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Whole blood platelet impedance aggregometry with the ROTEM platelet device: comparison of 2 anticoagulants and storage times for the establishment of canine reference intervals

Heimgartner, Lara Mercedes ; Stirn, Martina ; Kutter, Annette P N ; Sigrist, Nadja E ; Jud Schefer, Rahel

Abstract: The ROTEM platelet device, a point-of-care whole blood platelet impedance aggregometer, is an add-on to the rotational thromboelastometry ROTEM delta device. The latter has been validated in dogs. We examined whether canine whole blood is suited for analysis with the ROTEM platelet device using adenosine-5'-diphosphate (ADP) and arachidonic acid (ARA) as agonists for platelet activation, and if there are significant differences between sample storage times and anticoagulants used. Subsequently, we determined canine reference intervals (RIs) for the ROTEM platelet device for ADP and ARA. In a pilot study, we examined whole blood from 7 dogs after 15-min and 60-min storage of lithium-heparinized samples and 40-min and 80-min storage of hirudinized samples. Statistical analysis showed no significant differences between ROTEM platelet device results for both ADP and ARA in lithium-heparin and hirudin anticoagulated canine whole blood. Lithium-heparinized blood samples analyzed after 15-min storage had the lowest coefficient of variation. RIs were determined for heparinized whole blood samples from 49 dogs after 15 min of storage.

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1 Whole blood platelet impedance aggregometry with ROTEM® platelet: **Determination**
2 **of optimal sample storage time and anticoagulant for the establishment of canine**
3 **reference intervals**Comparison of 2 different anticoagulants and storage times for the
4 establishment of canine reference intervals

5
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20
21 Running head: Canine reference intervals for ROTEM® platelet

23 **Abstract**

24 The point of care whole blood platelet impedance aggregometer ROTEM® platelet is ~~an add-~~
25 ~~on device-extension~~ to the rotational thromboelastometry device ROTEM® delta. ~~The latter ,~~
26 ~~which~~ has been validated in dogs. We examined whether canine whole blood is suited for
27 analysis with ROTEM® platelet using adenosine 5' diphosphate (ADP) and arachidonic acid
28 (ARA) as agonists for platelet activation and if there are significant differences between
29 sample storage times and anticoagulants used. Subsequently, canine reference intervals for
30 ROTEM® platelet were determined for the beforementioned agonists. In the pilot study,
31 whole blood of 7 dogs was examined after 15 min and 60 min sample storage time for
32 lithium-heparinized samples and 40 min and 80 min for hirudinized samples, respectively.
33 ROTEM® platelet yielded results for both ADP and ARA in lithium-heparin and hirudin
34 anticoagulated canine whole blood at various sample storage times. Statistical analysis
35 showed no significant differences between groups. Lithium-heparinized blood samples
36 analyzed after 15 min sample storage time showed the lowest coefficient of variation.
37 Reference intervals were determined from heparinized whole blood samples of 49 dogs after
38 15 min of sample storage time. ~~Reference intervals of healthy dogs using heparinized whole~~
39 ~~blood are comparable to human ROTEM® platelet reference intervals.~~

40

41 **Key words:** dog, heparin, hirudin, primary hemostasis, thrombocytes

1 Introduction

Platelets play an important role in primary hemostasis. They are activated through exposed subendothelial surfaces or by binding of specific agonists like arachidonic acid (ARA), adenosine 5'-diphosphate (ADP), thrombin or thromboxane A2 (TXA2). Platelet activation leads to platelet adhesion, aggregation and release of granule contents that enable further hemostatic processes.^{8,19,46}

While thrombocytopenia is a common finding in various diseases in dogs, thrombocytopathia is less frequently identified.¹⁹ Thrombocytopathia is classified as primary (congenital) and secondary (acquired).¹³ Primary thrombocytopathia is seen with ~~von Willebrand's factor disease~~, Glanzmann thrombasthenia or Bernard-Soulier syndrome and is rare in dogs.¹³ There are various conditions leading to secondary thrombocytopathia in dogs, such as acute hemorrhage, septic peritonitis, uremia, hepatopathy, monoclonal gammopathy or treatment with non-steroidal anti-inflammatory drugs (NSAIDs).^{5,16,25,26,29,41,49-51} The opposite - an amplified activation of platelets, and thus an increased risk of thromboembolic disease - has been described in dogs with immune mediated hemolytic anemia, sepsis, heartworm disease, lymphosarcoma, parvoviral enteritis, and in critically ill dogs.^{4,27,30,34,39,45}

As the importance of thromboembolic disease is gaining more recognition in small animal medicine, antiplatelet drugs such as acetylsalicylic acid (inhibition of ARA-dependent platelet activation) and clopidogrel (inhibition of ADP-dependent platelet activation) are increasingly used.^{18,46} It is important to monitor the response to treatment, as for both ARA- and ADP-inhibitors individual differences in response to treatment have been described in dogs.^{5,11,41} Assessment of platelet function is furthermore desirable before undergoing elective surgical procedures or after experiencing trauma to assess the risk of excessive bleeding in patients under anti-platelet drug therapy.³⁸

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66 There are various methods to assess platelet function, such as flow cytometry, adhesion- or
67 aggregation assays.⁸ Light transmission platelet aggregometry serves as the gold standard and
68 was first described ~~by Born~~ in 1962.³ This method has many disadvantages like the need to
69 prepare platelet rich plasma ~~and the~~ requirement of large sample volumes, ~~and non-~~
70 ~~standardized incubation times.~~^{7,19}
71 Platelet impedance aggregometry using whole blood was first described in 1980.⁶ In 2006, a
72 point-of-care (POC) device using whole blood platelet impedance aggregometry (Multiplate®
73 analyzer, Roche Diagnostics ~~Germany GmbH~~) was introduced in human medicine and has
74 since been evaluated in different species such as dogs, cats, sheep, pigs and rats.^{1,10,15,17,18,28,48}
75 The utilization of whole blood for platelet function testing enables the interactions of platelets
76 with other blood cells in a physiological environment, prevents the loss of platelet
77 subpopulations during preparation of platelet rich plasma and requires less ~~training for trained~~
78 ~~personnel to be able to perform the analyses.~~^{10,12,15,28} ~~The w~~Whole blood platelet impedance
79 aggregometry ~~method~~ is based on a test cell containing two electrodes on which platelets
80 adhere and aggregate after activation with specific agonists. Platelet aggregation leads to a
81 change in impedance between the two electrodes. The increase in impedance is measured over
82 time and is used to quantify the degree of platelet activation.⁶
83 Recently, the new POC whole blood platelet impedance aggregometer ROTEM® platelet
84 (Tem International ~~GmbH~~) was released as an ~~extension to~~ add-on device to the ~~widely-used~~
85 ~~rotational thromboelastometry device~~ ROTEM® delta device (Tem International ~~GmbH~~). It
86 uses a method similar to the Multiplate analyzer, but while the Multiplate analyzer features
87 two pairs of electrodes in one test cell as an internal control, the ROTEM platelet only has one
88 pair of electrodes in each test cell. The ROTEM® platelet needs only a small sample of whole
89 blood and is user friendly due to an automated pipetting system. ROTEM® platelet allows
90 ARA (ara-tem (Tem Innovation ~~GmbH~~)), ADP (adp-tem (Tem Innovation ~~GmbH~~)) and

thrombin receptor activating peptide (TRAP) (trap-tem (Tem Innovation-GmbH)) dependent platelet activation. Based on manufacturer instructions, fresh whole blood used for analysis is anticoagulated with either citrate, hirudin or heparin (Calatzis A, Görlinger K, Spannagl M, Vorweg M. ROTEM Analysis Targeted Treatment of Acute Haemostatic Disorders. <https://www.ttuhsc.edu › internal › documents › ttim-manual>). While the Multiplate® analyzer using ADP, ARA and collagen as agonists for platelet activation has been validated in dogs, to our knowledge no studies have been conducted investigating the optimal sample storage time, anticoagulant and agonist for the use of ROTEM® platelet in dogs.^{10,15,28} Neither have corresponding reference intervals (RI) been determined.

The aim of the present study was twofold: A pilot study was conducted to determine the optimal anticoagulant and sample storage time for the ROTEM® platelet device and to determine the functionality of assays with canine blood. The second aim of the study was to establish canine reference intervals for ROTEM® platelet parameters in samples handled based on the results of the pilot study.

2 Materials and Methods

2.1 Study population

The study was designed as a prospective observational study and took part between February and September 2017 at the small animal clinic of the Vetsuisse Faculty (University of Zurich, Switzerland). It was approved by the local ethical committee on animal research (ZH 042/15). A total of 53 healthy staff- and client-owned dogs were recruited and informed owner-consent was obtained for each dog.

Dogs enrolled in the study had to be adult (age > 12 months) and weigh > 5 kg for the pilot study or > 3 kg for the determination of RIs respectively. Dogs were not enrolled if they had donated blood, were vaccinated, have been treated with NSAIDs, paracetamol, dipyrone,

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116 steroids, anti-platelet drugs (e.g. clopidogrel), tranexamic acid or colloids in the last 4 weeks
117 prior to blood collection, or if they had a history of coagulation problems (e.g. bleeding
118 during surgery). To avoid breed specific results, a maximum of 4 dogs of the same breed were
119 included in the study and no Cavalier King Charles Spaniels were enrolled due to known
120 inherited giant platelet disorder.⁹

121 Health status of each dog was verified by history, physical examination, complete blood cell
122 count (Sysmex XT-2000iV, Sysmex Europe-GmbH), microscopic evaluation of a blood
123 smear, serum biochemistry panel (Cobas 600, Roche Diagnostics-(Switzerland)-AG) and
124 venous blood gas analysis (RAPIDPoint® 500 Blood Gas System, Siemens Healthcare
125 Diagnostic-Inc.). Activated partial thromboplastin time (aPTT), prothrombin time (PT),
126 thrombin time (TT), Fibrinogen Clauss (Stago STart 4, Diagnostica Stago-S.A.S.) and
127 ROTEM® delta analyses were conducted for a concurrent study.²²

128 ~~Dogs were excluded from statistical analysis if moderate or massive amount of platelet~~
129 ~~aggregates were observed in the blood smear or any other significant laboratory abnormalities~~
130 ~~were detected.~~ Feathered edge and monolayer area of blood smears were reviewed by
131 experienced laboratory technicians for the presence of platelet aggregates; presence or
132 absence of aggregates was semiquantitatively graded as absent, mild, moderate or marked.
133 Dogs were excluded from statistical analysis in case of moderate or marked platelet
134 aggregation or if any other significant laboratory abnormalities were detected. Dogs with
135 spurious thrombocytopenia were not excluded.

136

137 2.2 Sampling

138 Dogs were fasted for at least 4 hours prior to blood collection. Applying minimal stasis of the
139 vein, blood was collected by jugular venipuncture with gentle aspiration using a 20 Gauge
140 needle and a 10 mL or 20 mL syringe. Dogs were either sitting or in lateral recumbency

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during blood withdrawal. If venipuncture showed to be problematic, the attempt was stopped and the other jugular vein was punctured after a brief pause for the dog to recover. If a satisfactory venipuncture was not possible, the dog was excluded from the study. Blood collection tubes were filled in the following order immediately after sampling in the following order: 3 mL of serum (Serum Tube 4.5 mL, Sarstedt-~~AG and Co.~~) for complete serum biochemistry profile, 2 x 1.3 mL sodium-citrate (Micro tube 1.3 mL 9NC, Sarstedt-~~AG and Co.~~) for traditional coagulation times (aPTT, PT, TT), Fibrinogen Clauss and ROTEM® delta analysis, 2 x 1.3 mL of lithium-heparin (Micro tube 1.3mL LH, Sarstedt-~~AG and Co.~~) for ROTEM® platelet and 0.5 mL of lithium-heparin for venous blood gas analysis, 2.7 mL of hirudin (S-Monovette 2.7 mL Hirudin, Sarstedt-~~AG and Co.~~) (only if the dog was part of the pilot study) for ROTEM® platelet analysis, and 0.5 mL of K3-EDTA (Microvette 500 K3E, Sarstedt-~~AG and Co.~~) for complete blood cell count and blood smears. Each tube was carefully inverted 10 times immediately after filling. All venipunctures and platelet impedance aggregometry analyses were performed by the same person.

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2.3 Platelet impedance aggregometry with ROTEM® platelet

Blood samples were held at room temperature in the designated space on the ROTEM® platelet device. Diluent (0.9 % sodium chloride with preservatives, Tem Innovation~~GmbH~~) was warmed to 37°C in the designated heating area prior to analysis. Analysis was performed as described by the manufacturer. Briefly, 0.15 mL of diluent and 0.15 mL of whole blood are pipetted into a single use cuvette containing a stirring bar and two electrodes (ROTEM® platelet cuvette, Tem Innovation-~~GmbH~~). The sample is incubated for 3 min while the device determines an impedance baseline value of the system. During this time, 0.02 mL of diluent are added to the reagent to reconstitute the lyophilized agonist for platelet activation. After determination of a baseline, platelets are activated by addition of 0.012 mL of reconstituted

166 reagent into the cuvette containing whole blood and diluent. The aggregation of the activated
167 platelets leads to an increasing electrical impedance, which is recorded over 6 min. The
168 increase of impedance is directly proportional to the extent of platelets involved in coating the
169 electrodes by aggregation. The recorded impedance slope is processed with specific software
170 and includes the following parameters: 1) the area under the aggregation curve (AUC
171 ($\Omega \cdot \text{min}$)) for overall platelet aggregation, 2) the amplitude of measured impedance at 6 min
172 (A_6 (Ω)) for the extent of platelet aggregation, 3) the maximum slope (MS (Ω/min)) for the
173 rate of aggregation ([Calatzis A, Görlinger K, Spannagl M, Vorweg M. ROTEM Analysis
174 Targeted Treatment of Acute Haemostatic Disorders. https://www.ttuhsc.edu › internal ›
175 documents › ttim-manual](https://www.ttuhsc.edu/internal/documents/ttim-manual)).

176 ARA and ADP were evaluated as agonists for platelet activation using ara-tem and adp-tem
177 reagents for ROTEM® platelet. Each ROTEM® platelet device features 2 channels (A and
178 B). As samples were run in duplicates for both chosen agonists, 2 ROTEM® platelet devices
179 were used in this study to enable a total of 4 parallel assays.

180

181 **2.3.1 Pilot study: Comparison of 2 different anticoagulants and storage** 182 **timesDetermination of optimal sample storage time and anticoagulant**

183 The first 8 dogs recruited were enrolled in the pilot study. Hirudin and lithium-heparin were
184 chosen as anticoagulants and analyses were performed after a storage time of 15 min and 30
185 min for lithium-heparin and 40 min and 80 min for hirudin. Sample storage times were chosen
186 based on the available literature as well as clinical and logistic reasons.^{23,28,33,35} Exact time
187 points varied ± 3 min - depending on order of analyses - due to the time needed to perform the
188 single test steps. At each sample storage time, analyses with ara-tem and adp-tem were
189 performed in duplicate.

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190 Reagents and order of analysis were randomly assigned (www.randomizer.org) to 1 of the 2
191 devices, but the same reagent was run in duplicate on the same device to assess differences
192 between the 2 channels.

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194 2.3.2 Determination of reference intervals

195 Based on the results of the pilot study, RIs were established with lithium-heparin
196 anticoagulated blood after a sample storage time of 15 min. If an error occurred and the test
197 had to be repeated, a maximum of 10 min delay from the predetermined timepoint was
198 tolerated (a maximum of 25 min after sampling). Similar to the pilot study, samples were
199 analyzed in duplicates simultaneously on 2 ROTEM[®] platelet devices in randomly assigned
200 order (www.randomizer.org).

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202 2.4 Statistical analysis

203 Statistical analysis was performed using Analyse-it (Analyse-it Software) add-in for
204 Microsoft Excel (Microsoft[®] Excel[®] 2016-MSO) and Reference Value Advisor V2.1 add-in
205 for Microsoft Excel macroinstruction set (Geffré A, et al. Reference Value Advisor: a new
206 freeware set of macroinstructions to calculate reference intervals with Microsoft Excel. Vet
207 Clin Pathol 2011;40:107-112). Statistical significance level was set at $p < 0.05$ for all tests.
208 Figures were generated with GraphPad Prism Version 9 (~~© 1992-2021~~ GraphPad Software;
209 ~~LCC~~).

211 2.4.1 Pilot study: Comparison of 2 different anticoagulants and storage 212 timesdetermination of optimal sample storage time and anticoagulant

213 Overall platelet aggregation quantified as AUC for each single channel as well as for means
214 of channel A and B for the same reagent and sample storage time was determined. Data was

215 tested for normality using Shapiro-Wilk test. For normally distributed data mean and standard
216 deviation (SD) as well as median and range were calculated and for non-normally distributed
217 data median and range were calculated. Comparison of storage time and anticoagulant was
218 performed using Levene's test for homogeneity of variances and Kruskal-Wallis test for
219 location.

220 Overall coefficient of variation (CV) of AUC was calculated for every group (designated by
221 storage time, anticoagulant and reagent). The percentage difference between simultaneous
222 measurements on channel A and B of every sample was calculated.

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224 2.4.2 Determination of reference intervals

225 Serving as a quality control, the percentage difference between simultaneous measurements of
226 AUC on channel A and B of every sample was calculated. If results differed more than 20%
227 from the mean of the 2 channels, the samples were excluded.

228 Normality was tested using Anderson-Darling test and outliers were identified by Dixon-Rees
229 and Tukey's test. The mean results of the 2 channels were determined for AUC, A6 and MS
230 and used for determination of RIs by the reference value advisor program. As determined by
231 the program, nonparametric RIs were reported and a bootstrap method was used for
232 determination of confidence intervals (CI). RIs are reported as mean, median, SD, range and
233 2.5th – 97.5th percentile with 90% CI.

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235 3 Results

236 3.1 Study population characteristics

237 Of the 53 dogs recruited for blood withdrawal, 3 dogs were excluded because of moderate
238 platelet aggregates and 1 dog because of anemia, resulting in 7 dogs included in the pilot
239 study and a total of 49 dogs for RI determination. Results of the pilot study for the chosen

storage time and anticoagulant were included into the determination of RIs. Mean platelet count of all included samples was 263'000 uL (range 122'000 – 513'000 uL), 17 of 49 samples showed minor platelet aggregates (35%).

The 49 dogs included 30 females (19 intact (39%), 11 spayed (22%)) and 19 males (7 intact (14%), 12 neutered (25%)). Median age was 48 months (range 12 – 116 months) and median weight was 24 kg (range 4.7 – 63 kg). Breeds represented were 15/49 (31%) mixed breed, 4/49 (8%) Flat Coated Retriever, 3/49 (6%) Border Collie, 2/49 (4%) of each Belgian Shepherd, Berger Blanc Suisse, Dutch Shepherd, German Pointer, Golden Retriever, Leonberger, Samoyed and 1/49 (2%) of each Alaskan Malamute, Beagle, Cocker Spaniel, Dalmatian, Great Dane, Greater Swiss Mountain Dog, Labrador Retriever, Lagotto Romagnolo, Little Lion Dog (Löwchen), Pug, Rottweiler, Siberian Husky and Welsh Corgi Pembroke.

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3.2 Pilot study: Comparison of 2 different anticoagulants and storage times

Determination of optimal sample storage time and anticoagulant

Mean platelet count in the dogs of the pilot study was 270'000 uL (range 160'000 – 372'000 uL). One dog showed mild platelet aggregation (14%). CV of all measurements in a group was lowest for assays using lithium-heparin at 15 min sample storage time with adp-tem (7.3%) and highest for hirudin at 40 min sample storage time using ara-tem and lithium-heparin at 60 min sample storage time using adp-tem respectively (20.3%). All sample groups featured results with a difference between measured AUC of channel A and B greater than 20%, except for analyses at 15 min sample storage time using lithium-heparin for both reagents (Table 1). Boxplots of the mean AUC of parallel analyses on two channels were created (Figure 1).

Levene's test for dispersion showed variances of populations to be equal for means ($p = 0.11$) and single channels alone ($p = 0.20$). Kruskal-Wallis test for location detected no significant differences between medians of populations ($p = 0.91$ for means and $p = 0.89$ for single channels). Therefore, no significant differences were detected between groups. Based on the low CV and the lack of AUC measurements with $> 20\%$ channel difference, the use of lithium-heparinized blood at 15 min sample storage time was chosen for the establishment of RIs.

3.3 Reference intervals

Results of AUC differing more than 20% between channel A and B resulted in the additional exclusion of values from 4 dogs each for the calculation of RIs with adp-tem and ara-tem, respectively. The affected measurements belonged to different dogs for adp-tem and ara-tem. RI for adp-tem and ara-tem were determined (Table 2).

Mean ROTEM® platelet parameters were not significantly different between female and female spayed dogs as well as between female and male/male neutered/female spayed dogs (data not shown).

4 Discussion

Evaluation of platelet function has become increasingly important in both clinical cases and research studies. Interpretation of test results require species-specific RIs and optimal sample handling. Our study results show that canine blood is suited for analysis with ROTEM® platelet ~~and yields results comparable to RI in people for both adp-tem and ara-tem tests~~. No significant differences were detected between different anticoagulants and sample storage times in a pilot study including 7 dogs, however heparinized samples analyzed with adp-tem after 15 min of storage time showed the lowest CV.

289 The storage time of samples and the effect of different anticoagulants on platelet function
290 testing have been studied in animals and in people on various devices, but to the authors
291 knowledge, there have been no investigations using ROTEM® platelet in dogs and only few
292 studies in people.^{33,36,37} ~~Nissen et al. (2020) compared the~~ The stability of human blood
293 samples anticoagulated with hirudin and citrate analyzed with ROTEM® platelet ~~were~~
294 ~~previously compared in people.~~³³ Although citrated blood is recommended by the
295 manufacturer for ROTEM® platelet determination of platelet function in people, citrated
296 blood yielded significantly lower results.³³ The same finding was detected in dogs using the
297 Multiplate® analyzer.^{10,24,28} Furthermore, ~~Defontis et al. (2013) detected as~~ spontaneous
298 aggregation in canine blood anticoagulated with citrate ~~may occur, and recommend~~ either
299 lithium-heparin or hirudin tubes for use with the Multiplate® analyzer ~~are recommended.~~¹⁰
300 Citrated blood was therefore not considered as anticoagulant in our study. Based on the
301 above-mentioned studies, lithium-heparin and hirudin were chosen as anticoagulants in the
302 pilot study for determination of canine platelet function using ROTEM® platelet analysis.
303 Although sodium-heparin showed accurate results for a longer sample storage time in
304 Multiplate® analyzer than lithium-heparin in people, lithium-heparin was preferred by the
305 authors of this study because it is routinely used and available at the clinic where the study
306 took place.²³ In people, aggregation in ara-tem and adp-tem ROTEM® platelet assays
307 increased from baseline to 15 min sample storage time and stabilized from 15 min to 60 min
308 sample storage time in hirudin tubes.³³ We therefore chose a resting time of at least 15 min for
309 our pilot study. Our results show that the use of hirudin offers no advantages over lithium-
310 heparin in canine whole blood samples analyzed with ROTEM® platelet after a storage time
311 of 15 min to 80 min. However, if longer storage times are a prerequisite, using hirudin as an
312 anticoagulant may be considered. Canine hirudinized but not heparinized samples showed
313 spontaneous aggregation in Multiplate® analyzer.²⁸ We did not ~~microscopically~~ evaluate for

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spontaneous aggregation ~~by means of ROTEM platelet aggregation without an activator~~, but none of the samples in this study showed ~~macroscopic moderate or marked~~ platelet aggregation ~~prior to analysis~~ evaluated on a blood smear prior to analysis.

3 different agonists for the activation of platelets are available as reagents for the ROTEM® platelet device: TRAP (trap-tem), ADP (adp-tem) and ARA (ara-tem) (~~Calatzis A, Görlinger K, Spannagl M, Vorweg M. ROTEM Analysis Targeted Treatment of Acute Haemostatic Disorders~~. [https://www.ttuhscc.edu > internal > documents > ttim-manual](https://www.ttuhscc.edu/internal/documents/ttim-manual)). Although thrombin is a strong agonist for platelet activation in vivo, a study with the Multiplate® analyzer has shown that the TRAP-6 reagent used in tests designed for people does not induce significant platelet activation in dogs, presumably caused by species differences in the thrombin receptor, and our own experience has shown the same for the ROTEM® platelet device.^{10,24} Trap-tem therefore has not been investigated in this study. ADP binds on specific transmembrane receptors of platelets, activates G-proteins and other secondary pathways, eventually leading to the release of ARA (which is then converted to TXA2), the secretion of platelet granules and the change of platelet shape and size.⁴⁶ An earlier study using light transmission platelet aggregometry with platelet rich plasma showed that a fraction of dogs does not respond to ADP as an agonist.¹⁴ In contrast to this, all samples were ~~aggregated activated~~ by ADP in our study, which is in accordance with other studies using platelet impedance aggregometry to measure platelet function in dogs.^{10,15,28,44} The agonist ARA is a precursor of TXA2, one of the most important physiologic agonists of platelet activation, and serves as an agonist in the activation of platelets.⁴⁶ It has been reported that tests based on ARA activation result in higher imprecision and interindividual variation than ADP in dogs as assessed with Multiplate® analyzer, which was however not confirmed by our pilot study.^{10,24} Similar to ADP, ARA was also described previously to inconsistently activate canine platelets in an

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earlier studies using platelet rich plasma in an optical aggregometer other methods than platelet impedance aggregometry, a finding not supported by the current study.^{20,21}

Canine AUC, A6 and MS measured from heparinized samples yielded results comparable to a preliminary study including 20 people. Compared to RI in people, canine AUC, A6 and MS yielded comparable results for heparinized samples (Calatzis A, Görlinger K, Spannagl M, Vorweg M. ROTEM Analysis Targeted Treatment of Acute Haemostatic Disorders. <https://www.ttuhsc.edu › internal › documents › ttim-manual>). To the authors knowledge, no studies have established canine ROTEM® platelet RIs for AUC, A6 and MS. ROTEM® platelet AUC is not comparable to the AUC of the Multiplate® analyzer and the Multiplate® analyzer does not yield results for A6 and MS.³⁷ While we and other studies in people report RIs for A6 and MS, these parameters do not seem to be commonly used in the interpretation of platelet function analysis in people and their use has yet to be determined in veterinary clinical and laboratory studies.³³

A common finding in all RIs established for platelet impedance aggregometry for people and animals is the relatively large range of RIs, which makes the clinical interpretation of results challenging. However, dogs treated with ADP- or ARA-antagonists such as clopidogrel or acetylsalicylic acid are expected to show a very low AUC in the respective test. It was shown that AUC decreased significantly in dogs treated with clopidogrel in Multiplate® analyzer using ADP as agonist, and therefore, distinguishing impaired from normal platelet function is still possible.^{42,47} Blois et al. (2015) found poor correlation of different methods of platelet function testing in dogs were identified when comparing thromboelastographic platelet mapping to whole blood platelet aggregometry with Multiplate® analyzer and platelet function analysis using citrated whole blood. They suggested that population based RIs are unsuitable for most platelet function tests, and that subject based RIs are preferable as biological variation in platelet aggregation should be considered.² In accordance, Shropshire

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et al. (2018) found subject based RIs for ADP- and ARA-induced platelet aggregometry in Multiplate® analyzer ~~are to be~~ more sensitive in detecting abnormalities in platelet function in dogs.⁴³

In people, gender influences whole blood platelet aggregation RIs for ROTEM® platelet and day-to-day variation may occur and may be more pronounced in citrate-anticoagulated samples in people.³³ Age, however, does not seem to influence platelet aggregation in the Multiplate® analyzer in people.⁴⁰ In our study, mean values of all variables were not significantly different between female and female spayed dogs as well as female and male/male neutered/female spayed dogs. This result has to be interpreted in view of the small group size and further studies are needed to determine if population-based RI's are clinically useful. RI's in ROTEM® delta parameters were shown to be impacted by female sex.²² It was previously described that results of platelet impedance aggregometry vary between different breeds of dogs, which is why a maximum of 4 dogs of one breed were included in this study.³²

There are several limitations of the present study: Blood was withdrawn and analyzed at the described storage times consecutively on 2 devices. This resulted in a difference between ara-tem and adp-tem measurements of up to 3 min. As the results of ara-tem and adp-tem were not directly compared, this is judged to be of little importance in the current study. Sample storage time was different for both anticoagulants in this study because of lack of additional analyzers. RIs were established from the means of parallel measurements on 2 channels, with results differing more than 20% between channels being excluded. This approach was chosen because ROTEM® platelet features only one pair of electrodes per test cell, while the Multiplate® analyzer contains 2 pairs of electrodes in each test cell serving as internal control by excluding results differing more than 20% from each other.³⁷ It was decided by the authors

that duplicate measurements were more important than uniform sample storage times between anticoagulants, since duplicate measurements can improve analytical performance.²

Sample collection may have an influence on results. We minimized this effect by using gentle suction, a large needle and jugular venipuncture as well as exclusion of patients where venipuncture was difficult. Additionally, samples were analysed for aggregation on blood smears and samples with moderate or severe platelet clumping were excluded. We did not exclude samples with minor platelet clumping because we observe mild platelet aggregation frequently in our laboratory, so the samples included in the study are consistent with our reference-population. A study examining the effect of storage conditions and anticoagulant on platelet clumping in healthy dogs observed 12.9 – 31.4% of EDTA anticoagulated samples to feature platelet aggregation. The authors concluded that platelet clumping is common in blood samples of healthy dogs and seems to be difficult to avoid.³¹

The following limitation is given by the device and could not be addressed by a different study design. The analysis time in ROTEM® platelet is limited to 6 min compared to 12 min with Multiplate® analyzer. The authors deemed this of little importance, since ~~Marsehner et al. (2012) concluded that~~ short analyses times of 6 min are preferable over 12 min on the Multiplate® analyzer when using canine whole blood.²⁸

Further studies are needed to examine if ROTEM® platelet test can reliably monitor antiplatelet drug therapy and recognize abnormal values in clinical patients.

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Conflicting interests

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414

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541

542 **Tables**

543 **Table 1.** Pilot study – Platelet impedance aggregation in 7 healthy dogs measured with ROTEM® platelet using adp-tem (ADP) and ara-tem

544 (ARA) as agonists in heparinized or hirudinized whole blood at different sample storage times.

545

Anticoagulant and sample storage time	adp-tem				ara-tem			
	AUC Mean ± SD	AUC Median (range)	CV	> 20%	AUC Mean ± SD	AUC Median (range)	CV	> 20%
Lithium-heparin 15 min								
Means of channel A and B	87 ± 15	82 (67-104)	7.3	0	95 ± 14	98 (77-113)	16.3	0
Channel A	89 ± 13	89 (70-104)			102 ± 17	101 (84-134)		
Channel B	85 ± 17	83 (64-103)			89 ± 18	91 (68-120)		
Lithium-heparin 60 min								
Means of channel A and B	90 ± 30	103 (46-119)	20.3	1	*	97 (82-152)	20.1	2
Channel A	92 ± 39	106 (20-131)			101 ± 29	101 (61-143)		

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Channel B	88 ± 26	95 (48-117)			102 ± 28	94 (77-160)		
Hirudin 40 min								
Means of channel A and B	104 ± 20	104 (79-137)	12.7	1	98 ± 16	101 (71-120)	20.3	1
Channel A	107 ± 18	108 (80-138)			93 ± 29	102 (45-137)		
Channel B	102 ± 26	104 (62-135)			102 ± 10	102 (91-116)		
Hirudin 80 min								
Means of channel A and B	97 ± 39	100 (31-144)	20.1	1	103 ± 40	96 (60-182)	14.4	1
Channel A	100 ± 39	101 (38-161)			*	102 (73-185)		
Channel B	94 ± 44	95 (23-160)			98 ± 45	90 (36-178)		

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ADP: adenosine 5' diphosphate, ARA: arachidonic acid, AUC: area under aggregation curve in Ω *minutes, SD: standard deviation, CV:

coefficient of variation in %, > 20%: number of measurements with more than 20% difference between channel A and B in each group. *not

normally distributed data.

Table 2. Reference intervals for ROTEM® platelet for adp-tem (ADP) and ara-tem (ARA), measured after 15 min sample storage time in lithium-heparinized whole blood samples of 45 healthy dogs.

Reagent	Parameter	Mean	Median	SD	Minimum	Maximum	Reference Interval		90% CI	
							Lower limit	Upper limit	Lower limit	Upper limit
adp-tem	AUC	93	99	21	47	140	49	137	41 – 59	128 – 146
	A6	23	25	5	11	35	12	34	10 – 14	32– 37
	MS	8	9	2	5	13	4	13	3 – 5	12 – 14
ara-tem	AUC	96	92	20	54	142	55	137	47 – 63	128 – 145
	A6	25	25	5	15	36	15	35	13 – 17	33 – 38
	MS	8	8	2	5	13	4	12	4 – 5	11 – 13

ADP: adenosine 5' diphosphate, ARA: arachidonic acid, AUC: area under the curve in Ω *min, A6: amplitude at 6 minutes (Ω), MS: maximal slope (Ω /min), SD: standard deviation, CI: confidence interval.

557 **Figures**

558 **Figure 1.** Boxplots of the mean AUC of parallel analyses on 2 channels measured with ROTEM® platelet using adp-tem (ADP) and ara-tem

559 (ARA) as agonists in heparinized or hirudinized whole blood samples of 7 dogs at different sample storage times. The box extends from 25th to

560 75th percentiles, the middle line shows the median. The whiskers extend from the lowest to the highest value.

561 ADP: adenosine 5' diphosphate, ARA: arachidonic acid, AUC: area under aggregation curve in Ω *minutes, hep: lithium-heparin

562 anticoagulated samples, hir: hirudin anticoagulated samples.

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