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Comparison of Multiplate, Platelet Function Analyzer-200, and Plateletworks in Healthy Dogs Treated with Aspirin and Clopidogrel

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Background: Platelet function testing may be warranted to assess response to aspirin and clopidogrel.

Hypothesis/Objectives: To evaluate the effects of aspirin, clopidogrel, or combination therapy using 3 platelet function tests: Multiplate Analyzer (MP), Platelet Function Analyzer-200 (PFA), and Plateletworks (PW).

Animals: Six healthy laboratory Beagles.

Methods: Randomized double-blind placebo-controlled study (crossover design). Dogs were given aspirin 1 mg/kg, clopidogrel 2 mg/kg, or combination therapy for 1 week each, with a washout period of 2 weeks. Platelet function was assessed on days 0 and 7 of each phase using MP (adenosine diphosphate [ADP], arachidonic acid [AA], collagen [COL] agonists), PFA (P2Y, COL-ADP [CADP], COL-Epinephrine [CEPI] cartridges), and PW (ADP, AA, COL agonists). Platelet counts were obtained with impedance and optical counters.

Results: For MP, mean aggregation was decreased for COL and AA with combination therapy and for ADP with all treatments. For PFA, mean CT was increased for the CEPI cartridge with aspirin; and for the P2Y and CADP cartridges with clopidogrel or combination therapy. More dogs receiving clopidogrel showed an increase in PFA CT using the P2Y than the CADP cartridge. For PW, mean aggregation was decreased for AA with all treatments; for ADP with clopidogrel or combination therapy; and for COL with clopidogrel. The PW results with the 2 hematology counters showed almost perfect agreement.

Conclusion and Clinical Importance: All platelet function tests detected treatment effects in some dogs and may have utility for monitoring therapy.

Key words: Aggregation; Aggregometry; Impedance; Thromboembolism.

In dogs, the antiplatelet agents clopidogrel and aspirin may be used prophylactically against thromboembolism in different diseases in which a hypercoagulable state is identified. These diseases include immune-mediated hemolytic anemia (IMHA), protein-losing enteropathy and nephropathy, hyperadrenocorticism, and hypothyroidism.[1] Despite use of these medications, dogs with IMHA continue to have a high mortality rate (50–70%), and thromboembolism is thought to account for up to 80% of the deaths.[2] Platelet inhibition after treatment with clopidogrel or aspirin might be variable as in humans,[3–7] and therefore, platelet function testing may be warranted to monitor response to treatment.

Aspirin inhibits cyclooxygenase (COX) hence preventing thromboxane A_2 synthesis.[8] Aspirin resistance has been well described in humans, with an estimated prevalence of 8–45%.[3,4,9] Different mechanisms have been proposed, including genetic polymorphisms of COX or thromboxane A_2 synthase, increased platelet reactivity to platelet agonists other than arachidonic acid (AA), or alternative pathways of thromboxane synthesis.[4,5,7] In veterinary medicine, a study evaluating platelet function identified complete inhibition of platelet aggregation in only one-third of dogs when aspirin was administered at a dosage of 1 mg/kg/d for 10 days.[10]

Clopidogrel causes irreversible inhibition of the adenosine diphosphate (ADP) receptor $P2Y_{12}$.[8] Despite adequate dosing, clopidogrel resistance occurs in 4–30% of humans.[6,7,11] Different mechanisms have been proposed to explain this lack of response, including genetic polymorphisms of the $P2Y_{12}$ receptor and cytochrome P450.[7]

Abbreviations:

% Agg	% aggregation
AA	arachidonic acid
ADP	adenosine diphosphate
AUC	area under the curve
CADP	collagen/adenosine diphosphate
CEPI	collagen/epinephrine
COL	collagen
COX	cyclooxygenase
CT	closure time
H	hour
Min	minutes
MP	multiplate
PFA	Platelet Function Analyzer-200
PW	plateletworks
Sec	seconds
U	units

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Platelet function is measured by adding agonists to fresh blood products and observing the extent of platelet aggregation. Different methods have been developed using this principle. The Multiplate analyzer[a] (MP) is an impedance aggregometer.[12] The instrument is designed for point-of-care patient evaluation, is automated and simple to operate, and is the first impedance aggregometer to be approved for therapeutic monitoring in humans. In recent years, several studies have optimized and validated the use of this instrument in dogs.[13–15]

The Platelet Function Analyzer[b] (PFA) measures the time for platelets to close a small aperture under high shear stress conditions, mimicking blood flow in a blood vessel. The first generation of this instrument (PFA-100) has been used for over 2 decades for point-of-care platelet function testing of various hematologic conditions.[16] The new generation of this device (PFA-200) has been specifically modified to use the novel INNOVANCE P2Y cartridge to better enable detection of $P2Y_{12}$ receptor blockade in humans receiving clopidogrel.[17] The use of PFA-200 and this new cartridge has not yet been reported in dogs.

Plateletworks[c] (PW) is a method for evaluating platelet function that uses hematology counters that are routinely available in general and referral practices. The principle of this test is to compare platelet counts before and after the addition of an agonist. It has been validated for use in cats.[18–20] The use of Plateletworks has not yet been reported in dogs.

We aimed to evaluate the effects of aspirin, clopidogrel, or combination therapy, in healthy laboratory Beagles using 3 platelet function tests: MP, PFA-200 (including the INNOVANCE P2Y cartridge), and PW (using impedance-based and optical-based hematology counters).

Materials and Methods

Animals

Six healthy purpose-bred beagles (3 spayed females, 3 castrated males) with a median age of 5.1 years (range, 4.8–5.4 years) and weight of 11.1 kg (range, 8.3–14.4 kg) were used. None of the dogs had received any prior treatments with drugs affecting platelet function for a minimum of 2 weeks before to the start of the study. The health status of the dogs was confirmed by physical examination and CBC, serum biochemistry profile, urinalysis, and coagulation profile (prothrombin time, partial thromboplastin time, and fibrinogen concentration) using standard methods in the Animal Health Laboratory, University of Guelph. Dogs were housed at a laboratory facility and transported by motor vehicle to the Ontario Veterinary College on testing days. Dogs were fed a dry canine maintenance food twice daily and had free access to water. Animal use was approved by the University of Guelph Animal Care Committee. The study was performed in accordance with the standards of the Canadian Council on Animal Care.

Study Design

Dogs received clopidogrel[d] (2 mg/kg, PO) and a placebo[e] (PO, capsule), aspirin[f] (1 mg/kg, PO) and a placebo[g] (PO, liquid), or a combination of clopidogrel (2 mg/kg, PO) and aspirin (1 mg/kg, PO), once daily for 7 consecutive days. Drugs were compounded to individual doses based on body weight by the Pharmacy of the Ontario Veterinary College Health Sciences Center. Drugs were administered at approximately the same time each morning. Dogs were observed at the time of dosing for any abnormal behaviors, which were recorded on individual forms. The investigators and the technicians administering the drugs were blinded to the intervention received by each dog until the end of the study. Drugs were administered in a randomized crossover design. A washout period of 14 days occurred between phases.

Blood Sample Collection

Blood collection was performed after a 12-hour overnight fast. For each phase of the study, blood samples were collected before treatment (T0) and after 7 consecutive days of treatment (T7), which began the following day (T1). The order of blood collection was randomized at the first visit (toss of a coin), and that order was maintained throughout the study. At every visit, 2 blood samples were collected. Before blood collection, the hair was clipped over venipuncture sites, and topical lidocaine[h] was applied. The first sample was collected by left jugular venipuncture using a 20-ga Vacutainer[i] set and vacuum tubes. Blood was collected into tubes in the following order: 1 × 3-mL serum tube (for biochemistry profile on the first visit only, discarded on the following visits), 4 × 1.8-mL 3.2% citrate tube (prothrombin time, partial thromboplastin time, and fibrinogen on the first visit only, PFA-200), 1 × 4-mL heparin tube (MP, clopidogrel and aspirin drug metabolite concentrations), and 1 × 2-mL EDTA tube (CBC, baseline platelet count for PW). Every tube was agitated 4 times by inversion and then allowed to rest until analysis. A second blood sample was collected by right jugular venipuncture using a 6-mL plastic syringe and 20-ga needle. The needle of the syringe was removed, and 1 mL of blood was transferred into each PW agonist tube (which does not contain a vacuum) in the following order: ADP tube, collagen (COL) tube, and AA tube. The tubes were agitated by inversion following the manufacturers' recommendations: 15–20 times for all tubes, followed by rest until analysis for the ADP tube; 5 times every minute until analysis for the COL tube, and 1 time every 8–10 seconds for 2 minutes followed by rest until analysis for the AA tube. For all venipunctures, quality (graded 1–3; 1 = no redirection, 2 = 1–2 redirections, 3 = 3–4 redirections) and stoppage of blood flow (graded 1–3; 1 = no stoppage, 2 = very brief stoppage, 3 = substantial stoppage) were recorded. A blood smear prepared from the EDTA sample was examined for platelet clumping. A maximum of 20 mL of blood was collected from each dog at each visit.

Platelet Function Testing

Multiplate

The agonists ADP,[j] COL,[k] and AA[l] were used. Agonist solutions were prepared according to the manufacturers' recommendations by adding 1 mL distilled water to a lyophilized agonist stock. The agonists were stored at −30°C for a maximum for 4 weeks (ADP and AA) or at 4°C for a maximum of 7 days (COL). The test was performed as recommended by the manufacturer. Briefly, 300 μL of 0.9% NaCl solution preheated to 37°C was added in the test channel. The heparin tube was agitated 4 times, and 300 μL of blood was added in the test channel. After 3 minutes incubation and stirring at 37°C, 20 μL of agonist was added to the diluted sample to a final concentration of 6.5 μmol/L ADP, 3.2 μg/mL COL and 0.5 mmol/L AA. The aggregation was measured for 6 minutes, which resulted in the generation of 2 curves (measured by 2 independent sensor units), and results were displayed as area under the curve (AUC) and expressed in units

(U). If Pearson's correlation coefficient was <0.98 or if the difference of each curve from the mean was >20% (both automatically calculated by the MP), the analysis was repeated. Following the manufacturers' recommendations, all samples were analyzed between 30 minutes (min) and 3 hours (h) after venipuncture.

PFA-200

The cartridges P2Y,[m] COL/ADP[n] (CADP), and COL/Epinephrine[o] (CEPI) were used. The cartridges were stored at 4°C and allowed to warm to room temperature in their pouches before usage. The citrate tubes were agitated 4 times before each analysis, and 800–900 μL of blood was pipetted into the different cartridges. At the beginning of the study, 800 μL was used, following the manufacturer's recommendations. However, during the course of the study, it was noted that a "no closure, maximum syringe travel" message was sometimes generated before reaching the limit of measurement of the analyzer, which is 300 seconds. Examples of such results would be >270 and >280 seconds. This was thought to be due to an insufficient amount of blood in the cartridge, and following additional manufacturer's recommendations, the amount of blood was increased to 900 μL during the last phase of the study. For results that reached "no closure" before reaching the upper limit of 300 seconds, the maximum number reached was used for analysis (e.g, 270 seconds if >270 seconds was obtained). The A Channel was used for all analyses; the order of analysis was as follows: P2Y, CADP, CEPI. Each sample was analyzed in duplicate; duplicate analyses were performed sequentially. The time required to close the aperture was recorded as the closure time (CT). For CADP, a reference interval of 45–109 seconds previously had been determined at our institution's laboratory using PFA-100.[21] If a sample error message was recorded (e.g, flow obstruction), the sample was analyzed a third time. Following the manufacturer's recommendations, all samples were analyzed between 10 minutes and 4 hours after venipuncture.

Plateletworks

Agonist tubes containing ADP, COL, and AA were used. Platelet counts were determined using 2 hematology counters, 1 using impedance[p] , and 1 using an optical method[q] . The filled agonist tubes were agitated before each analysis according to the manufacturer's recommendations (4 times for ADP, 5 times for COL, 2 times for AA). The order of analysis was as follows: COL tube, AA tube, ADP tube, and EDTA tube. Platelet count in each tube was obtained in duplicate before the next tube was analyzed. Because of the longer time of analysis with the impedance-based hematology counter, the platelet count was first measured once with that machine, then in duplicate with the optical-based hematology counter, and finally a second time with the impedance-based hematology counter. Platelet aggregation (% Agg) was calculated as follows: (EDTA platelet count − agonist platelet count)/EDTA platelet count × 100. All samples were analyzed within 30 minutes of venipuncture.

Drug Metabolite Concentrations

Heparinized blood obtained on days 0 and 7 of each phase was centrifuged within 10 minutes of blood collection, and serum was kept frozen at −80°C. At the end of the study, drug metabolite concentrations were measured by validated assays using high-performance liquid chromatography as previously described.[20,22] For aspirin, the metabolite salicylate was measured. For clopidogrel, the inactive metabolite SR 26334 was measured. For both metabolites, the limit of quantification was 0.1 μg/mL. These assays were performed in the Clinical Pharmacology Laboratory, College of Veterinary Medicine, North Carolina State University.

Statistical Analysis

All statistical analyses were performed using statistical software.[r] Data were checked for normality using a Shapiro–Wilk test. For variables that were not normally distributed, log transformation was performed, and if the distribution was normalized, log-transformed data were used in analysis. A general linear mixed model accounting for carryover and treatment period was performed to determine if there were visit (T0 versus T7) or drug (aspirin, clopidogrel, combination) effects. If the overall F-test was significant for a visit or drug effect, pairwise comparisons (*t*-test) were performed. If a significant interaction was detected, post hoc *t*-tests were Tukey-adjusted. For PW, results obtained with the 2 hematology counters were compared using Pearson's correlation coefficients to test for an overall association, and Lin's concordance correlation was used to test for agreement. A Mann–Whitney Wilcoxon test was performed to compare drug metabolite concentrations obtained between T0 and T7. $P \leq 0.05$ was considered significant at a power of 80%.

Dogs were classified with respect to magnitude of response as follows: for MP and PW, dogs were classified into 3 groups as described previously[6]: <25% decrease from baseline, 25–49% decrease from baseline, >50% decrease from baseline. For PFA, dogs were assessed according to 2 different classifications: prolongation of CT above the reference interval when a reference interval was available (CADP), or no closure.

Results

Blood Collection

Venipuncture scores were recorded on 70/72 occurrences. The median score for quality was 1 (range 1–3), and the median score for stoppage of blood flow was 1 (range 1–3), with over 80% of venipunctures achieving a score of 1 in both categories for both the Vacutainer (venipuncture 1) and syringe draw (venipuncture 2). Only 1/70 venipunctures scored 3. This score was obtained in both categories (quality and stoppage) for dog 6 in the clopidogrel group at T0 during venipuncture 1. No outlying values for platelet function results were noted for venipunctures that had higher scores.

Platelet Function Tests

All results were normally distributed except those for the PFA P2Y and CADP cartridges and PW COL. Log transformation of P2Y data resulted in the data having less kurtosis; distribution was not normal (Shapiro–Wilk, $P = 0.01$) but was sufficiently adjusted for the analysis to be valid. Platelet function test results are summarized in Table 1. There were no significant differences for any of the platelet function tests between treatment groups at T0.

Multiplate

Samples were analyzed at a median time of 50 minutes after venipuncture at T0 (range, 47–67 minutes) and 49 minutes at T7 (range, 45–95 minutes). On 6 occasions, a quality control flag was displayed because of the correlation coefficient being <0.98, the difference of each curve from the mean being >20% or both. Those tests were repeated and yielded results within quality control parameters.

Table 1. Results before (TO) and after 7 days (T7) of treatment with aspirin, clopidogrel, and combination therapy in 6 healthy dogs. Results shown are mean ± SD (range) and are expressed in units (for MP), seconds (for PFA), and % aggregation (for PW). Results shown for PW were obtained with the impedance hematology counter (Vetscan HM5). The *P* value refers to the difference between results for each platelet function test at T0 and T7.

	Aspirin			Clopidogrel			Combination		
	T0	T7	*P* value	T0	T7	*P* value	T0	T7	*P* value
MP									
COL	15 ± 10 (3–24)	10 ± 10 (0–25)	0.14	18 ± 10 (4–38)	15 ± 10 (5–26)	0.37	19 ± 10 (6–39)	6 ± 10 (0–21)	0.023
AA	36 ± 15 (4–53)	32 ± 15 (13–51)	0.93	41 ± 15 (21–67)	20 ± 15 (7–53)	0.07	44 ± 15 (25–57)	9 ± 15 (3–25)	0.003
ADP	62 ± 8 (54–73)	52 ± 8 (33–68)	0.028	59 ± 8 (45–66)	13 ± 8 (9–17)	<0.001	58 ± 8 (50–71)	12 ± 8 (7–20)	<0.001
PFA									
P2Y	111 ± 72 (38–245)	139 ± 89 (57–300)	0.54	95 ± 47 (41–171)	300 ± 0 (300–300)	<0.001	119 ± 88 (46–300)	300 ± 0 (300–300)	<0.001
CADP	67 ± 61 (55–102)	70 ± 60 (54–88)	0.99	65 ± 60 (50–80)	185 ± 60 (52–300)	0.006	68 ± 61 (53–111)	215 ± 60 (64–300)	0.001
CEPI	167 ± 66 (105–283)	235 ± 66 (113–300)	0.015	159 ± 66 (104–256)	160 ± 66 (103–300)	0.95	184 ± 66 (78–295)	234 ± 66 (103–300)	0.066
PW									
AA	80 ± 7 (20–98)	7 ± 7 (0–25)	<0.001	92 ± 7 (82–95)	6 ± 7 (0–18)	<0.001	74 ± 7 (29–97)	8 ± 7 (0–28)	<0.001
COL	17 ± 10 (1–51)	13 ± 10 (0–26)	0.37	18 ± 10 (0–37)	8 ± 10 (0–23)	0.029	14 ± 10 (0–28)	11 ± 10 (0–22)	0.55
ADP	47 ± 31 (14–86)	44 ± 31 (0–88)	0.93	44 ± 31 (1–88)	8 ± 31 (0–20)	0.0071	44 ± 31 (0–86)	6 ± 31 (0–22)	0.0049

MP, Multiplate; PFA, Platelet Function Analyzer-200; PW, Plateletworks; COL, Collagen; AA, Arachidonic acid; ADP, Adenosine diphosphate; CADP, Collagen/adenosine diphosphate; CEPI, Collagen/epinephrine.

Comparing T0 to T7 results within each treatment group (Table 1), mean aggregation was decreased for COL and AA in dogs receiving combination therapy, but not for dogs receiving either drug alone. Mean aggregation was decreased for ADP in dogs of all treatment groups.

PFA

Samples were analyzed for P2Y at a median of 52 minutes after venipuncture at T0 (range, 44–73 minutes) and 54 minutes at T7 (range, 43–95 minutes); for CADP at a median of 66 minutes at T0 (range, 56–119 minutes) and 71 minutes at T7 (range, 61–117 minutes); and for CEPI at a median of 81 minutes at T0 (range, 69–109 minutes) and 88 minutes at T7 (range, 75–138 minutes). On 7/216 occasions, a message error was generated as a consequence of flow obstruction. Those tests were repeated. A second flow obstruction occurred for 2 of those repeated tests. For those results, only 1 measurement was included in the final analysis. On 16/216 occasions using the CADP or CEPI cartridge, the analyzer generated a "no closure" message before reaching the upper limit of 300 seconds. Only 2 of those results were obtained at T0, and 14 were obtained at T7. After increasing the amount of blood pipetted into the cartridge to 900 μL upon the manufacturer's recommendation, this type of error occurred an additional 3 times but on those 3 occasions the CT was very close to the upper limit of 300 seconds (>297, >294, >294 seconds).

Comparing T0 to T7 results within each treatment group (Table 1), the mean CT was increased for the PY2 and CADP cartridges in dogs receiving clopidogrel alone or combination therapy. The mean CT was increased for the CEPI cartridge in dogs receiving aspirin alone.

Plateletworks

Samples were analyzed for COL at a median of 11 minutes after venipuncture at T0 (range, 8–18 minutes) and 10 minutes at T7 (range, 7–16 minutes); for AA at a median of 17 minutes at T0 (range, 15–21 minutes) and 16 minutes at T7 (range, 14–19 minutes); for ADP at a median of 24 minutes at T0 (range, 21–29 minutes) and 23 minutes at T7 (range, 20–27 minutes). No platelet clumping was observed on microscopic examination of blood smears of any EDTA sample.

Comparing T0 to T7 results within each treatment group (Table 1), mean AA-induced aggregation was decreased in all treatment groups. Mean ADP-induced aggregation was decreased in dogs receiving clopidogrel alone or combination therapy. Mean COL-induced aggregation was decreased in the clopidogrel group only.

Results obtained with both hematology analyzers showed substantial to perfect agreement for all variables (ρ_c, 0.77–0.98), except for COL % Agg (ρ_c, 0.34), which showed only fair agreement.

Aspirin and Clopidogrel Responders

Results are summarized in Table 2. As previously discussed, several CADP and CEPI results reached no closure before 300 seconds. As reported in Table 2, the classification of dogs as responders based on CADP or CEPI tests changed if those values were considered equivalent to 300 seconds.

Considering the less strict criteria for each classification (>25% decrease from baseline for MP and PW, CT above the reference interval for PFA), the tests that detected drug response most consistently were as follows: for aspirin, PFA CEPI (up to 4/6 dogs) and PW AA (6/6); for clopidogrel, MP AA (5/6), MP ADP (6/6), PFA P2Y (6/6), PFA CADP (4/6), PW AA (6/6), PW COL (4/6), and PW ADP (5/6); and for combination therapy, MP COL (6/6), MP AA (6/6), MP ADP (6/6), PFA P2Y (6/6), PFA CAPD (5/6), PFA CEPI (up to 5/6), PW AA (6/6), and PW ADP (6/6). Using a more strict definition for treatment response and considering the stronger criteria only for each classification (>50% decrease in baseline for MP and PW; CT > 300 seconds for PFA), the tests that detected drug response most consistently were as follows: for aspirin, PW AA (6/6); for clopidogrel, MP AA (4/6), MP ADP (6/6), PFA P2Y (6/6), PFA CADP (4/6), PW AA (6/6), and PW ADP (5/6); and for combination therapy, MP COL (4/6), MP AA (5/6), MP ADP (6/6), PFA P2Y (6/6), PW AA (6/6), and PW ADP (6/6).

Drug Metabolite Concentrations

In the aspirin and combination treatment groups, the median salicylate concentration was 0 μg/mL for dogs before receiving aspirin (range, below the limit of quantification of 0.1 μg/mL to 1.11 μg/mL) and 1.33 μg/mL for dogs after receiving aspirin (range, below the limit of quantification to 3.69 μg/mL). This difference was significant ($P = < 0.001$). In the clopidogrel and combination therapy groups, the median SR 26334 concentration was 0 μg/mL for dogs before receiving clopidogrel (all results below limit of quantification of 0.1 μg/mL) and 0.25 μg/mL for dogs receiving clopidogrel (range, 0.1–0.35 μg/mL). This difference was significant ($P < 0.001$).

Discussion

All 3 platelet function tests detected aspirin and clopidogrel effects in some dogs and may have utility for monitoring antiplatelet therapy. More dogs receiving clopidogrel showed an increase in PFA CT using the P2Y cartridge (6/6 dogs) than the CADP cartridge (4/6 dogs receiving single-agent clopidogrel and 5/6 dogs receiving combination therapy). Results obtained with both hematology counters were similar for PW. Although the manufacturer's recommendations are to use impedance-based platelet counts, our study shows that it is valid also to use optically based platelet counts in dogs, as has been previously reported in cats.[19,20]

Although significant results were obtained for several tests when examining mean values obtained at T0 compared to T7, results were variable for individual dogs for some of those tests. Tests for which every dog's individual results followed the changes in the mean result included MP ADP with clopidogrel and combination therapy; PFA P2Y with clopidogrel or combination therapy; PW ADP with clopidogrel or combination therapy; and PW AA with all treatments. Drug metabolite concentrations were assessed to evaluate if

Table 2. Number of dogs classified as aspirin, clopidogrel, or combination therapy responders after 7 days of treatment. Classification based on 25% or 50% decrease in AUC (MP); 25% or 50% decrease in % Agg (PW); no closure or CT > upper reference interval (PFA). Upper reference interval (upper ref) for PFA was defined as 109 seconds for CADP.

	Aspirin			Clopidogrel			Combination		
	<25%	25–49%	>50%	<25%	25–49%	>50%	<25%	25–49%	>50%
MP									
COL	2/6	2/6	2/6	4/6	2/6	0/6	0/6	2/6	4/6
AA	3/6	1/6	2/6	1/6	1/6	4/6	0/6	1/6	5/6
ADP	4/6	2/6	0/6	0/6	0/6	6/6	0/6	0/6	6/6
PW									
COL	2/6	1/6	3/6	2/6	1/6	3/6	4/6	1/6	1/6
AA	0/6	0/6	6/6	0/6	0/6	6/6	0/6	0/6	6/6
ADP	4/6	1/6	1/6	1/6	0/6	5/6	0/6	0/6	6/6

PFA	No Closure	CT > Upper Ref	No Closure	CT > Upper Ref	No Closure	CT > Upper Ref
P2Y	1/6	–	6/6	–	6/6	–
CADP	0/6	0/6	4/6	4/6	3/6 (4/6)[a]	5/6
CEPI	1/6 (4/6)[a]	–	2/6		–	2/6 (5/6)[a]

AUC, Area under the curve; MP, Multiplate; Agg, Aggregation; CT, Closure time; PFA, Platelet Function Analyzer-200; PW, Plateletworks; Sec, seconds; COL, Collagen; AA, Arachidonic acid; ADP, Adenosine diphosphate; CADP, Collagen/adenosine diphosphate; CEPI, Collagen/epinephrine.

[a]Represent number of dogs classified as responders when values that did not reach a closure time but did not attain 300 seconds because of insufficient sample volume were included.

individual differences could have been due to problems with drug administration or decreased absorption of the drugs from the gastrointestinal tract, or if they were truly due to individual differences in platelet response to those drugs. Drug metabolite concentrations confirmed that all dogs received and absorbed the drugs. However, drug metabolite concentrations could not explain the variation observed in platelet function test results. This finding may be a consequence of individual differences in response to those drugs, individual responses to platelet agonists, or because the inactive metabolites are not good surrogate markers of the active drugs.[22]

The true definitions of aspirin or clopidogrel resistance in humans are not well described. Different definitions of resistance have been proposed, including clinical resistance (defined as the occurrence of thromboembolism despite antiplatelet medication) and laboratory resistance (defined as the failure of antiplatelet medication to inhibit platelet function as assessed by in vitro methods).[4,5,7,23] None of the 6 dogs in this study had laboratory resistance, because response to therapy could be confirmed in all cases by changes in at least 1 test result (i.e, all dogs had a decrease % Agg > 50% with PW AA regardless of treatment group). However, some dogs' test results were inconsistent. For example, for dog 5, postaspirin AA % Agg decreased >50%; but this dog's PFA CEPI postaspirin CT did not reach "no closure". For dogs 4 and 5, postclopidogrel AA % Agg and ADP % Agg decreased by >50%, their PFA P2Y CT reached >300 seconds, and their MP ADP decreased by >50%; but both dogs' PFA CADP post-treatment CT did not reach "no closure" nor did their results go above the upper limit of the reference interval. Some of the tests that detected a treatment response consistently for all dogs, such as PW AA, or PFA P2Y for clopidogrel, may be too sensitive and may reach the upper limit of that test with clinically subtherapeutic drug concentrations in the animal. Such a test may only be useful to confirm owner compliance with administration and absorption of the drug. Such a test also may be useful to suggest risk for clinical aspirin or clopidogrel resistance if that test fails to detect a response. Alternatively, some of the tests that detected a treatment response infrequently, such as MP ADP for aspirin and MP COL and PFA CEPI for clopidogrel, may be too insensitive. The most useful test may be 1 that shows a graded response, which then can be correlated to clinical therapeutic response in future longitudinal studies. A combination of different tests, with different sensitivities to detect antiplatelet drug effect may be required to predict an acceptable decrease in risk for thrombosis.

Drug metabolite measurements were used as surrogate markers of plasma drug concentrations. For aspirin, salicylate was used because aspirin itself has a very short plasma half-life, which made its measurement logistically unfeasible for our study, and a validated assay was not available. Two of the dogs had measurable salicylate concentrations at T0. Laboratory, pharmacy, and animal facility records were reviewed in an effort to identify clerical, analytic, or medication errors, and none were identified. However, the platelet function test results for these dogs at T0 were not consistent with aspirin effect; therefore, it is believed that the salicylate concentrations are erroneous. For clopidogrel, the inactive metabolite SR 26334 was measured as a surrogate marker. This was done because the parent drug and active metabolite usually are not detectable in plasma with currently available assays.[22,24] However, the metabolic pathway for clopidogrel activation has not been characterized in dogs, and it is possible that plasma concentrations of SR 26334 do not correlate with concentrations of the clopidogrel active metabolite.[22] Recently, stabilization of the clopidogrel active metabolite and quantification using high-performance liquid chromatography in cats have been described.[25] If developed in dogs, this technique may better assess clopidogrel concentrations in the future.

For most of these tests, no reference interval has been established in dogs. This makes it more challenging to assess a dog's response to therapy. Previous studies have used different ways to assess response to therapy, such as a significant change in the mean from baseline,[6,26] a CT > 300 seconds for PFA,[10,23] or a CT > upper limit of the reference interval.[27] For PFA CADP, a reference interval of 45–109 seconds previously had been determined at this institution's laboratory using PFA-100,[21] and was used in our study to help classify responders versus nonresponders. No reference interval was available for any of the other platelet function tests evaluated. A previous study[28] performed at our institution evaluated biologic variability of different platelet function tests, including MP (COL, AA, ADP agonists) and PFA (CAPD, CEPI cartridges), and indicated that use of population-based reference intervals was inappropriate for most platelet function tests because of high biologic variability. Based on that study, the authors suggested that subject-based reference intervals may be more appropriate. This is consistent with what was observed in our study. Baseline results obtained with PFA CEPI, for example, ranged between 78 and 295 seconds. In the study that evaluated biologic variability, results obtained with PFA CEPI ranged between 54 and 300 seconds,[28] and in another study performed at the same institution, results obtained with that same platelet function test ranged between 62 and 300 seconds.[29]

The aspirin dosage chosen for our study is a common antiplatelet dosage.[10] A previous study performed at this institution indicated that 1 mg/kg/d was the lowest dosage that inhibited platelet function as assessed by PFA-100 and optical aggregometry.[30] However, a recent report suggested that an aspirin dosage of 2 mg/kg may be more reliable for platelet inhibition.[31] In that study, the 2 mg/kg dosage resulted in a mean decrease in maximum amplitude of 81.3% with turbidimetric aggregometry and a CT >300 seconds in 62.5% of dogs with PFA-100, compared to 41.3% and 25%, respectively, for an aspirin dosage of 1 mg/kg. Therefore, the dosage used in our study may have been insufficient to cause maximal inhibition of platelet function.

The agonist concentrations for MP used in our study were those recommended by the manufacturers. Several

studies have optimized MP impedance aggregometry and have described different agonist concentrations.[13–15,26] Analyses performed using a wide range of concentrations showed that a maximum attainable aggregation response was obtained with 10 μmol/L ADP, 5 μg/mL COL, and 1 mmol/L AA.[13] However, maximum aggregation response is not necessarily desirable because it can lower diagnostic sensitivity.[14] For this reason, the agonist concentrations used in our study were those currently recommended by the manufacturer for human platelets.

The PW manufacturer recommends that all agonist tubes be analyzed within 10 minutes of venipuncture. This could not be achieved in our study because the samples had to be agitated in a precise manner and then physically transported to the laboratory (about 6 minutes), then analyzed in duplicate using 2 different hematology counters. Samples were analyzed at a median of 10 minutes for COL, 16 minutes for AA and 23 minutes for ADP, with no sample being analyzed >29 minutes after venipuncture. A previous study in cats[18] showed that the ADP % Agg was stable up to 30 minutes after venipuncture, but decreased significantly between 30 and 60 minutes. Pilot studies performed at our institution (data not shown) suggested that the same was true for dogs, with a significant drop in ADP % Agg observed between 30 and 45 minutes postvenipuncture. However, further data obtained after completion of this study indicated that in some dogs significant spontaneous disaggregation may occur after 10–15 minutes (data not shown). No data are available for COL or AA in dogs or cats. Because COL is considered by the manufacturer to be the most susceptible to spontaneous disaggregation, it was decided to analyze the COL sample first, which allowed obtaining a median time to analysis of 10 minutes. However, some results were analyzed up to 18 minutes after venipuncture. This may explain in part why results obtained with this agonist were less consistent both among dogs and duplicate samples. Because AA is considered by the manufacturer to be a weaker agonist than ADP, the AA samples were analyzed before the ADP samples. The times to analysis for AA also were longer than recommended by the manufacturer. It is not known how much the delays in time to analysis for AA and ADP affected magnitude of responses, but they did not decrease the ability of the AA assay to detect aspirin or clopidogrel effects (100%) and of the ADP assay to detect clopidogrel effect (100%) and did not appear to decrease the precision of the assays. It is not known if the delay in analysis decreased the ability of ADP to detect aspirin effects. Further studies are warranted to determine the maximum time to analysis that can be used for the different agonists, but such a time constraint may render this test less usable in some clinical settings. Pending such studies, it is recommended that the time to analysis be as short as possible and consistent pre- and post-treatment.

A slight change in protocol was adopted for PFA during the third phase of the study after it was noted that a "no closure" message was sometimes generated before reaching the maximum of 300 seconds. The amount of blood loaded in the cartridge was increased to 900 μL, which allowed the acquisition of more precise data. However, several results obtained before this change of protocol lacked precision because the analyzer stopped measuring the CT before complete closure and before the limit of 300 seconds was reached. Examples include results such as >270 seconds, where the actual result is known to be above 270 seconds, but a more precise result could not be obtained. For some or all of those results, the limit of 300 seconds may have been obtained if the blood sample volume had been sufficiently large to allow completion of the analysis. Statistical analysis was performed using the maximum number reached (e.g, 270 seconds if >270 seconds was obtained) but also was repeated after all results that reached no closure before attaining 300 seconds were converted to 300 seconds to investigate if this would have impacted our results. No difference was noted in the significance of results.

Consideration needs to be given to the small number of dogs included in this study. Because of the strong treatment effect, which was expected with MP, PFA, and PW with some agonists based on previous studies,[10,17,18] a power calculation showed that 4 dogs would be enough to detect a significant difference with a power of 0.8. This proved to be accurate, but it is possible the study was underpowered to detect more subtle effects.

Dogs used in our study were all healthy Beagles. As described previously,[26] platelet function test results may vary among different breeds, and results obtained in our study may not directly apply to different breeds or to dogs with clinical diseases that may affect platelet function.

In conclusion, all 3 platelet function tests were useful to detect aspirin and clopidogrel effect in some dogs. More dogs receiving clopidogrel showed an increase in PFA CT using the P2Y than the CADP cartridge. Plateletworks using AA was the only test that detected all drug effects in all dogs. Results obtained with both hematology counters were similar for PW. All platelet function tests used in our study may be useful for monitoring antiplatelet response in dogs treated with aspirin or clopidogrel, but additional studies are needed to determine the optimal tests and agonists for use in dogs with different diseases.

Footnotes

[a] Diapharma Group, Inc, West Chester, OH
[b] Siemens Healthineers, Erlangen, Germany
[c] Helena Laboratories, Beaumont, TX
[d] Teva Clopidogrel Bisulfate 75 mg tablets, compounded into a liquid using OraBlend suspending agent (Galenova Inc, St-Hyacinthe, QC, Canada)
[e] Gelatin capsule (size #4, Capella Enterprises, Carleton Place, Ontario, Canada) filled with lactose monohydrate powder (Galenova Inc, St-Hyacinthe, QC, Canada)
[f] Acetylsalicylic Powder USP and lactose monohydrate powder (Galenova Inc, St-Hyacinthe, QC, Canada) in gelatin capsule (Capella Enterprises, Carleton Place, Ontario, Canada)
[g] OraBlend suspending agent; Galenova Inc, St-Hyacinthe, QC, Canada

[h] Maxilene; RGR Pharma, LaSalle, ON, Canada
[i] Vacuette Multiple Use Drawing Needles; Greiner Bio-One GmbH, Kremsmunster, Austria
[j] ADPtest kit; Verum Diagnostica GmbH, Munich, Germany
[k] COLtest kit; Verum Diagnostica GmbH, Munich, Germany
[l] ASPItest kit; Verum Diagnostica GmbH, Munich, Germany
[m] INNOVANCE PFA P2Y; Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany
[n] Dade PFA Collagen/ADP Test Cartridge; Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany
[o] Dade PFA Collagen/EPI Test Cartridge; Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany
[p] Vetscan HM5; Abaxis Veterinary Diagnostics, Union City, CA
[q] ADVIA 2120i; Siemens Healthineers, Erlangen, Germany
[r] SAS 9.3; SAS Institute Inc, Cary, NC

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