Effects of platelet clumping on platelet concentrations measured by use of impedance or buffy coat analysis in dogs

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Objective—To determine whether platelet clumps are homogeneously distributed in blood samples, and whether platelet concentrations (PC) obtained by use of impedance and buffy coat analysis can be considered minimum values when platelet clumps are present.

Design—Prospective study.

Sample Population—50 blood samples obtained from 30 dogs.

Procedure—10 blood samples containing platelet clumps were used and 10 smears were made from each sample; amount of platelet clumping was graded for all 100 smears. Blood from each of 20 healthy dogs was divided between 2 EDTA tubes before and after platelet clumping was induced by adenosine diphosphate (ADP). The PC for each ADP-treated and untreated sample were measured, using impedance and quantitative buffy coat analyzers.

Results—Platelet clumps were evident in all 100 blood smears, but the amount of clumping varied considerably within some samples. Using the impedance analyzer, the PC of ADP-treated samples were significantly lower and never higher than the PC of untreated samples. Using the buffy coat analyzer, some ADP-treated samples had increased PC; however, significant differences were not detected between treated and untreated samples.

Conclusions and Clinical Relevance—Platelet clumping was not homogeneous within blood samples. When platelet clumps were identified by direct examination of blood smears, the PC detected by use of the impedance analyzer could be considered minimum values. In contrast, the PC detected by use of the buffy coat analyzer were sometimes increased. Useful information can be obtained by measuring PC in blood with platelet clumps; values obtained by use of impedance can be considered minimums, and values obtained by use of buffy coat analysis may be either minimum values or reasonable estimates of PC. (*J Am Vet Med Assoc* 2001;219:1552–1556)

Bleeding disorders are common in domestic animals and may result from thrombocytopenia, thrombocytopathy, coagulopathy, or vasculopathy. Because severe thrombocytopenia is the most common cause of spontaneous hemorrhage in small animals (ie, dogs and cats), accurate assessment of **platelet concentrations (PC)** is essential for determining appropriate diagnostic and therapeutic plans in patients with bleeding disorders. In addition, although mild-to-moderate thrombocytopenia does not result in spontaneous or excessive bleeding, thrombocytopenia may be an important diagnostic clue to the presence of occult infection, inflammation, or neoplasia.[1] Unfortunately, in vitro platelet clumping is a common[1-3] problem that interferes[1,3,4] with the accurate measurement of PC. The inaccurate reporting of PC may have important clinical consequences, such as unnecessary and expensive testing (eg, rickettsial titers, coagulation profiles, bone marrow aspirates) or treatments (eg, doxycycline, heparin).

To avoid reporting inaccurate PC resulting from platelet clumping, a single blood smear made from an aliquot of well-mixed anticoagulated blood is usually examined microscopically. Veterinary diagnostic laboratories vary in the way PC are reported when platelet clumping is detected. Some laboratories report only that the platelets were clumped and provide no number or estimation of the PC. Others report an estimate and note that platelets were clumped or equate platelet clumping with adequate PC.[2,5] Still other laboratories report the PC generated by automated hematologic analyzers as minimum values despite platelet clumping. However, if the size and distribution of platelet clumps in a sample are not homogeneous, the test aliquot could have a disproportionately high number of platelets found within clumps, compared with the entire sample. If platelet clumps break apart in the analyzer, allowing previously clumped platelets to be counted individually, the values determined may be greater than the actual PC.

To our knowledge, there have been no studies confirming the consistent distribution of platelet clumps on every blood smear made from a sample containing clumps. Likewise, despite the common practice of reporting automated analyzer-derived PC as minimum values in the presence of platelet clumping, reports supporting this practice are lacking. The study reported here was designed to help determine an appropriate method of reporting PC values obtained from automated hematologic analyzers when samples contain platelet clumps. We hypothesized that when platelet clumps are present in EDTA-anticoagulated blood samples, these clumps are distributed homogeneously within the sample, and the PC obtained by an impedance analyzer[a] and a quantitative buffy coat analyzer[b] do not overestimate the true PC.

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Materials and Methods

Samples—Homogeneity of platelet clumping was evaluated using 10 EDTA-anticoagulated canine whole blood samples submitted to the University of Missouri Veterinary Clinical Pathology Laboratory for routine hematologic analysis. The platelets in each sample were determined to be clumped by trained laboratory technologists. Samples were used within 1 hour of submission to the laboratory.

A different set of samples was used to evaluate the accuracy of automated PC obtained for samples with platelet clumps. Twenty healthy dogs of both sexes, each > 6 months old and owned by hospital personnel, were identified. A 6-ml sample of blood was collected from each dog by atraumatic jugular venipuncture. The needle was removed from the syringe, and 3 ml of blood was immediately transferred to an opened EDTA collection tube, which was mixed thoroughly by gentle inversion. **Adenosine diphosphate (ADP,** 30 µl; 10 µ*M* final concentration) was added to the remaining 3 ml of blood in the syringe to induce platelet clumping, and the blood was gently mixed by inversion. After 60 seconds, the blood was transferred to a second EDTA collection tube. In this way, platelet clumps were induced in samples for which the actual PC were known.

Smear preparation and evaluation—Each blood smear was prepared from a separate microhematocrit tube. Microhematocrit tubes were filled (after mixing the sample tube with 10 slow inversions) to approximately two thirds full by capillary action, and smears were made, using standard methods. Ten smears were prepared from each blood sample, and 4 smears were made from each of the 20 ADP-treated and untreated samples.

After Wright-Giemsa staining, each slide was scanned microscopically at low magnification (100X) from the feathered edge to the monolayer, and the width of the monolayer was then traversed twice while viewing at a greater magnification (500X). One investigator (SLK) performed all smear evaluations and interpretations. The size of the largest platelet clump for each smear was determined objectively with an ocular micrometer. Size was defined by the clump diameter or by averaging the length and width for elongate clumps. Groups of only 2 to 5 platelets were placed in a "borderline" category, and other clumps were classified as small (< 25 µm), medium (25 to 100 µm), or large (> 100 µm). A subjective scale was used to indicate the concentration of platelet clumps (approx number per unit area) on each smear. Concentration was classified as 3+ (with clumps in almost every 100X field), 2+ (clumping between 3+ and 1+), 1+ (3 to 10 clumps/smear), rare (fewer than 3 clumps/smear), or 0 (no clumps seen). The 10 smears from each sample were examined consecutively.

In addition to the previously described systematic evaluation, blood smears from the healthy dogs were scanned over the entire perimeter to confirm the absence of platelet clumps in the untreated samples. The ADP-treated samples from these dogs were classified into 1 of 2 categories based on the amount of platelet clumping induced by ADP. If at least 1 of the 4 smears from a single ADP-treated sample contained long strings of platelet clumps and 1 or more large (> 100 µm) clumps, clumping was considered severe. All other ADP-treated samples containing platelet clumps were considered to have mild-to-moderate clumping.

Platelet quantification—The impedance and buffy coat analyzers were operated according to manufacturers' directions. Each of the 40 sample tubes was mixed by 10 slow inversions immediately prior to analysis. The impedance analyzer determines PC by detecting particles within a given size interval as they pass through an aperture and impede current across it. Impedance is proportional to size, although cell shape and orientation can affect it. The buffy coat analyzer determines PC by assessing the thickness of the platelet layer in the buffy coat after centrifugation. The thickness of the platelet layer is correlated with platelet number, although platelet size and packing may also affect it.

Statistical analyses—Descriptive analysis was used to compare concentration and size of platelet clumps between smears created from separate aliquots of blood from the same blood sample. A paired *t*-test was used to compare the PC of ADP-treated versus untreated samples. A *t*-test was used to compare the amount of change in PC in samples analyzed by impedance when ADP induced mild-to-moderate versus severe clumping. Analyses were performed by use of commercially available software.[c] Values of $P < 0.05$ were considered significant.

Results

Smear evaluation—Twenty-six blood samples were drawn from 20 healthy dogs. Six of these were not included in the study, because platelet clumping was detected in samples before addition of ADP, and second attempts at phlebotomy from the same dogs produced samples with little or no platelet clumping. Of the 20 untreated samples used in this study, no platelet clumps were detected in 10. However, small clumps distributed at a 1+ concentration were identified in 8 samples, and rare borderline clumps were identified in 2 additional samples. Because clumping was mild when present, all 20 samples were included in the study. For the corresponding samples treated with ADP, all contained clumping that was easily detected at 100X magnification. The amount of platelet clumping was considered severe in 11 samples and mild-to-moderate in 9. The ADP-induced platelet clumps usually consisted of loose groups of granulated platelets, which were individually identifiable. In 1 sample, platelet clumps appeared cohesive, and individual platelets were not distinct.

Clumps or borderline clumps were detected in 100 of 100 blood smears made from blood samples previously identified as containing platelet clumps, although borderline and some small clumps were not readily identified under 100X microscopic power. For 16% of the smears, borderline (n = 9) or rare (7) clumping was the only evidence of platelet clumping (**Fig 1 and 2**). Smears with only borderline clumps came from samples for which other smears usually had a rare or 1+ concentration of borderline or small clumps. The size of the largest platelet clump detected in separate smears of the same sample varied considerably, with 50% of the samples producing smears spanning at least 3 categories. For example, smears made from 1 blood sample included 1 smear with only borderline clumping but another with large clumps. Homogeneity of maximum platelet clump size was evident in only 1 sample. The concentration of clumping also varied considerably among separate aliquots of the same sample, with 40% of the samples producing smears with at least 3 different grades.

Platelet concentration—The PC determined by use of the impedance analyzer were significantly ($P < 0.001$) lower in the ADP-treated samples than in the corresponding untreated samples (**Fig 3**). The ADP-

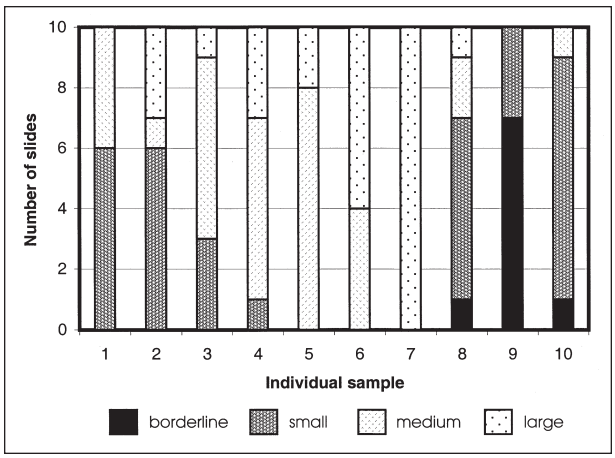

Figure 1—Variation in size of the largest platelet clump in samples of canine blood. Ten blood smears were made from separate aliquots of each of 10 blood samples containing platelet clumps. Platelet clumps were measured with a micrometer, and size categories were defined as follows: borderline (2 to 5 platelets/clump); small (< 25 µm); medium (25 to 100 µm); or large (> 100 µm). The size of the largest clump on each slide remained uniform in 1 sample but varied considerably (over 3 to 4 size categories) in 5 of the samples.

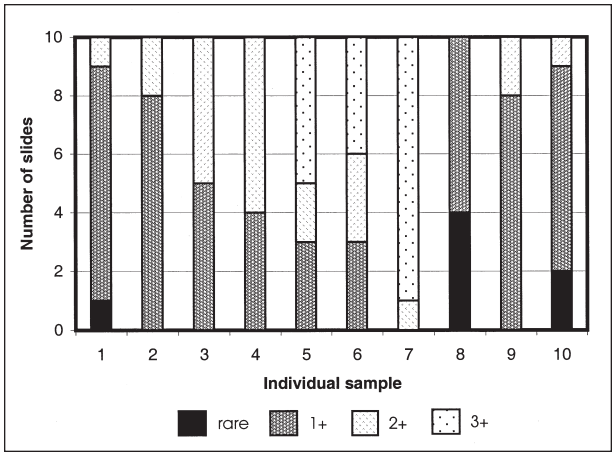

Figure 2—Variation in concentration of platelet clumping (number of clumps per unit area) in blood smears made from samples of canine blood containing platelet clumps. Ten smears were made from separate aliquots of each of 10 blood samples. The concentration of platelet clumps was determined microscopically and classified as the following: 3+ (clumps in almost every 100X microscopic field); 2+ (clumping between 3+ and 1+); 1+ (3 to 10 platelet clumps/blood smear); or rare (fewer than 3 clumps/smear). For all 10 blood samples, the concentration of clumps was inconsistent among the 10 smears. The concentration of clumping varied considerably (over 3 categories) in 4 of the samples.

induced change ranged from +3% to –92%, with a mean and median of –39% and –46%, respectively. The +3% change was within the limits of precision for this analyzer (coefficient of variation = 3.1%).[d] The mean PC prior to addition of ADP was 258,000/µl (median, 257,000/µl); after addition of ADP, the mean PC was 160,000/µl (median, 131,000/µl). After induction of platelet clumps, the PC decreased to < 200,000/µl in 12 of 20 samples and to < 100,000/µl in 6 of 20. When severe platelet clumping was induced by ADP, the percentage change in PC was significantly ($P = 0.005$) greater than that for samples with only mild-to-moderate ADP-induced clumping (**Fig 4**).

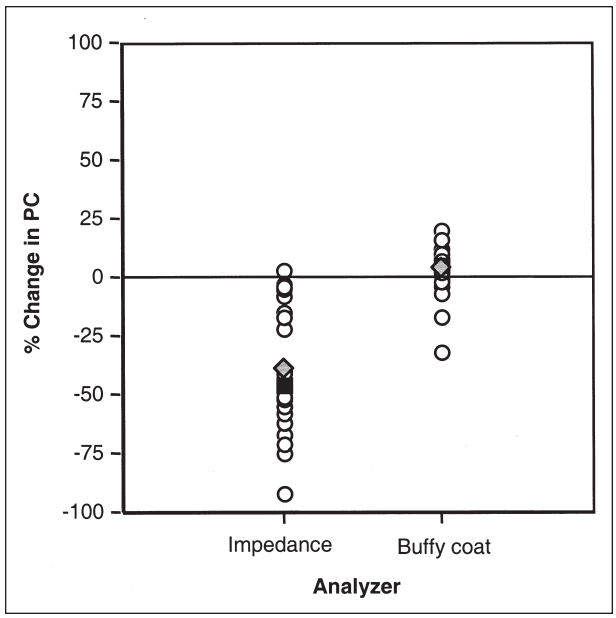

Figure 3—Effect of platelet clumping on platelet concentrations (PC) measured by impedance[a] and quantitative buffy coat[b] analyzers. Blood drawn from healthy dogs was divided, and adenosine diphosphate (ADP) was added to 1 aliquot to induce platelet clumping. The PC were measured from paired samples, with and without (or with minimal) platelet clumping. The PC decreased significantly (paired *t*-test, $P < 0.001$) in the ADP-treated samples when measured by impedance. Using impedance, PC of the ADP-treated samples were always less than or similar to (± 3%) the PC of the corresponding untreated samples (n = 20). Using buffy coat analysis, PC were not significantly changed (paired *t*-test, $P = 0.109$) by ADP-induced platelet clumping; 80% of samples changed by < 10% and 95% of samples changed by < 20%. The PC obtained by buffy coat analysis for ADP-treated samples were frequently greater than the actual PC. Shaded diamond = Mean PC. Black square = Median PC.

In contrast to the significant decreases in PC detected by use of the impedance analyzer, the change in PC detected by use of the buffy coat analyzer ranged from +20% to –32%, with mean and median changes of +4% and +5%, respectively (Fig 3). The mean of the untreated sample PC was 324,000/µl (median 326,000/ml), and the mean of the ADP-treated samples was 339,000/µl (median 338,000/µl). The PC in ADP-treated samples were not significantly different from the PC of the corresponding untreated samples ($P = 0.109$). The changes induced by ADP were usually much greater than the amount of change that could be explained by the intra-assay **coefficient of variation** (**CV**; 1.3%) for the buffy coat analyzer. This CV was determined by performing 10 repeated tests from 2 different blood samples with platelet concentrations similar to the test samples. Platelet clumping induced by ADP decreased PC only 5 of 20 times and only once by > 10 % (when samples were evaluated by buffy coat analysis). The smear examination for the sample with the greatest decrease (–32%) revealed large cohesive clumps of platelets rather than the more typical looser clumps of platelets that could be individually identified in all other instances of ADP-induced platelet clumping. Using the buffy coat analyzer, ADP-induced platelet clumping was associated with an increased PC in 13 of 20 samples.

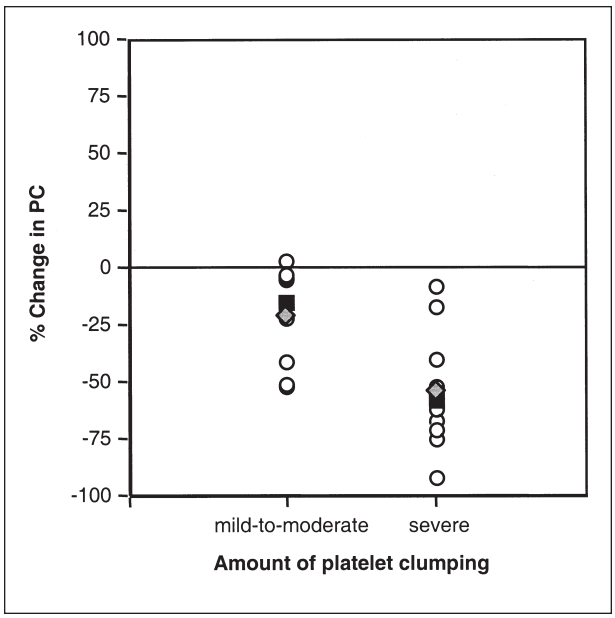

Figure 4—Effect of the amount of ADP-induced platelet clumping on impedance[a]-derived PC. Blood drawn from healthy dogs was divided, and ADP was added to 1 aliquot to induce platelet clumping. The PC were measured from paired samples, with and without (or with minimal) platelet clumping. The amount of platelet clumping was determined by analysis of smears made from the ADP-treated samples. The decrease in PC was significantly greater in samples containing severe platelet clumping, compared with those containing only mild-to-moderate clumping (*t*-test, $P = 0.005$). *See* Figure 3 for key.

Discussion

Despite the finding that platelet clumping is not homogeneous within blood samples containing platelet clumps, we found that PC measured by use of impedance analysis can be considered minimum values when platelet clumps are present. In contrast, PC derived from use of buffy coat analysis are not always minimum values but may offer a clinically reasonable estimate of the actual PC when platelets are loosely clumped.

By any method of platelet analysis, the most accurate PC are determined from a blood sample free of platelet clumps, but collection of such samples may not be possible in every clinical situation. In vitro platelet clumping is minimized by techniques that reduce platelet activation, such as atraumatic venipuncture of large veins, prompt transfer of blood to the anticoagulant, and a 2-tube method of sample collection, which reduces platelet activating factors in the sample by discarding the first and using the second of 2 sequential collection tubes obtained by vacuum methods.[5,6] Despite appropriate sample collection techniques, platelet clumping remains a common problem in veterinary medicine.[1-3] In the present study, samples from the 20 healthy dogs were collected by experienced phlebotomists from the jugular veins of cooperative patients and expediently transferred to anticoagulant, yet 14 of 26 (54%) blood draws contained at least mild platelet clumping. In clinical situations, many obstacles confound our ability to attain samples that are free of platelet clumping. Factors such as patient size, level of cooperation, hemodynamic status, and available venous access may impede collection of an ideal sample. Additionally, by the time platelet clumping is detected by laboratory analysis, an animal may no longer be available for repeated sample collection. Thus, collection of a blood sample lacking platelet clumps may not be feasible in every clinical situation, and estimates of PC may be desired despite the presence of platelet clumps.

Our results revealed that evaluation of a single blood smear will not always be an accurate reflection of the overall degree of platelet clumping within a particular sample. The variability in concentration and size of platelet clumps between different smears made from the same sample indicate that the distribution of platelet clumps within EDTA-anticoagulated blood samples is not always homogeneous. Despite this, platelet clumps were present on all smears made from samples known to contain them. This finding supports the clinical practice of evaluating a single blood smear for the identification of a sample containing platelet clumps as long as the definition of platelet clumping includes borderline and small clumps. Detection of clumps was likely increased by the careful and systematic examination of a greater proportion of the smear, compared with the more cursory clinical method. Less rigorous inspection criteria may have failed to identify clumping in smears containing only borderline (9%) or rare (7%) clumps. Although minimal platelet clumping (rare-to-mild and borderline-to-small clumps) was evident in 10 of 20 untreated samples, it is possible that all 10 of these samples would have been considered free of platelet clumps in a clinical setting. The finding that platelet clumping was not homogeneous allows for the possibility that automated methods may overestimate the actual PC, so this prospect was explored. It also indicates that clumping may be present in a blood sample and may not be recognized with routine clinical detection methods. We did not, however, investigate whether samples considered free of platelet clumping by laboratory technologists consistently produced smears without clumps.

Automated hematologic analyzers that use electrical impedance for identification of platelets are found in large veterinary diagnostic laboratories and private clinics. With this method, platelet clumps may go uncounted or be misidentified as leukocytes or erythrocytes because of their greater volume than individual platelets.[4] In this study, the impedance analyzer did not overestimate the actual PC when clumps were present, and reductions in PC (as a result of platelet clumps) were statistically and clinically significant. If a slide had not been examined, or if platelet clumping had not been reported, more than half of the samples with platelet clumps may have been considered thrombocytopenic, and one third of them would have appeared severely thrombocytopenic. The difference between the actual and measured PC was more likely to be clinically important in samples with severe platelet clumping.

In contrast to large commercial laboratories, private veterinary practices may measure PC by use of buffy coat analysis. According to the owner's manual,[e] small platelet aggregates do not affect measured PC,

but larger aggregates can falsely decrease PC by not separating with the individual platelets in the buffy coat after centrifugation. When many or large platelet clumps collect at the end of the float (which is used to expand the buffy coat), the analyzer alerts (flags) the operator that the reported PC are lower than the true values. In this study, there were no such flags, and we found no significant difference ($P = 0.109$) between PC with or without ADP-induced platelet clumps. Our findings indicated that the buffy coat analyzer often reports a reasonable approximation of the true PC when analyzing samples with platelet clumps. Mild-to-moderate increases and decreases (+20% to –32%) may have been attributable to altered packing in the small space around the float as a result of platelet clumps. Although the buffy coat analyzer we used did not flag any of our samples, it should be stated that blood samples with platelet clumps may generate flags and, thereby, report PC as minimum values.

We found that platelet clumping is not homogeneous within blood samples, and size and concentration of platelet clumps may vary from inconspicuous clumping to obvious large clumps in aliquots of blood obtained from the same anticoagulated sample. Despite this inherent variability in the severity and distribution of platelet clumps, it is reasonable to consider the PC obtained by use of the impedance analyzer, which detects electrical impedance, as equal or less than the true PC. In contrast, the buffy coat analyzer appeared to be less sensitive to platelet clumping; 95% of the time, the PC in ADP-treated samples were within 20% of the PC determined before addition of ADP. Although PC measured by buffy coat analysis may be greater or less than the true value when platelet clumps are present, increases in PC that were attributable to platelet clumping were not great enough to alter diagnostic and therapeutic plans in the dogs we tested. Additionally, considerable platelet clumping is expected to generate flags by the analyzer, indicating the reported value should be considered a minimum. The measured PC determined by buffy coat analysis were likely to be more accurate estimates of the actual PC than were the PC determined by the impedance analyzer in canine blood samples containing platelet clumps.

[a]CELL-DYN 3500 System, Abbott Laboratories, Abbott Park, Ill.
[b]QBC VetAutoread Hematology System, IDEXX Inc, Westbrook, Me.
[c]Sigma Stat, SPSS Science Corp, Chicago, Ill.
[d]Abbott CELL-DYN 3500 System Operator's Manual, Abbott Park, Ill.
[e]QBC VetAutoread Hematology System Owner's Manual, Westbrook, Me.

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