recommended approach. Firstly, there is no consensus in the veterinary or human literature regarding quality specifications for TEG, and TE_a was generated for the purpose of this study. Although we followed best practice guidelines and used biological variation for our calculations, the TE_a used here may be too strict. For dogs, and for K for horses, estimates of CV_i for kaolin-activated TEG were not available and therefore CV_i was not method-specific. Intra-individual variation has been shown to differ for different activation methods, at least in horses.[27] In addition, CV_a was calculated from results of internal quality control and not from duplicate measurements. Commercial quality control material (QCM) does not have the same matrix as canine or equine blood, and CV_a derived from QCM may not reflect the analytical variation specific to each species or to each condition of storage. Duplicate analysis of samples at each time point would have shown whether there was a change in CV_a with different storage conditions. Thirdly, our sample size was low and our study was most likely underpowered.

Published storage studies for kaolin-activated TEG in veterinary species (horses, cows and Asian elephants) present conflicting results, probably due to the variation in storage times and species differences. There are no published storage studies for kaolin-activated TEG in dogs. Machackova et al. (2018) evaluated kaolin-activated TEG sample stability at RT in seven healthy horses (breed undefined) and found no statistically significant changes in R, K, α or MA at 6 hrs, compared to a 2 hr baseline.[15] Statistically significant decreases in R time were found at 12 and 24 hrs, which is similar to the trend seen in our study for both dogs and horses. The significant decrease in K from our study was not reported by Machackova et al. Three R values were below, two K values were above, and two α values were below that laboratory's reference intervals after 6 hrs of storage. These changes were not noted in our study to the same degree. Sommerey et al. (2014) evaluated specimen stability for kaolin-activated TEG in healthy dairy cows.[14] Samples were stored at RT and measured first at 2 hrs post-collection, then again once or twice from 16 to 100 hrs post-collection. R was once again significantly decreased after the 2 hr measurement but only two R values were slightly below the reference interval. Perrin et al. (2018) recorded significant differences representing a tendency towards hypercoagulability in kaolin-activated TEG samples from 44 Asian elephants after 24 hrs of FT storage (samples were left to come to RT for 30 minutes after 23.5 hours of FT storage), compared to a 60 minute baseline.[13] R, K and MA were significantly decreased and α was significantly increased. Some R and MA values were below, and some α values were above the reference intervals. Similarly, in our study, unacceptable negative biases were found for R and MA in dogs and for R in horses at 22.5 hrs FT. , which may be indicative of species-specific variations in hemostasis.

Published storage studies for kaolin-activated TEG in human medicine also report contradicting findings, reporting either no differences in R or MA [29], or decrease in R, increase in α and no difference in MA [30], or decrease in R and MA and no difference in K, α and LY30 [31], or decrease in K, increase in α and no difference in R, MA and LY30.[29-32] However, clinically relevant differences were not assessed. Two storage studies for tissue factor-activated TEG (one in dogs and the other in horses) report corroborative statistically significant decreases in R and K and increases in α and MA, which is similar to some of the trends seen in our study.[33,34] Clinically relevant differences were also not assessed.

Apart from a decrease in R, which seems consistent across species and studies, it is therefore not possible to identify an expected storage time-related trend in other variables, which makes interpretation of data and formulation of recommendations difficult. The sample size in most studies, including ours, is small, and outliers representing random

error may skew results. High inter-operator variability has been reported for kaolin activated TEG in veterinary medicine, even though the procedure is standardized.[35] High CVs have also been reported within and between veterinary laboratories for kaolin-activated TEG run on canine platelet-rich plasma.[36] These sources of variation may be a reason for the differing results found between studies and species.

As previously mentioned, R represents time between clot initiation and production of initial insoluble fibrin strands ("clot") and is proportional to coagulation protein concentration.[7] In our study, biases for R were often unacceptable and were usually negative (i.e. R was longer). Coagulation time tests, prothrombin time (PT) and activated partial thromboplastin time (aPTT), have been reported to positively correlate with R values.[37] PT and aPTT have also been reported to be respectively prolonged and shortened over 24 hours of canine citrated whole blood storage at RT, but remain unchanged in blood stored at FT.[38,39] Some activation of coagulation via the contact pathway is expected to occur during sample collection. A trend towards hypercoagulability with increased activity of the procoagulant factors XII and XIII has been described in equine and canine blood stored for up to 30 minutes and activated by recalcification only.[40,41] This effect was however not present when samples were activated with the contact activators kaolin or celite. The effect of *ex vivo* contact activation on procoagulant activity with longer storage times has not been evaluated, but could potentially result in a longer R, although the use of a strong activator like kaolin may negate this effect.

In terms of room or refrigerator storage temperature, the latter appeared to have an advantage. The effects of cold temperatures on platelet structure, effectivity and longevity have been thoroughly explored and subsequently established. Firstly, soon after refrigeration, platelet shape starts changing from discoid to spherical, indicating activation.[42-44] Secondly, platelet metabolic activity drops due to a decrease in anaerobic glycolysis.[45] Thirdly, aggregation and clot strength have been shown to be enhanced by cold temperatures in multiple *in vitro* and *in vivo* studies [46-50], although, this conclusion has been contradicted by another *in vivo* study that reported equal effectivity of cold and room temperature on platelets for up to 24 hrs.[50] If 24 hour cold temperature storage does indeed enhance platelet function, it would be expected to influence TEG variables which correlate with platelet count and function: K and MA.[51] However, the expected increased MA and shorter K [51] was not noted in the refrigerated samples of our study. Studies directly comparing cold and room temperature storage of human blood reported no changes in R, K, α

or MA between samples stored for 24 hrs under these two conditions.[45,52] These two studies did not compare results to reference intervals or baseline 30 minutes values. Studies evaluating the effects of long-term cold temperature storage in citrate dextrose anticoagulated or citrate phosphate dextrose anticoagulated human blood reported R, K, α and MA variables within laboratory reference intervals in samples stored for 3 days[53] and 10 days[54]. These two studies also did not compare results to baseline 30 minutes values.

The percentage of results WTAAEL was generally much higher for dogs than for horses. This was particularly evident for α (90-100% WTAAEL in dogs versus 20-60% WTAAEL in horses) and MA (60-100% WTAAEL in dogs versus 20-70% WTAAEL in horses) and is in part due to the much stricter TE_a used for these variables for horses (TE_a α 6.4%, MA 3.5%) compared to dogs (TE_a α 20.0%, MA 14.4%). The difference in TE_a arises because dogs have a higher CV_i for these variables than horses do, based on results of biological variation studies.

In other studies using TE_a and calculating the percentage of results WTAAEL, maximum stability time (T_{max}) was defined as the point at which at least 95% of results were WTAAEL.[17,19] The justification for using the 95% criterion was that interpretation of results generated at T_{max} would be similar to the principle of interpreting results compared to a reference interval, which is established for 95% of a reference population - it is considered to be acceptable that one in 20 healthy individuals will have a result outside of a reference interval. Our study lacks sufficient numbers to use the 95% criterion as we only evaluated 10 subjects for each species. In dogs, 90% of results were WTAAEL at 2hr FT and this storage condition may be acceptable, although higher numbers of specimens are needed to confirm this. In horses, no storage conditions appeared to be acceptable when comparing results to TE_a.

Although LY30 and LY60 values were not assessed against TE_a, it was noted that LY30 and LY60 results were increased above the upper reference limit in several dogs across all storage conditions. Although sufficient evidence for the clinical utility of these lysis variables in dogs is lacking [55], they have been used in dogs to identify hyperfibrinolysis (with or without concurrent hyper- or hypocoagulability) which may accompany trauma [56-58], liver disease [59,60], systemic inflammatory response syndrome (SIRS) [61] or disseminated intravascular coagulopathy (DIC) [62]; or used to monitor fibrinolytic treatment. For previous storage studies, LY30 was either not mentioned [14,29,30,33] or LY30 values were not compared [13,15,34] or LY30 values were compared but showed no statistically significant difference.[31,32] Based on the

results of our study, should a clinician specifically want LY30 and LY60 values for dogs, citrated samples should not be stored for longer than 30 min at RT before kaolin-activated TEG analysis.

Limitations of this study include the small sample size, the lack of some TEG data for some subjects and that samples were not analyzed in duplicate, all of which diminish the power of the statistical analysis. In addition, although animals were apparently healthy, full diagnostic and hemostatic testing was not performed to confirm this. In terms of the horses, population-based reference intervals for Nooitgedacht horses were lacking and the CVi used here from a previous publication may not be applicable to our Nooitgedacht population. The longest storage time for samples from two horses was 24 hrs, versus 22.5 hrs for the other eight horses. Finally, the results presented here apply to TEG performed on healthy subjects and cannot necessarily be extrapolated to diseased animals, particularly those with hemostatic derangements.

5 Conclusion

In healthy dogs, our findings suggest that R, K, α and MA variables could be assessed using kaolin-activated TEG in citrated whole blood samples stored for up to 2 hours at refrigerator temperature, although further evaluation with more samples is needed to confirm this.

In healthy horses, our findings suggest that storage for more than 30 minutes at room or refrigerator temperature results in unacceptably large changes in TEG variables. Breed-specific TEG reference intervals and biological variation should be investigated in Nooitgedacht horses (and possibly other horse breeds as well).

The purpose of this study was to define the effects of storage time and temperature on TEG in healthy dogs and horses. Many of the changes seen in this study cannot easily be explained and contradict results from other storage studies. This variability infers an unpredictability when extending TEG sample storage past the currently recommended 30 minutes.

Follow-up storage studies should be performed using larger populations of healthy dogs and horses, as well as animals suffering from hemostatic defects. Incorporating other hemostasis assays like functional fibrinogen concentration and

platelet aggregometry could assist in the interpretation of findings in future studies. In addition, consensus on total allowable analytical error specifications for veterinary species is needed.

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