Flow cytometric detection of circulating platelet-derived microparticles in healthy adult horses

Nora L. Springer, DVM; Eliza Smith, BVSc; Marjory B. Brooks, DVM; Tracy Stokol, BVSc, PhD

Objective—To develop a flow cytometric assay to quantify platelet-derived microparticles (PMPs) in equine whole blood and plasma.

Sample—Citrate-anticoagulated whole blood from 30 healthy adult horses.

Procedures—Platelet-poor plasma (PPP) was prepared from fresh whole blood by sequential low-speed centrifugation (twice at 2,500 × g). Samples of fresh whole blood and PPP were removed and stored at 4° and 24°C for 24 hours. Platelet-derived microparticles were characterized in fresh and stored samples on the basis of the forward scatter threshold (log forward scatter < 101) and labeling with annexin V (indicating externalized phosphatidylserine) and CD61 (a constitutive platelet receptor). A fluorescent bead-calibrated flow cytometric assay was used to determine microparticle counts. Platelet counts, prothrombin time, and activated partial thromboplastin time were measured in fresh samples.

Results—Significantly more PMPs were detected in fresh whole blood (median, 3,062 PMPs/µL; range, 954 to 13,531 PMPs/µL) than in fresh PPP (median, 247 PMPs/µL; range, 104 to 918 PMPs/µL). Storage at either temperature had no significant effect on PMP counts for whole blood or PPP. No significant correlation was observed between PMP counts and platelet counts in fresh whole blood or PPP or between PMP counts and clotting times in fresh PPP.

Conclusions and Clinical Relevance—Results indicated that the described PMP protocol can be readily used to quantify PMPs in equine blood and plasma via flow cytometry. Quantification can be performed in fresh PPP or whole blood or samples stored refrigerated or at room temperature for 24 hours. (Am J Vet Res 2014;75:879–885)

Received April 12, 2014. Accepted June 26, 2014.
From the Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853.
Supported by the 2010 American Society for Veterinary Clinical Pathology Share the Future Research Grant.
Presented in part at the 46th Annual Meeting of the American Society for Veterinary Clinical Pathology and the 62nd Annual Meeting of the American College of Veterinary Pathologists, Nashville, Tenn, December 2011.
The authors thank Alexa Fland of the Cornell University Equine Research Park for technical assistance.
Address correspondence to Dr. Springer (nls67@cornell.edu).

ABBREVIATIONS

aPTT Activated partial thromboplastin time
CV Coefficient of variation
FITC Fluorescein isothiocyanate
FL1 First fluorescence detector
FL2 Second fluorescence detector
FL3 Third fluorescence detector
PE Phycoerythrin
PMP Platelet-derived microparticle
PPP Platelet-poor plasma
PT Prothrombin time

Microparticles (also sometimes referred to as microvesicles) are small (approx 0.1 to 1 µm in diameter) vesicles shed from cell membranes by diverse mechanisms, including cellular activation, differentiation, senescence, and apoptosis.1,2 They are derived from many cell types and retain membrane antigen and cytoplasmic components from their cell of origin. Particular attention has been given to PMPs because of the essential role of the platelet membrane in coagulation. Platelets activated under conditions of sustained high intracellular calcium express phosphatidylserine, a negatively charged phospholipid, on their outer membrane leaflet. Platelet-expressed phosphatidylserine (originally referred to as platelet factor 3) is critical for generation of thrombin by providing binding sites for coagulation factors, promoting their assembly into highly active complexes, and protecting them from inhibitors.3,4 One study5 has shown that the procoagulant activity of a single PMP is approximately equal to that of an activated platelet, despite the difference in respective surface area. The same study5 suggested that PMPs have a procoagulant activity 50 to 100 times as great as activated platelets. The importance of PMP in thrombin generation is illustrated by Scott syndrome, an inherited bleeding disorder in people and German Shepherd Dogs characterized by inefficient thrombin formation caused by an inability to exteriorize phosphatidylserine or generate phosphatidylserine-expressing
microparticles. Increased circulating PMP concentrations have been described in thrombotic conditions such as thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, stroke, deep vein thrombosis, and pulmonary embolism in human patients, suggesting a pathogenic role for PMPs in these disorders. For example, a study in patients with a first episode of acute coronary syndrome found that high PMP concentrations were an independent predictor for a secondary thrombotic event and poorer clinical outcome at 1 year. Additionally, circulating PMP concentrations positively correlated to thrombus weight in a model of venous thrombosis in mice. Therefore, detection and quantification of PMPs may be useful in predicting patient risk for thrombosis. 

Early identification of nonovert coagulopathies is essential in management of critically ill equine patients. In particular, thrombosis is a common and potentially fatal consequence of several disorders in horses, such as gastrointestinal disease, neonatal sepsis, laminitis, and equine herpesvirus infection. The underlying cause of thrombosis is unknown, but it is likely related to systemic inflammation, given that inflammatory cytokines trigger coagulation. Because of the proinflammatory effects of activated coagulation factors and platelets, a positive feedback loop between inflammation and coagulation ensues, which results in a hypercoagulable state that can lead to microthrombosis and eventually culminate in overt disseminated intravascular coagulation. Considering the relationship between inflammatory disease and thrombosis in critically ill equine patients, it has been recommended that any equine patient meeting criteria for systemic inflammatory response syndrome should undergo testing for subclinical coagulopathies, particularly a hypercoagulable or prethrombotic state.

Unfortunately, there is a current lack of suitable diagnostic assays for assessing a hypercoagulable state in horses. Given that PMPs have been detected in various thrombotic syndromes in humans and numbers have been shown to be associated with additional thrombotic events, it is possible that quantification of PMPs will be a clinically useful marker of hypercoagulability. To evaluate the role of PMPs in thrombotic disorders and the diagnostic utility of PMP counts for detecting horses at risk for thrombosis, a method to detect and quantify PMPs in equine blood and plasma is needed. Flow cytometry is the most commonly used technique for quantification of PMPs in humans, and recent studies have used flow cytometry to detect microparticles in healthy dogs and dogs with Scott syndrome and in stored canine RBC concentrates. Flow cytometry was also used recently by our group for semiquantifying PMPs in agonist-stimulated equine leukocyte- and platelet-rich plasma before and after clopidogrel administration to healthy horses. The aims of the study reported here were to use a bead-calibrated flow cytometry assay to standardize enumeration of PMPs in equine citrate-anticoagulated whole blood and PPP; determine whether there is any association between PMP counts in whole blood and PPP and routine screening coagulation tests, specifically platelet counts, PT, and aPTT; and evaluate the effect of sample storage for 24 hours at 4° or 24°C on PMP counts in whole blood and PPP.

### Materials and Methods

#### Samples

Blood samples were collected from university-owned healthy horses housed at the Cornell University Equine Research Park or Research Park Annex. All horses were deemed to be in good health by clinical assessment by their caretakers, examination by a single author (NLS), and hemogram findings within reference intervals. No horses had received any medications in the 10 days prior to sample collection. The study was approved by the Cornell University Animal Care and Use Committee.

#### Blood collection and preparation of samples for PMP measurement

All samples were collected between August 2011 and May 2012 by atraumatic jugular venipuncture by means of vacuum assistance with a 20-gauge blood collection needle and 4.5-mL citrate blood collection tubes. Nine milliliters (two 4.5-mL tubes) of citrated whole blood was collected from each horse, after discarding the first few milliliters of blood from the needle. Samples were maintained at room temperature (22° to 24°C) for 30 minutes prior to processing into PPP in the laboratory. After combining the blood from both tubes, samples of whole blood were removed for same-day PMP analysis or for storage at 4° or 24°C for PMP analysis at 24 hours. To obtain PPP, the remaining whole blood sample was centrifuged at 2,500 X g for 10 minutes at 20°C. The harvested supernatant was centrifuged again to create PPP. Similar to whole blood, samples of PPP were removed for same-day PMP analysis or stored at 4° or 24°C for 24 hours.

#### Screening coagulation tests

Platelet counts were measured in both fresh and stored whole blood and PPP samples with an automated hematology analyzer prior to flow cytometric PMP analysis. Platelet counts were corrected for citrate dilution by multiplying the automated count by a factor of 1.1. Blood smears were made from whole blood samples to verify the automated count and assess for the presence of platelet clumps, which were scored according to a previously reported method. Prothrombin time and aPTT were measured in fresh PPP with an automated coagulation analyzer, and aliquots of a pooled equine control plasma were also assayed as reagent controls.

#### Flow cytometric quantification of PMPs

The PMPs were defined by their small size (log forward scatter, < 10³) and dual labeling with annexin V-FITC (a protein that binds to outer membrane phosphatidylserine) and CD61-PE (an antibody directed against the platelet membrane antigen GpIIbIIIa), as previously described. Quantification was performed by simultaneous analysis of 7.7-µm fluorescent counting beads on fresh whole blood and PPP and samples stored for 24 hours at 4° or 24°C. Analysis was performed on fresh whole blood and PPP within 4 hours after blood sample collection. For analysis, 3 µL of PPP or 2 µL of whole blood and 5 µL of glycine-proline-arginine-proline (an inhibitor of fibrin polymerization; final concentration, 1mM) were added to annexin-binding buffer (10mM...
HEPES, 150 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, and 1.8 mM calcium chloride) to a final reaction volume of 100 µL. The samples were then incubated with annexin V-FITC (1:600 final concentration) and CD61-PE (1:20 final concentration) in the dark at room temperature for 30 minutes. A negative control consisted of whole blood or PPP incubated in annexin-binding buffer with an isotype IgG. The reaction was quenched with 600 µL of annexin-binding buffer and 25 µL of fluorescent counting beads added to each sample after vigorous vortexing to disperse bead aggregates. Each sample was analyzed in duplicate, and a positive control of frozen platelets was included with each run. Platelets fragment after a freeze-thaw cycle, and the fragments, whose size overlaps with that of PMPs, usually express CD61 and phosphatidylserine. Platelets for freezing were derived from platelet-rich plasma by low-speed centrifugation (250 × g for 10 minutes) of leukocyte- and platelet-rich plasma, which was obtained after 20 minutes of gravity sedimentation of whole blood from a single horse. The platelet-rich plasma was then frozen at −20°C in aliquots and thawed at room temperature before use.

Flow cytometric analysis was performed on a flow cytometer with the manufacturer's software package and the following settings: forward scatter, side scatter, and fluorescence were performed on log mode; voltage for FL1 (annexin V-FITC) was 670 V; voltage for FL2 (CD61-PE) was 540 V; voltage for FL3 (beads) was 530 V; forward scatter threshold was 20 U; side scatter threshold was 100 U; compensation for FL1 was 4% of FL2; and compensation for FL2 was 12% of FL1. A microparticle gate was created on a forward scatter versus side scatter dot plot, and a fluorescent counting bead gate was created on a FL3 versus a forward scatter dot plot (Figure 1). Platelet-derived microparticles were defined as those events that were double-positive for CD61 and annexin V on a dual fluorescence quadrant plot of the microparticle gate. Events were collected under low flow rates, and acquisition ceased when 2,500 events were counted in the fluorescent bead gate. The absolute number of PMPs/µL was calculated by the following formula:

\[
\text{Absolute number of PMPs/µL} = \frac{\text{number of events in test sample} \times \text{number of beads per sample}}{\text{volume of test sample initially used}}
\]

There were 2,500 bead events and 25,506 beads/25 µL for the lot number used in this study.

An intra- and interassay CV for PMP quantification was determined by analyzing both pooled PPP (representing a sample with a low number of events) and frozen-thawed pooled platelet-rich plasma (representing a sample with a high number of events). Both

![Figure 1—Gating strategy and representative scatter dot plots for the flow cytometric detection and enumeration of PMPs. A—Forward versus side scatter dot plot for whole blood sample. Notice that the 7.7-µm-diameter fluorescent counting beads are not distinguishable from RBCs. B—Dot plot for PPP sample containing 7.7-µm-diameter fluorescent counting beads. The region containing counting beads and residual RBCs is indicated (circle). C—Forward scatter versus FL3 plot to define the region containing 7.7-µm-diameter fluorescent counting beads (circle) on the basis of their size and high intensity fluorescence. D—Dual fluorescence dot plot of the microparticle (R1) gate in whole blood from panel A. Events that are double positive for CD61 and annexin V are found in the upper right quadrant and were classified as PMPs (percentage of events in this quadrant are shown). E—Dual fluorescence dot plot of the microparticle (R1) gate in PPP from panel B (percentage of double positive CD61 and annexin V events in the R1 are shown). F—Dual fluorescence dot plot of frozen-thawed platelets, which were used as a positive control sample. FSC = Forward scatter. R1 = Microparticle gate defined by log forward scatter < 10. R2 = Platelet gate. SSC = Side scatter.](image-url)
samples were stored at −20°C in individual aliquots and thawed immediately prior to use. For the intra-assay CV, a single aliquot of both samples were analyzed in 10 replicates on a single day. For the interassay CV, each sample was analyzed on 10 consecutive days, with a new aliquot on each day of analysis.

**Statistical analysis**—Statistical analysis was performed with the aid of commercially available software. Datasets for coagulation tests and PMP numbers were assessed by a Shapiro-Wilk test for normality and found to have parametric and non-parametric values, respectively; values for coagulation tests are reported as mean ± SD, and PMP counts are reported as median (range). Mean platelet counts in whole blood in the tested samples were compared by means of repeated-measures ANOVA, followed by a paired t-test, with Bonferroni correction for pairwise comparisons (P < 0.017). Similarly, median PMP counts were compared in tested whole blood or PPP by means of a Friedman test, followed by a Dunn test (P < 0.017). Median PMP counts in fresh whole blood and PPP were compared by means of a Wilcoxon signed rank test (P < 0.05). Spearman rank correlation coefficients were used to measure the association between PMP counts in fresh whole blood and PPP (P < 0.05) and between coagulation test results in fresh whole blood and PPP (P < 0.017).

**Results**

The sampled population of horses (n = 30) consisted of 23 mares, 4 stallions, and 3 geldings. The mean age was 14 years, with a range of 8 to 25 years, for horses. The sampled population of horses (n = 30) consisted of 23 mares, 4 stallions, and 3 geldings. The mean age was 14 years, with a range of 8 to 25 years, for horses. The mean age was 14 years, with a range of 8 to 25 years, for horses. The mean age was 14 years, with a range of 8 to 25 years, for horses.

Corrected platelet counts were significantly higher in fresh whole blood (126 ± 31 × 10^3 platelets/µL; reference interval, 94 ± 31 × 10^3 platelets/µL) to 232 ± 31 × 10^3 platelets/µL than either whole blood stored at 4°C (110 ± 33 × 10^3 platelets/µL; P = 0.001) or whole blood stored at 24°C (116 ± 23 × 10^3 platelets/µL; P = 0.004). However, this difference was within the analytic variation of the assay for the analyzer used in this study and unlikely to be biologically relevant. Platelet counts did not differ significantly (P = 0.17) between whole blood stored at 4°C or 24°C. Platelet clumping was observed in samples from individual horses in fresh and stored whole blood (Table 1), and significantly more clumps were seen in whole blood stored at 4°C versus whole blood analyzed when fresh (P = 0.001) or after storage at 24°C (P = 0.013). No significant (P = 0.35) difference was found in platelet clumping between fresh whole blood and whole blood stored at 24°C. The platelet count was 7.3 ± 4.3 × 10^3 platelets/µL in fresh PPP, confirming depletion of platelets in the prepared plasma. The PT in fresh PPP was 12.4 ± 4.2 seconds (reference interval, 11.0 to 15.0 seconds) and the aPTT was 47.0 ± 4.0 seconds (reference interval, 43 to 61 seconds).

Platelet-derived microparticles were detected in both fresh whole blood and PPP, but median counts were significantly (P < 0.001) higher in fresh whole blood (Table 2). No correlation (R = 0.11; P = 0.57) was found between the PMP count in fresh whole blood versus PPP. No correlation was found between the PMP count in fresh whole blood and the PT or aPTT (data not shown). A weak negative correlation (R = −0.41; P = 0.03) was found between the PMP count in fresh whole blood and platelet count. No correlation was found between the PMP count in fresh PPP and the platelet count, PT, or aPTT (data not shown).

Compared with fresh whole blood, median PMP counts in whole blood samples was lower at 24 hours at both storage temperatures; however, these results were not significantly different (Table 2). In contrast, median PMP counts were slightly, but not significantly, higher in PPP stored for 24 hours at 4°C, with minimal changes occurring in median PMP counts in PPP stored at 24°C.

The intra-assay CVs of the PMP counts in pooled PPP and dual-labeled low forward scatter events in the positive control frozen PPP samples were 28% and 18%, respectively. The interassay CV was 22% and 31% for pooled PPP and frozen platelet-rich plasma, respectively.

**Discussion**

This report describes a standardized flow cytometric method for characterizing and enumerating PMP in equine fresh and stored whole blood and PPP on the
basis of forward scatter thresholding, labeling with an anti-CD61 antibody and annexin V, and comparison to particle counts with a fluorescent bead calibrator. The flow cytometric protocol was adapted from one used for quantifying MPs in canine blood. This protocol differs from a previously described procedure in horses. The procedure used in the present study involved measuring PMP in different samples (whole blood and PPP vs platelet-rich plasma) and the use of fluorescent counting beads designed for absolute cell counting to quantify MPs rather than the relative number of MPs/s, 7500 CD61-positive events in a platelet gate, as in that previous study. The technique used in the previous study is not directly applicable for PPP samples because platelet numbers are greatly diminished from these samples. In the present study, PMP counts in fresh equine whole blood or PPP did not correlate or correlated weakly to traditional hemostasis tests (platelet counts, PT, and aPTT). The effects of storing whole blood or PPP at 4° or 24°C for 24 hours on PMP counts was also assessed because it is likely that PMP quantification by flow cytometry will be a referral rather than a point-of-care test. The number of MPs in whole blood or PPP was not significantly affected by sample storage at either temperature.

The PMP counts in equine whole blood and PPP overlapped but were generally lower than that observed in canine samples that were similarly analyzed. The PMP samples in the canine report were collected from a single centrifugation step and may have contained more large MPs or small platelets. Alternatively, the difference may reflect the inherently lower platelet counts and smaller platelet size in horses, compared with dogs. As described for both human and canine samples, there was substantial variation in the absolute numbers of PMP quantified in whole blood and PPP in individual horses. This variation may be explained by inherent in vivo differences in PMP counts, differences in blood collection and processing with ex vivo generation of MPs (potentially resulting from activation or mechanical shearing of some platelets), and analytic variation, which was quite high for pooled PPP and frozen platelets. Several preanalytic variables, particularly the time between sample collection and processing, physical transport of the sample (degree of agitation), and centrifugation technique (duration, number of steps, and centrifugation force), substantially influence microparticle counts. In fact, a study revealed that there was substantial variation in PMP counts provided by different laboratories, even when the same instrumentation and sample (frozen PPP) were used. This variability with the same sample type could be the result of differences in sample handling, reagents (use of filtered or nonfiltered solutions for diluting and labeling samples), and instrument resolution, calibration, and maintenance. To facilitate comparisons among laboratories and minimize preanalytic influences on PMP counts, a standardized protocol for PMP quantification in human plasma has recently been proposed. Unfortunately, these recommendations were published just prior to completion of the present study.

The high analytic variation of PMP counts could be the result of an inability of the analyzer to discriminate between low numbers of small-sized events. A recent study found that newer generation flow cytometers have greater sensitivity for quantification of PMP in human PPP than older models, and analyzer sensitivity should be considered when evaluating results. Because of the high variation in PMP counts, it is possible that this flow cytometric–based assay is less sensitive and may not be able to discriminate between those patients that are or are not at risk of thrombosis. However, the diagnostic utility of PMP quantification remains to be tested in defined cohorts of sick animals (eg, horses with colitis).

In this study, PMP counts were far higher in whole blood than in PPP and no correlation was found between PMP counts in fresh whole blood and PPP, indicating that PMP counts in PPP cannot be predicted from counts in whole blood in horses. This is in contrast to the situation in dogs, in which there is a weak but significant positive correlation between PMP counts in whole blood and PPP. The higher counts in whole blood versus PPP could be caused by inclusion of small or fragmented activated platelets in the microparticle gate, which were subsequently removed during centrifugation, or loss of microparticles by binding to other cells during centrifugation. By use of the available instrumentation and fluorescent markers, it was impossible to discriminate between activated small or fragmented platelets and true microparticles generated in vivo from physiologic processes. Although efforts were taken to minimize ex vivo platelet activation in this study (eg, samples were maintained at room temperature for 30 minutes before processing), it is possible that platelets were activated during venipuncture, sample transport, or preparation for analysis.

A single outlier (markedly higher PMP counts) was found among each of the samples of fresh whole blood and PPP, although the outlier values were not obtained from samples from the same horse. When the original flow cytometric data were reexamined to determine a cause for these 2 higher results, a higher number of total events were observed for these outliers, compared with results from the other horses. Additionally, a greater number of CD61-negative, annexin V-positive events were seen in the fresh whole blood outlier, suggesting this horse had circulating microparticles from other cell types. It is possible that the higher number of total events for both outlier values was the result of fragmentation or activation of platelets during sample processing, despite the care taken to minimize platelet activation or shearing. This could be the result of the use of evacuated tubes to obtain the blood samples, with the vacuum possibly generating turbulence, which could activate or damage individual platelets. However, evacuated tubes have been used in multiple studies for quantifying MPs in human patients, and evacuated tubes are commonly used for obtaining blood samples from horses in clinical practice. The data were reanalyzed after excluding these 2 outliers, and the results were unchanged.

In this study, the PMP counts in whole blood or PPP were not correlated with PT and aPTT (measured by routine screening coagulation assays) in healthy horses. This is not surprising because these assays
are designed to detect deficiencies or decreased function of coagulation factors, not platelet activation. Also, these assays are performed in a cell-free system with an excess of phospholipid and any contribution from PMP-associated phosphatidylserine is likely to be minimal. Future studies are needed to assess the association of PMP counts to PMP apoptosis or activation. It is possible that the negative correlation between platelet and PMP counts in equine whole blood in this study was caused by fragmentation or activation of platelets (which will decrease the platelet count and increase the PMP count) or inclusion of small platelets within the PMP gate. Some platelet clumps were observed in fresh whole blood samples, which could also have affected the observed association between PMP counts and platelet counts.

Median PMP counts did decrease at both storage temperatures in whole blood, albeit not significantly. Storage-associated platelet clumping may be contributing to the decrease in PMP counts by reducing the platelet surface area available for shedding of PMP. However, this is unlikely to be the sole explanation, considering that there was little difference in the median PMP count between samples of whole blood stored at 4° or 24°C, despite the fact that significantly more clumping was seen in smears from whole blood stored at 4°C than fresh whole blood or whole blood stored at 24°C. Alternative mechanisms include disruption of microparticles (so they were too small to be discriminated by the analyzer) or PMPs becoming bound to other blood cells (leukocytes or erythrocytes) during storage and subsequently not being detected in the defined microparticle gate. In contrast, a mild increase in PMP counts was seen in PPP stored at 4° versus 24°C, although the change in median PMP counts was not significant. We attributed the increase in PMP counts in PPP stored at 4° versus 24°C to cold-induced activation of the few remaining platelets in this sample type.26 Regardless, this study shows that citrate-anticoagulated whole blood or PPP samples can be maintained at 4° or 24°C for up to 24 hours for PMP quantification, although 24°C would be the optimal storage temperature to minimize the potential for cold-induced activation.

In summary, this study characterized PMPs in whole blood or PPP for use in evaluating the role of PMPs in thrombotic and other conditions in horses. We determined that PMPs could be detected in equine whole blood and PPP and that storage for 24 hours at either 4° or 24°C did not significantly affect PMP counts. The stability of PMPs in stored blood increases the likelihood that PMP counts could be used as a diagnostic test because samples shipped via overnight courier could be tested. However, additional studies are needed to evaluate PMP in well-defined cohorts of ill horses and to define any association of PMP counts to patient outcomes and relate PMPs to other laboratory parameters of hypercoagulability, such as calibrated automated thromboelastography27 and viscoelastic testing.28 The protocol developed in this study will provide a foundation for future studies on the pathophysiologic relevance of circulating PMPs in horses.

References


