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Evaluation of kaolin-activated thromboelastography and sample stability in healthy horses

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ABSTRACT: Thromboelastography is an accurate alternative to routine coagulation testing for the monitoring of haemostasis. However, its use in equine medicine is limited not only by the lack of reference interval values for kaolin-activated citrated samples, but also by the limited accessibility of the test for field practitioners within the 2-hour storage time recommended by the manufacturer. To address this issue, we here evaluated kaolin-activated thromboelastography using a TEG[®] 5000 Thrombelastograph[®] Hemostasis System in 36 healthy horses, and sample stability was evaluated at four timepoints post collection in seven horses. Reference values were established as follows: reaction-time 5.0–16.0 min, K-time (period in which the clot strength reaches 20 mm of amplitude) 1.1–5.2 min, α -angle (speed of fibrin cross-linking) 36.5–79.0°, maximal amplitude 44.5–69.7 mm, fibrinolysis 30 minutes after maximal amplitude was reached 0.0–2.8%. During storage, the trends in the changes of values were similar for most parameters, and values remained mostly within the reference intervals. Thromboelastography is thus useful in defining thrombohaemorrhagic complications in horses but can be sensitive to preanalytical factors and storage.

Keywords: haemostasis; TEG; physiological values; coagulation; stability

Thromboelastography (TEG) is a viscoelastic method commonly used in humans for blood coagulation and fibrinolysis testing (Thakur and Ahmed 2012). However, the method is not as common in veterinary medicine as in human medicine even though the test is not species-dependent. In recent years, the number of studies on equine thromboelastography has been slowly rising (e.g., Epstein et al. 2009; Mendez-Angulo et al. 2010; Epstein et al. 2011; Hyldahl-Laursen et al. 2013; Scruggs et al. 2016), but the physiological values required for the clinical use of this method are still unknown. Further testing, including comparison of thromboelastography and standard coagulation testing, is required.

In contrast to routine coagulation testing, which provides information on isolated parts of the coagulation cascade, thromboelastography measures

both soluble and cellular components and thus provides a global overview. Because of its complexity, it can be useful not only in monitoring coagulation, but can also provide information that can help in the differential diagnosis of blood-linked diseases (Mallett and Cox 1992; Kol 2012). Several values are obtained from the TEG measurement: R-time (reaction time), the time period from the start of testing to the time when fibrin is first formed, which correlates with the activity of soluble factors; K-time and α -angle, which correlate with platelets, fibrinogen and soluble factors: K-time represents the period in which the clot strength reaches 20 mm of amplitude and α -angle represents the speed of fibrin cross-linking. Maximal amplitude (MA) is linked with fibrinogen and platelets and correlates with clot strength and LY30 describes fibrinolysis 30 minutes after MA was reached (Zuckerman et

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al. 1981; Thakur and Ahmed 2012). Other values are obtained from TEG analysis. Those values are calculated from the aforementioned values and serve mostly as an estimate of the final value. For this reason, those were not evaluated in the study. According to Epstein et al. (2009), R-time correlates with prothrombin time in healthy horses. Kol (2012) reported that TEG is suitable for use in veterinary medicine but that it is important to minimise differences in preanalytical factors such as storage time and blood collection. Besides these factors, thromboelastography is also influenced by many clinical conditions, for example, packed cell volume, haemoglobin concentration and corticosteroid usage (Kol and Borjesson 2010). However, the graphical outcome (TEG tracing) is quite easy to read and can be used in diagnostics too.

The crucial aspect of thromboelastography appears to be the stability of samples. Since there are only a few studies on thromboelastography in horses and kaolin-activated TEG in particular is not well described, sample stability is usually taken from human studies or the manufacturer's guide. The aim of this study was thus to evaluate the use of kaolin-activated thromboelastography in horses and to establish physiological values for this particular method. Secondly, the stability of the samples for the thromboelastography examination was evaluated.

Our hypothesis was that the stability of the samples would be shorter than that in dogs or cows, which was described to reach up to 24 hours at laboratory temperature (Sommerer et al. 2013), since the number of equine platelets in citrated samples tends to decrease rapidly (Prins et al. 2009).

MATERIAL AND METHODS

Blood was collected from 36 clinically healthy horses sampled for a preventive examination for equine infectious anaemia. The test group consisted of 18 mares and 18 geldings, with an average age of 16 years (min. 3, max. 25 years). Eight of the horses were Arabian horses, seven were Thoroughbreds, two were Haflingers and the rest ($n = 19$) were Czech warmbloods.

Haematological examination, standard coagulation tests and thromboelastography were performed. Blood was collected from the jugular vein into EDTA tubes using 18G needles (Dispolab,

s.r.o., Czech Republic). Platelet counts (PLT) were then measured and examinations were performed within 30 minutes on a Celltac alpha impedance haematological analyzer (Nihon Kohden, Japan).

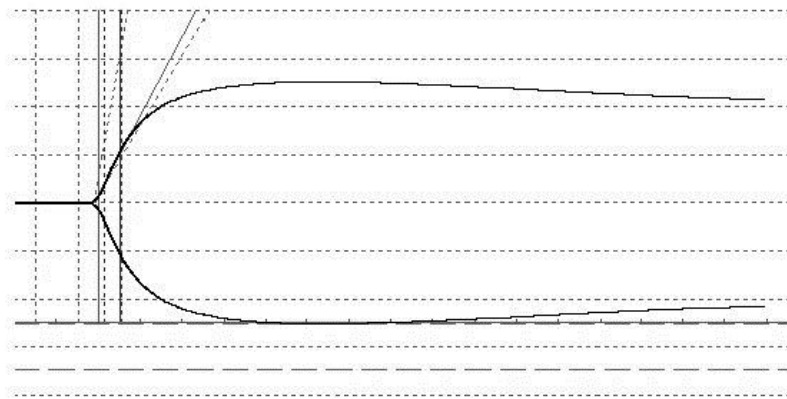
Routine coagulation tests were performed using citrated plasma. Blood was collected into sodium citrate tubes at a 1 : 10 dilution (Dispolab, s.r.o., Czech Republic) and centrifuged for 15 minutes at 1000 g. Tests were performed within one hour after sampling using a two-channel analyser (Coatron M2, Tecco, Germany). Prothrombin time (Thromboplastin-S, Dialab, s.r.o., Czech Republic), activated partial thromboplastin time (APTT-S, Dialab, s.r.o., Czech Republic; 0.025 M CaCl_2 , Dr. Kulich Pharma, s.r.o., Czech Republic) and fibrinogen levels (Bovinni trombin 100 NIH IU/ml, Dialab, s.r.o., Czech Republic) were measured. Citrated plasma was also used for D-dimer measurement, which was performed using NycoCard (Axis-Shield PoC, Norway – previously Nycomed, Norway) (Stokol et al. 2005).

Thromboelastography was performed using a TEG[®] 5000 Thromboelastograph[®] Hemostasis System (TEG System, Haemonetics, USA). As for quality control procedures, the E-test (Electronic testing) was performed each time after turning on the system as recommended by the manufacturer (Haemonetics, USA). A mechanical check and calibration were carried out by the owner of the system prior to our study. One millilitre of citrated blood was poured into a kaolin-coated vial. After mixing, 340 μl of this blood were added to a preheated (37 °C) cup filled with 20 μl of 0.2 M CaCl_2 in the TEG analyser. All samples were measured by the same operator within 1–1.5 hours after collection. R-time, K-time, α -angle and MA were analysed.

Reference intervals were determined using MedCalc software. A 95% double-sided reference interval was used, and outliers were detected using the Tukey method. The robust method was used as recommended for small sample sizes (MedCalc software bvba, Belgium).

To determine sample stability, blood was collected from seven horses at the Brno equine clinic (three mares, four geldings). The blood was collected into citrate tubes and thromboelastography was performed within two hours after collection and again after six, 12 and 24 hours. Blood (2 ml) was collected into five test tubes and stored at laboratory temperature (21 °C) between runs. One millilitre of blood from each test tube was used for thromboe-

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R	K	Angle	MA	PMA	G	EPL
min	min	deg	mm		d/sc	%
9.8	2.6	58.2	50.1	0.0	5.0	1.2

Figure 1. An example TEG tracing from a citrated kaolin-activated whole blood sample from one of the healthy horses

Angle = α -angle (speed of fibrin cross-linking), EPL = estimated percent lysis, calculated value (estimates LY30 in advance, changes to LY30 when reached), G = shear modulus strength, calculated value (log-derivation of the MA, represents clot strength in Kdynes/cm²), K = K-time (period in which the clot strength reaches 20 mm of amplitude), MA = maximal amplitude, PMA = projected MA (estimates if MA will reach the minimal normal value), R = reaction time (time from start of the test to first detectable change)

lastography, and the remainder was centrifuged and used for conventional coagulation testing. All tests were performed by the same operator on the same machine. The results were compared statistically using the Wilcoxon matched pairs test in MedCalc (MedCalc software bvba, Belgium).

RESULTS

Standard coagulation

Standard coagulation data as well as the number of platelets were within the reference intervals given by the laboratory in all the tested animals.

Thromboelastography

The TEG results of horses in this study yielded the following reference interval values: R-time 5.0–

16.0 min, K-time 1.1–5.2 min, α -angle 36.5–79.0°, MA 44.5–69.7 mm, LY30 0–2.8%.

Figure 1 presents an example of a TEG tracing from one of the healthy horses.

Sample stability

Seven horses were used for the stability evaluation. In the evaluation of standard coagulation tests, there were no significant differences between any of the samples. The results after two, six and 12 hours are shown in Table 1.

The evaluation of TEG revealed significant differences only in one of the parameters: R-time at 2 h vs 12 h and vs 24 h (Table 2). The narrowest range appeared to be in MA. The TEG tracing over time is depicted in Figures 2–4 (horse No. 3). Although there were no significant differences between results other than those mentioned above, the changes in the curve

Table 1. Standard coagulation test results in citrated plasma samples from seven healthy horses stored at 21 °C and analysed two, six and 12 hours after collection (reported as median)

Time (h)	PT (s)	aPTT (s)	FBG (g/l)	DD (mg/l)
2	14.5	64.9	1.05	0
6	15.1	68.8	1.14	0
12	15.2	66.8	1.05	0.05

aPTT = activated partial thromboplastin time, DD = D-dimers, FBG = fibrinogen, PT = prothrombin time

Table 2. Thromboelastography parameter values in citrated kaolin-activated whole blood samples from seven healthy horses stored at 21 °C and analysed after two, six, 12 and 24 hours (reported as median)

Time (h)	R-time (min)	K-time (min)	α -angle (°)	MA (mm)
2	9.3	2.7	52.9	56.0
6	10.6	3.2	49.8	52.7
12	6.5*	2.4	57.6	55.6
24	7.5*	2.4	58.5	58.3

MA = maximal amplitude

*Significant difference $P < 0.05$

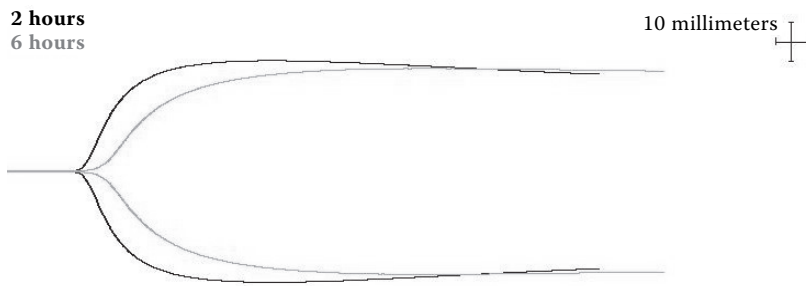


Figure 2. Graphical comparison of changes in thromboelastography values (horse No. 3) during storage of citrated whole blood using kaolin-activated thromboelastography. The result measured after two hours is represented by the black line; the result measured after six hours is represented by the grey line

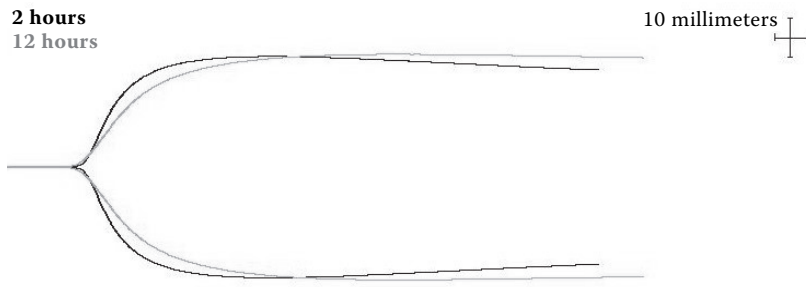


Figure 3. Graphical comparison of changes in thromboelastography values of horse No. 3 during storage of citrated whole blood using kaolin-activated thromboelastography. The result measured after two hours is represented by the black line; the result measured after 12 hours is represented by the grey line

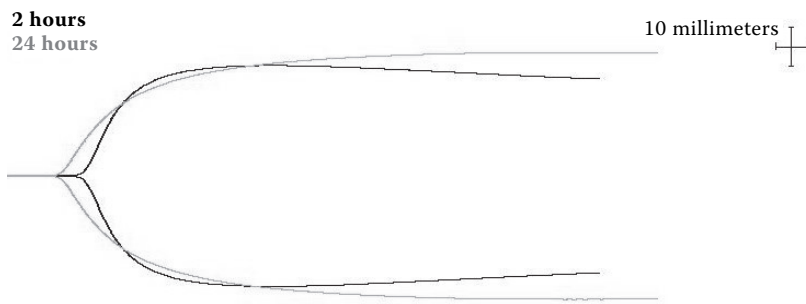


Figure 4. Graphical comparison of changes in thromboelastography values of horse No. 3 during storage of citrated whole blood using kaolin-activated thromboelastography. The result measured after two hours is represented by the black line; the result measured after six hours is represented by the grey line

are visible on the TEG tracing. We did not compare LY30 values because the values were too low.

Figures 5–8 show the graphical results of TEG parameter values for each of the seven samples ana-

lysed between two and 24 hours after collection. Some values for R-time, K-time and alpha angle tended to drift outside of the reference intervals, most often after six and 12 hours of storage.

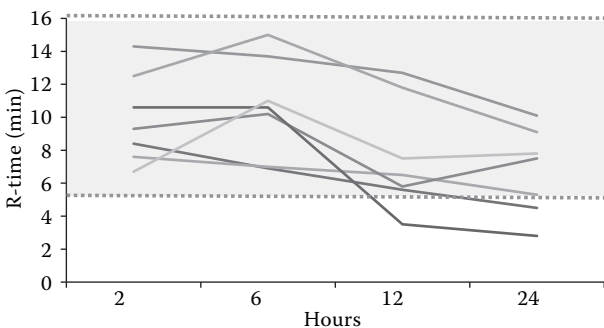


Figure 5. Changes in R-time values of seven healthy horses during storage of citrated whole blood using kaolin-activated thromboelastography. The established reference interval for the parameter is represented by the grey zone

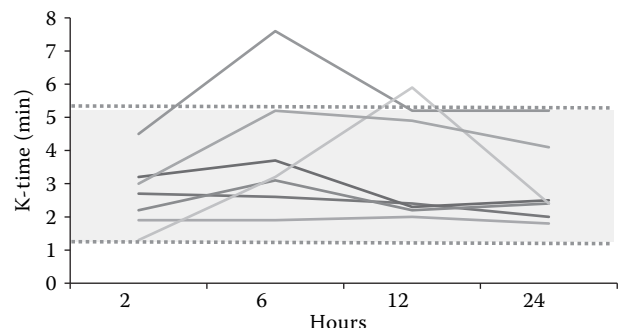


Figure 6. Changes in K-time values of seven healthy horses during storage of citrated whole blood using kaolin-activated thromboelastography. The established reference interval for the parameter is represented by the grey zone

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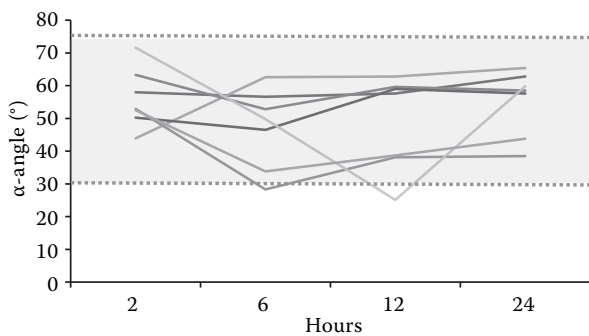


Figure 7. Changes in α -angle values of seven healthy horses during storage of citrated whole blood using kaolin-activated thromboelastography. The established reference interval for the parameter is represented by the grey zone

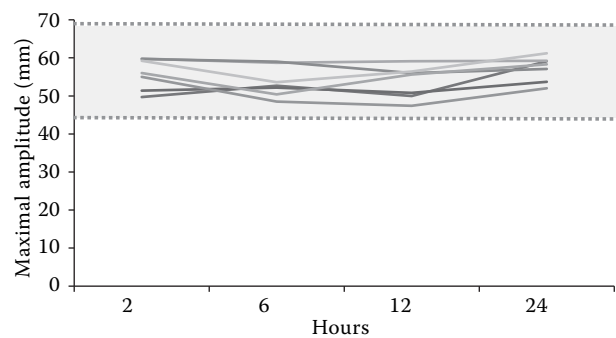


Figure 8. Changes in maximal amplitude values of seven healthy horses during storage of citrated whole blood using kaolin-activated thromboelastography. The established reference interval for the parameter is represented by the grey zone

DISCUSSION

Coagulation disorders in horses have been described in several studies (Monreal et al. 2000; Dunkel et al. 2010). Since they can occur as a severe complication of any critical illness in horses, it is important to examine not only the haematological and biochemical status of critically ill patients but also the coagulation status, as disseminated intravascular coagulation or thrombosis can complicate convalescence. Since horses with acute illnesses that require immediate coagulation testing (e.g., colic) can be admitted in the evening or during the night, inconsistencies among receiving clinicians and operators of blood sample analysers in clinics mean that methods that are least influenced by preanalytical factors should be selected.

A few studies have described thromboelastography in horses and a few used kaolin-activated TEG (Epstein et al. 2009; Leclere et al. 2009). To our knowledge, there is only one study evaluating kaolin-activated TEG in healthy horses. Hyldahl-Laursen et al. (2013) compared native TEG, tissue-factor-activated TEG and kaolin-activated TEG in 20 healthy horses. The results were as follows (median values (range min.–max.)): R 11.52 (7.25–16.65) min, K 3.45 (2.30–5.55) min, α 47.38 (34.1–58.75)°, MA 55.75 (48.55–60.80) mm, LY30 0 (0–0.1) mm. In our study, the results were similar although the ranges were wider for all measured parameters. The differences between our study and that of Hyldahl-Laursen et al. (2013) may be due to different preanalytical factors, but the most significant parameter – clot strength – was similar in both. Moreover, sample size in our study was

36 horses (vs 20 horses in the aforementioned study). We did not run samples in duplicate as in the aforementioned study, because the multiple samples from private stables enabled us to mainly include horses from private stables. On the other hand, that could also lead to statistical differences between the two studies.

According to Kol (2012), it is important to minimise the influence of preanalytical factors when using TEG as a diagnostic tool. Hyldahl-Laursen et al. (2013) reported that kaolin-activated TEG showed the smallest intra-individual variations (compared to tissue-factor-activated and non-activated TEG). As for the stability, White et al. (2009) reported that in humans all variables except LY30 differed significantly between samples measured after 15 and 120 minutes. We did not evaluate this parameter because it was very low or zero in the tested horses (0–2.5%, median 0.3%). On the other hand, Sommerey et al. (2013) performed a similar test on cows and dogs and according to their results the TEG parameters appeared to be stable for up to 36 hours after sampling. The results of our study were similar, with the only significant difference being in R-time, which was significantly shorter after storage. This can indicate a simulation of the hypercoagulative state since R-time is the time from the start of the test to the appearance of the first fibrin strands (Kaufmann et al. 1997; Kol 2012; Thakur and Ahmed 2012). Similar results were observed in horses with ischaemic or inflammatory gastrointestinal disease (subtle changes in R-time with non-consistent changes in other parameters, mostly K-time, relative to healthy horses), so storage conditions may result in samples from oth-

erwise healthy animals exhibiting signs of one of these states (Dunkel et al. 2010). Prins et al. (2009) stated that the number of platelets in equine blood decreases rapidly when stored in sodium citrate, as used in our study, which could render a hypo-coagulable result (Meng et al. 2003; Wolberg et al. 2004). This was not found in this study. White et al. (2009) reported significant differences in R-time (shortened) and α -angle (decreased) between samples taken at 15 and 120 minutes (together with significantly decreased MA). Hypercoagulation due to storage was seen in other studies, even though it was not statistically confirmed (Vig et al. 2001; Zambruni et al. 2004). Bowbrick et al. (2000) reported statistically significant hypercoagulation of the stored samples based on K-time and α -angle. Zambruni et al. (2004) suggested the repeated use of a single sample as the probable activator of the coagulation cascade, as contact with any surface can serve as the activator. That was not the case in our study, since we used different vials for every TEG run. Much more probable is the explanation provided by Camenzind et al. (2000), who detected increased amounts of thrombin-antithrombin complex (TAT) and prothrombin fragment 1 + 2 in citrated plasma after storage, which means that citrate does not completely stop thrombin generation in samples. The increase was detected already after 30 minutes and continued during the 8-hour storage period. Increased activation of soluble factors was observed in this study as well as a significant decrease in R-time values during storage. Other parameters reflecting not only coagulation factors but also platelet activation (K-time, α -angle) were not significantly changed, suggesting a decreased ability of platelet activation or decreased number of platelets (due to the citrated storage mentioned above). A possibility for future research would be to try different storage conditions to prevent this.

Despite the absence of significant differences between most of the parameters, some of them tended to slip out of the reference intervals. Such changes could be more significant in the non-activated TEG method since the kaolin-activated TEG was reported to be less influenced by preanalytical factors (Hyldahl-Laursen et al. 2013). However, the trends in the results were similar in all stored samples; it might also be possible to establish reference ranges depending on the time elapsed between the collection and measurement of samples. This would be useful for clinicians who do not have access to

the TEG system but still want to use the test and have to send the samples to a reference laboratory.

In conclusion, thromboelastography is a sensitive method that tests all components of the coagulation cascade and therefore can be helpful in establishing whether or not there is any coagulation disorder complicating primary disease. Kaolin-activated thromboelastography was shown to be a quick method for monitoring the coagulation system, and physiological values were established using 36 clinically healthy horses. The stability of samples is rather short-lived, and the sample should be measured within two hours after collection; if this is not possible, procoagulative changes, mainly in R-time, should be taken into consideration.

The limitation of this study is obvious: preanalytical factors such as transportation could have influenced the reference intervals. On the other hand, the conditions were close to those of the practicing equine veterinarian. For future studies, we would recommend running samples in duplicate to eliminate statistical errors.

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