

Determining optimal storage time and temperature for the detection of red blood cell and platelet surface associated immunoglobulin by flow cytometry in healthy horses

by

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Abstract

Immune-mediated destruction of red blood cells or platelets is an important consideration when approaching severe anemia or thrombocytopenia, respectively. Differentiating immune-mediated disease from other causes of these cytopenias can be challenging. While the magnitude of decrease in hematocrit or platelet count is helpful, subjectively, this is not always a reliable indicator as other disease processes can also cause marked anemia or thrombocytopenia. A flow cytometric assay used to detect surface-associated immunoglobulin on red blood cells and platelets has been developed to aid in this differentiation in dogs and horses; however, with limited use and a lack of assay standardization. There is a need to standardize assay protocols so that there is consistency between laboratories. It is important for clinicians to understand the results and the possible pre-analytical variables that may affect the reliability of the assay. Currently, there are vast differences in equipment, reagents, sample conditions, and how results are reported. The limited availability of this assay requires samples to be shipped to one of the few reference laboratories equipped to perform it, and there is limited knowledge on how pre-analytical variables, such as the effects of sample storage or shipping conditions, may have on assay performance. Few isolated studies have evaluated the effects of sample storage conditions on the assay results in dogs; however, to the author's knowledge this has not been assessed in horses. The objective of this study is to identify optimal storage time and temperature of equine whole blood for the detection of red blood cell and platelet surface-associated immunoglobulin concentrations via flow cytometry. Both assays were performed on samples at time of collection (0 hr), and then again at 4, 24, 48, and 72 hours post collection. The red blood cell samples were stored at 4°C and the platelet samples stored at 4°C and room temperature.

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Chapter 1 - Immune-mediated disease in veterinary species

Pathophysiology

Immune-mediated diseases encompass a wide variety of conditions recognized in both people and veterinary species. These diseases occur when the immune system reacts inappropriately towards the body's own cells by recognizing them as "foreign" as opposed to "self", and ultimately leading to their destruction. Immune-mediated destruction of circulating red blood cells and platelets are the most common examples of immune-mediated disease in veterinary species. This occurs due to the formation of autoantibodies targeting epitopes on the surface of the red blood cell or platelet, leading to receptor-mediated hemolysis or phagocytosis and destruction via macrophages (Figure 1). The presence of cell surface-associated antibodies is attributed to spontaneous production of autologous antibodies directed towards unaltered red blood cells or platelets (primary), or against red blood cells or platelets that have been antigenically altered via secondary causes.¹ These secondary causes include neoplastic, drug-associated, and infectious etiologies. The incidence of primary versus secondary immune-mediated disease, as well as the most common secondary causes, varies across species.²

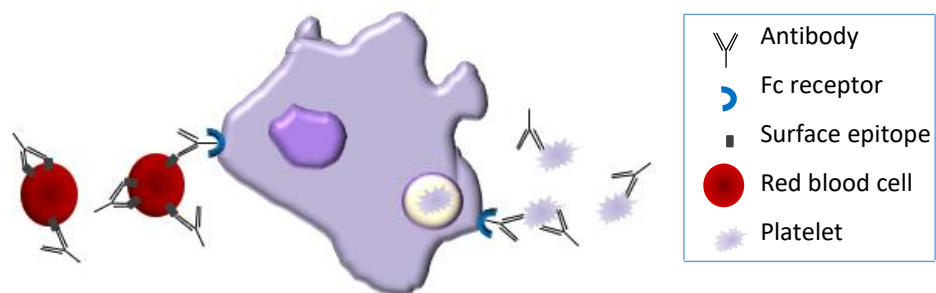


Figure 1: Autoantibodies targeting surface epitopes on red blood cells (left) and platelets (right). The autoantibodies bind to Fc receptors on macrophages (middle), leading to receptor mediated hemolysis or phagocytosis and destruction in patients with immune-mediated hematologic disease.

Immune-mediated thrombocytopenia (ITP) in veterinary species is not entirely understood and therefore most of the current literature is extrapolated from studies on the human condition. In this autoimmune disease, the autoantibodies target glycoproteins on the surface of the platelet, which leads to clearance by the mononuclear phagocytic system, primarily of the spleen.³ Antibody-mediated destruction is not the only mechanism resulting in platelet clearance. Patients with ITP cannot maintain self-tolerance due to dysfunctional T and B regulatory cells. This loss of self-tolerance results in the generation of autoantibody producing B cells as well as autoreactive cytotoxic T cells that directly induce platelet destruction.³

Immune-mediated hemolytic anemia (IMHA) and thrombocytopenia in horses

Anemia in horses is associated with hemorrhage (loss), decreased production of red cells by a multitude of mechanisms, or increased peripheral destruction of red blood cells via immune-mediated hemolysis. Thrombocytopenia in horses may result from decreased bone marrow production, increased utilization or consumption, sequestration, or increased peripheral (often immune-mediated) destruction. Identifying the cause of anemia or thrombocytopenia in horses is often challenging, therefore making treatment and management difficult. In horses, a suspected regenerative anemia with no clinical evidence of hemorrhage is commonly attributed to an immune-mediated process; however, criteria defining this diagnosis in horses is lacking. For severe thrombocytopenia, an immune-mediated disease is often among the top considerations; however, confirmatory testing is limited, making it a diagnosis of exclusion. The ability to recognize immune-mediated disease in these patients is crucial for prognosis and treatment. While information on IMHA and ITP in horses is mostly limited to case reports or small case series, it is concluded that these horses typically respond positively to immunosuppressive therapy and in cases of primary disease, have prolonged survival times when treated.⁴

Methods for diagnosis

Diagnosis of IMHA in horses is made based on clinical evidence of anemia and the detection of antibodies bound to the surface of the red blood cells (RBC-Ig). The most common, and widely available assay to aid in this diagnosis is the direct antiglobulin test (DAT or Coombs test). The Coombs test involves washing the patient's red blood cells and adding them to an antiglobulin reagent that binds the surface-associated antibodies, that if present, results in agglutination. The presence of agglutination represents a positive test suggesting the presence of autoantibodies. The benefits of this assay include its availability, its relatively low cost, and the lack of specialized equipment needed to perform this assay. The endpoint of the assay, agglutination, requires an optimal ratio of red blood cell antibody to antiglobulin to result in cross-linking of red blood cells or "lattice" formation. Therefore, a limitation of this assay is the high incidence of false negatives resulting in a low sensitivity and poor negative predictive value. Reported sensitivities range from 53% to 82% in dogs ^{1,2,5} with a specificity of 94% to 100%.² The same studies calculated a positive predictive value of 100% and a negative predictive value of 62% and 68%.^{1,5} Several factors can contribute to a false negative result, including an insufficient quantity of antibody or complement on red blood cells, poor technique, such as excessive washing of red blood cells or delayed processing, or most notably, a phenomenon called the prozone or postzone effect.^{1,6} The amount of reagent added must be carefully controlled to avoid these effects. The prozone effect refers to an excess in antibody and the postzone effect refers to an excess of reagent. If the proportion of reagent to antibodies is too low or too high, there will be an interference with the lattice formation resulting in a lack of agglutination, and therefore a false negative test. Sensitivity and specificity data in horses is lacking. Another potential limitation of the Coombs test is that it does not readily identify the

class of antibody bound to the erythrocyte surface. Differentiation between IgG and IgM requires incubation at 4°C and 37°C, with the assumption that IgM is involved in cold-reacting agglutination and IgG is involved in warm-reacting agglutination.¹ The limitations of this assay warranted development of more sensitive diagnostic methods using monospecific antibodies.^{1,5}

Other methods have been explored, including indirect antiglobulin tests, a direct enzyme-linked antiglobulin test, and quantitative radioimmunoassay; however, these assays are not widely used in veterinary medicine due to low sensitivities or inconsistent performance without a skilled laboratory technician.¹

Currently, there are no widely available assays for the diagnosis of ITP in veterinary species. The detection of platelet surface-associated immunoglobulin (PSA-Ig) has been assessed by enzyme-linked immunosorbent assays (ELISA), immunofluorescence, and immunoradiometric methods; however, none of these are commonly used in veterinary medicine.^{7,8} Typically, ITP is a diagnosis of exclusion, based on the severity of the thrombocytopenia and the response to immunosuppressive therapy. Immunosuppressive therapy is not a benign treatment so ideally an immune-mediated process is confirmed prior to initializing therapy. The lack of widely available assays for the reliable detection of immune-mediated anemia and thrombocytopenia demonstrates a clear need for an assay capable of detecting and quantitatively assessing red blood cell or platelet surface-associated immunoglobulins, thereby confirming the presence of immune-mediated disease in order to refine diagnoses and guide additional diagnostic efforts and treatment.

Flow cytometric detection of RBC-Ig and PSA-Ig

Flow cytometry is used to assess the physical and functional characteristics of cells in a fluid suspension and has a wide range of applications in both human and veterinary medicine.

When a sample is run through a flow cytometer, the cells are rapidly interrogated within a fluid stream (Figure 2). The cells within the fluid stream pass through a laser resulting in immediate analysis of its light scatter properties, including size, which correlates to forward scatter, and complexity which correlates to side scatter. The use of fluorescent reagents, such as fluorescent dyes conjugated to an antibody, allows the cytometer to identify subpopulations of cells. This data is then transferred and displayed in the form of scatterplots and histograms demonstrating the presence and characteristics of the cell population of interest. The ability to identify and interpret a specific population of cells requires the use of appropriate controls. Unlabeled cells are used to assess for background autofluorescence, so that it can be accounted for when interpreting sample data. Isotype controls are used to assess for nonspecific antibody binding. An isotype control should be identical to the primary antibody, but specifically target an antigen that is not found within the cell population being analyzed.

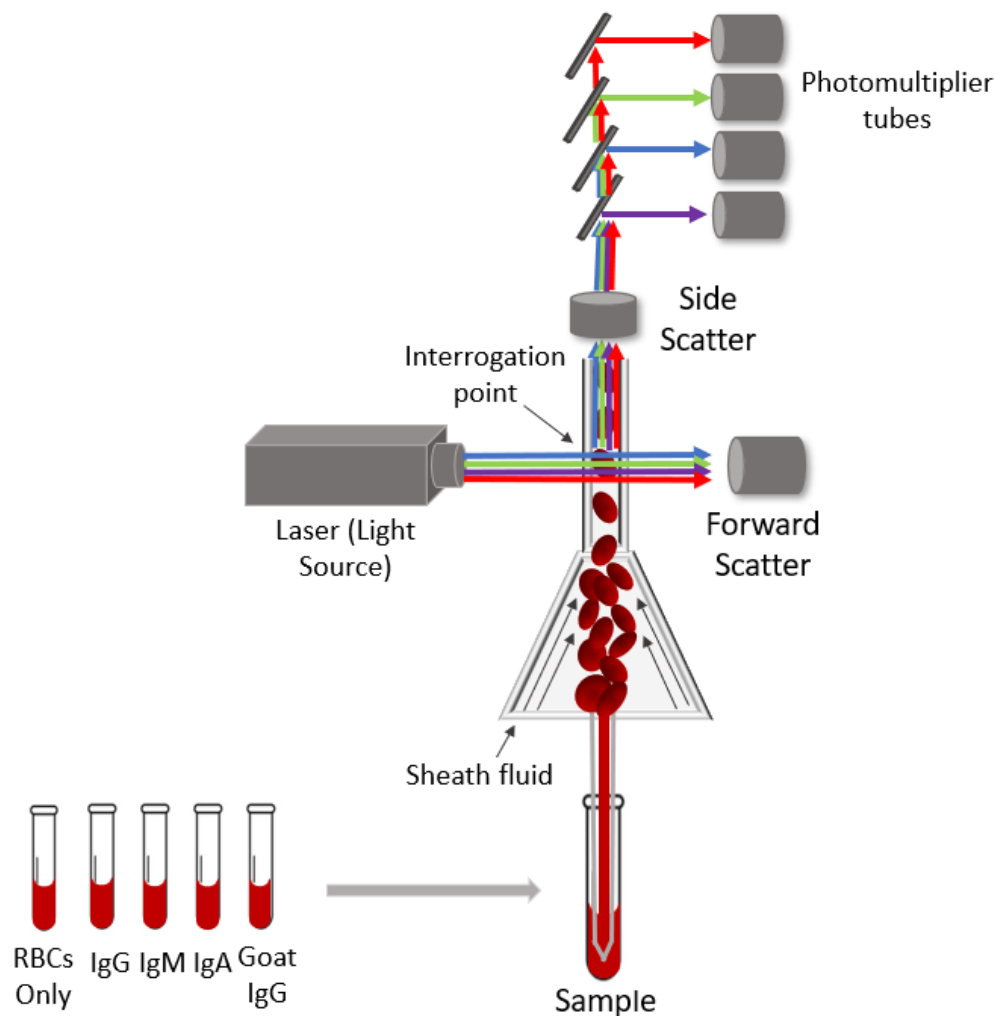


Figure 2: Components of the flow cytometer. The cells of interest are aspirated, arranged single file in a fluid stream, and interrogated by a laser source. The scattered light and fluorescence are collected by the photomultiplier tubes and converted into electronic signals for computer analysis.

Common applications in veterinary medicine include blood cell enumeration and differentiation, immunophenotyping for the diagnosis of leukemia and lymphoma or identification of subsets of lymphocytes, and quantifying DNA content in normal and neoplastic cells to assess ploidy and proliferative activity.⁹ Flow cytometry and direct immunofluorescence labeling techniques have also proven useful in detecting red blood cell and platelet surface-

associated antibodies on canine and equine red blood cells and platelets in cases of immune-mediated disease.⁹ These antibodies can be detected and quantified by incubating a patient sample with fluorescent-labeled class-specific antibodies before interrogation by the flow cytometer. The data is presented as a percentage of positively labeled cells (Figure 3). Multiple studies have shown this assay to be highly sensitive in the detection of RBC-Ig in dogs, with sensitivities of 100% and 92% and a negative predictive value of 100%.^{1,5} Additionally, flow cytometry has proven useful in quantitatively identifying the specific classes of surface-associated immunoglobulin on dog and horse erythrocytes in cases of IMHA.¹ A study identifying RBC-Ig isotypes in dogs with IMHA concluded that dogs with IMHA with ≥ 2 Ig erythrocyte bound isotypes, particularly IgG and IgM, were likely to have a more severe anemia, spherocytosis, and agglutination.¹⁰ Horses with IMHA are most commonly associated with IgG. IgG has been deemed responsible for neonatal isoerytholysis as well as agglutination of penicillin-coated equine red blood cells in horses with IMHA.¹¹ IgA has been associated with autoimmune diseases in dogs and has been theorized to be involved in the development of purpura hemorrhagica with subsequent IMHA.¹² Due to the differing roles of the class-specific isotypes in various causes of immune-mediated disease and the proposed association with severity of disease in dogs with IMHA, an assay able to detect and quantify class-specific isotypes is beneficial. Overall, this assay is quantitative, rapid, relatively simple, and has improved sensitivity over the Coombs test, as it is not influenced by the prozone effect.⁹ Major limitations of flow cytometry include lack of standardization across laboratories, the variety, cost, and availability of reagents and equipment, and sample handling and viability requirements.

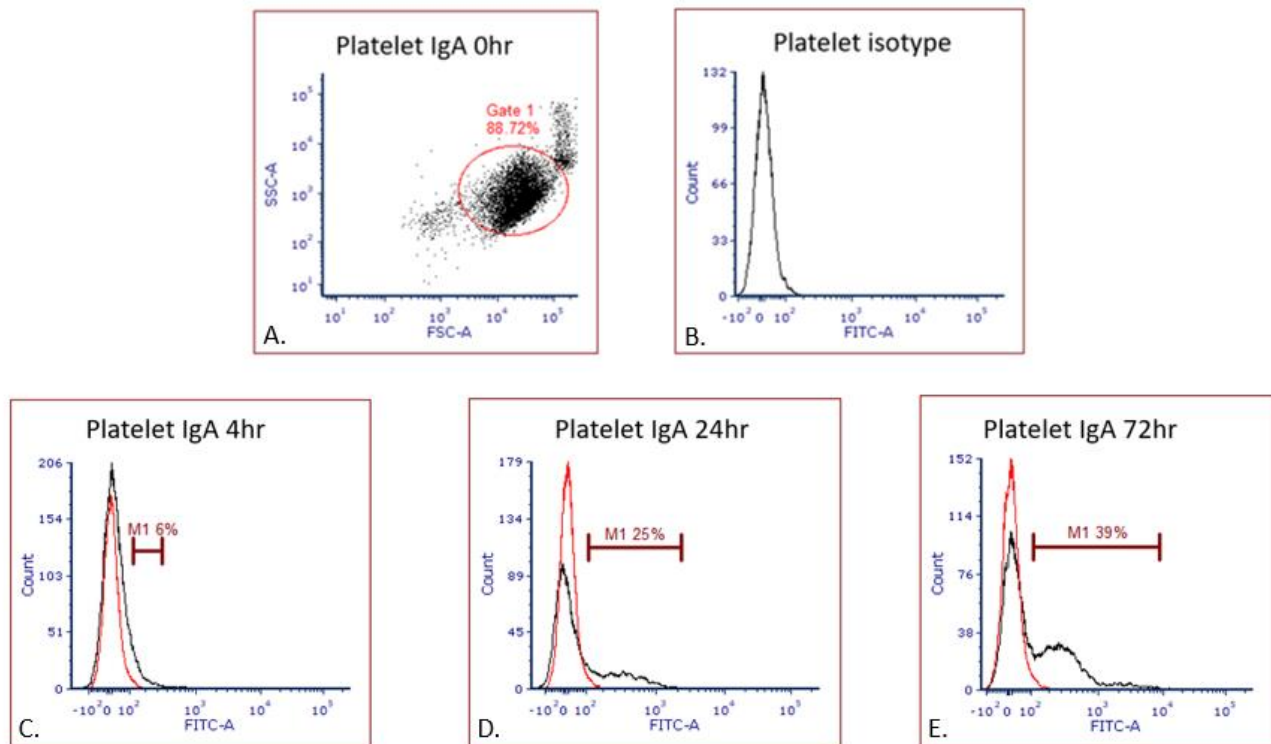


Figure 3: Scatter plot illustrates gating of population of interest based on forward (FSC-A) and side scatter (SSC-A) properties (A) from a single horse over time. Histograms demonstrate fluorescence intensity on the x-axis versus number of events on the y-axis (B-E). Isotype control-stained platelets illustrate weak to absent fluorescence intensity (B) whereas patient platelets stained with anti-IgA antibody demonstrate increasing percent positive platelets over time (black curve, panels C-E) relative to isotype control (red curve, C-E).

Critical barrier

When proposing the utility of a diagnostic assay in veterinary medicine, a major consideration in veterinary species, especially in large animal medicine, is sample handling. Previous studies have reported conflicting results regarding acceptable sample storage times without decreasing the reliability of the results. One study found that the percentage of PSA-IgG increased from 1.4% to 8% in 24 hours and increased 13% in samples stored for 48 and 72 hours in 4°C.¹³ This increase in PSA-IgG was attributed to the redistribution of IgG during storage and

platelet activation. Prior to that, Lewis et al, found there to be no statistically significant difference in PSA-IgG concentration when samples were kept on ice packs for up to 72 hours; however, a significant difference was reported when stored at room temperature.¹⁴ A more recent study found storage times up to 72 hours (4°C) to cause statistically significant differences in PSA-IgG concentrations in healthy dogs, but not in thrombocytopenic dogs.⁷ Additionally, they concluded there was no clinically significant difference in results when compared to fresh samples. Additionally, that study used a slightly higher cutoff value than previous studies ($\leq 10\%$ antibody binding) to distinguish between positive and negative samples. This 10% cutoff value was determined by serial direct flow evaluations in a healthy dog.⁷ All of these studies measured IgG concentrations in canine samples at storage times up to 72 hours, and except for Lewis et al., with storage at 4°C. There is minimal data on the effects of storage conditions on red blood cell and platelet surface associated immunoglobulins, IgG, IgM, and IgA in equine samples. Therefore, the main objective of this study was to identify optimal storage time and temperature of equine whole blood for the detection of these red blood cell and platelet surface-associated immunoglobulin concentrations using flow cytometry. This included first assessing assay precision and determining appropriate clinical decision thresholds using a cohort of clinically healthy horses.

Chapter 2 - Determining the effects of storage conditions on RBC-Ig and PSA-Ig concentrations in healthy horses

Introduction

The detection of surface immunoglobulin by direct immunofluorescence flow cytometry has become increasingly utilized in animals with suspected immune-mediated hematological disease, especially in anemic patients that are direct antiglobulin test (DAT or Coombs) negative. In people with suspected immune-mediated erythrocyte destruction, flow cytometry is preferred to avoid false negatives due to sensitivity issues with the direct antiglobulin test, as previously discussed.¹⁵ In a veterinary study comparing flow cytometry to DAT in dogs with IMHA, the flow cytometric assay demonstrated a more rapid, cost-effective, sensitive, and objective method to quantitate RBC-Ig⁵ which has been postulated to aid in patient diagnosis and monitoring effectiveness of treatment.¹⁶ A disadvantage of flow cytometry over DAT is the lack of standardization and limited knowledge of sample handling requirements. Studies optimizing sample handling conditions for this assay are specifically lacking in horses. It is known that samples for DAT can be stored at 4°C for 7 days without significantly affecting the reliability of the result.¹⁷ This is crucial information for submitting practitioners and similar standardized guidelines are needed for the flow cytometric assay. The details of appropriate sampling handling for flow cytometry are important because many of these samples must be mailed into reference laboratories, making it imperative to provide specific guidelines for the submitting veterinarian in order to optimize the viability of the sample and the reliability of the results. Current literature provides somewhat conflicting information, with acceptable storage times ranging from 24 hours to 72 hours. The majority of these studies focused exclusively on canine whole blood samples with only sparse reports of proposed storage conditions in horses. The

objective of this study is to identify optimal storage time and temperature of equine whole blood for the detection of red blood cell and platelet surface-associated immunoglobulin concentrations via flow cytometry. In this study we assessed for alterations in red blood cell and platelet surface-associated immunoglobulin (IgG, IgM, and IgA) concentrations due to prolonged sample storage times by repeated analysis at 0 hr, 4 hr, 24 hr, 48 hr, and 72 hr timepoints. Red blood cell samples were stored at 4°C consistent with previous studies.¹⁵ Platelets are known to be activated by cold temperatures¹⁸; therefore, platelet samples were stored at both 4°C and room temperature to see if eliminating the exposure to lower temperatures would improve the assay. We hypothesized there would be no statistically significant difference in the detectable percentages of red blood cells that are positive for surface bound IgG, IgM, or IgA with storage up to 72 hours. We also hypothesized that the samples for the PSA-Ig detection would be more stable when stored at room temperature as opposed to 4°C.

Methods

Animals and sample storage

Whole blood samples were collected into two 10 ml EDTA tubes from 10 clinically healthy horses from the Kansas State College of Veterinary Medicine teaching population. An IACUC protocol is in place for housing, care, and blood sampling from these horses. The blood was collected via jugular venipuncture and the tubes were inverted several times for adequate mixing. A complete blood count (CBC) and blood smear evaluation was performed on each horse at time of collection and each subsequent day in storage to ensure adequate (within RI) red blood cell and platelet densities. Following collection, 500 µL aliquots were placed into 12 x 75 mm polystyrene tubes for immediate processing to represent the 0 hr timepoint for both the red

blood cell and platelet flow cytometric assays. The remaining whole blood samples were stored at 4°C or room temperature awaiting analysis via direct flow cytometry at set time points, including 4 hr, 24 hr, 48 hr, and 72 hr post collection (Figure 4).

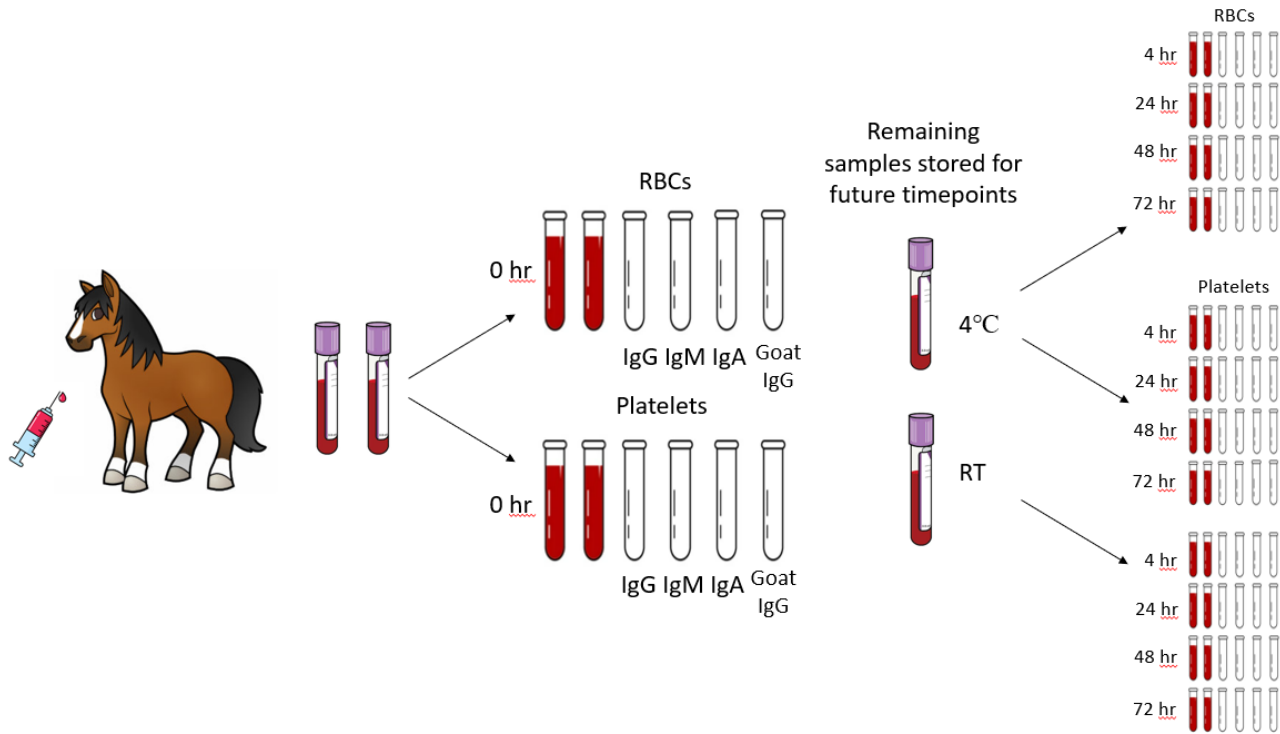


Figure 4: Freshly collected whole blood samples aliquoted for immediate analysis and stored at appropriate temperatures (4°C for red blood cells and both 4°C and room temperature [RT] for platelets) for future analysis.

Direct flow cytometry for RBC-Ig

At each timepoint, two 500 µL aliquots of anticoagulated whole blood stored at 4°C were placed into two 12 x 75 mm polystyrene tubes with 3 ml of phosphate buffered solution (PBS). The samples were centrifuged at 3400 rpm 1000 x G for 1 minute to pellet the red blood cells. After decanting the supernatant, the red blood cells were suspended in 3 ml of PBS. The red blood cells were washed 2 additional times for a total of 3 washes. After the last wash, the two tubes were combined and resuspended in 9 ml of PBS to create an approximate 2% suspension of

RBC. The suspension was separated into 5 new tubes of 100 μ L each. Tube 1 contained only the red blood cell suspension to serve as a control for erythrocyte autofluorescence. In addition to the 100 μ L of red blood cell suspension, tube 2 contained 100 μ L of FITC-labeled anti-equine IgG F(ab')₂ (Jackson Immuno Research Labs #108-096-003), tube 3 contained 100 μ L of FITC-labeled anti-equine IgM (Bethyl Labs #A70 14F), tube 4 contained 100 μ L of FITC-labeled goat anti-equine IgA (Bethyl Labs, #A70-103F), and tube 5 contained 100 μ L of isotype control FITC-labeled goat IgG (Fab μ) (Table 1). All tubes were incubated at 4°C in the dark for 30 minutes. After incubation, the samples were washed as described previously and the pellet was resuspended in 500 μ L of PBS. The samples were immediately analyzed by flow cytometry (BD LSR Fortessa™ X-20 SORP). Linear settings for FSC and SSC were used for initial gating of red blood cells. Log settings were utilized for fluorescence intensity. Data acquisition stopped when 10,000 events were acquired in the red blood cell gate. Percent positive events for each antibody isotype were quantified after adjusting for background and non-specific fluorescence based on isotype control antibody. Each sample was run 5 times at each time point to assess the precision of the assay via calculating the coefficient of variation.

Table 1: Sample preparation for both red blood cells and platelets

Tube	Sample	IgG	IgM	IgA	Goat IgG
1	100uL				
2	100uL	100uL			
3	100uL		100uL		
4	100uL			100uL	
5	100uL				100uL

Direct flow cytometry for PSA-Ig

For each timepoint, platelet-rich plasma was prepared from anticoagulated whole blood samples stored at 4°C and at room temperature. Two aliquots of 500 µL were placed into 12 x 75 mm polystyrene tubes and 3 ml of phosphate buffered saline (PBS) were added to each tube. Samples were centrifuged at 1800 rpm for 3 minutes. The platelet rich plasma was removed and placed into new polystyrene tubes and centrifuged at 3400 rpm; 1000 x G for 3 minutes to pellet the platelets. After decanting the supernatant, the platelets were resuspended in 3 ml of PBS and mixed by vortex. Platelets were washed 2 additional times for a total of 3 washes. After the final wash, the platelets were resuspended in 500 µL PBS. The platelet suspension was separated into 5 new tubes of 100 µL each. Tube 1 contained only the platelet suspension to be used as a control for platelet autofluorescence. In addition to the 100 µL of platelet suspension, tube 2 contained 100 µL of FITC-labeled anti-equine IgG F(ab')₂ (Jackson Immuno Research Labs #108-096-003), tube 3 contained 100 µL of FITC-labeled anti-equine IgM (Bethyl Labs #A70 14F), tube 4 contained 100 µL of FITC-labeled goat anti-equine IgA (Bethyl Labs #A70-103F), and tube 5 contained 100 µL of FITC-labeled goat IgG (Fab) (Jackson Immuno Research Labs #005-090-006) (Table 1). All tubes were incubated at room temperature in the dark for 30 minutes. After incubation, samples were washed twice as previously described and then resuspended in 400 µL PBS. Samples were then immediately analyzed by flow cytometry (BD LSR Fortessa™X-20 SORP). Linear settings for FSC and SSC were used for initial gating of platelets. Log settings were utilized for fluorescence intensity. Data acquisition stopped when 10,000 events were acquired in the platelet gate. Percent positive events for each antibody isotype were quantified after adjusting for background and non-specific fluorescence based on

isotype control antibody. Each sample was run 5 times at each time point to assess the reliability of the assay via calculating the coefficient of variation.

Statistical analysis

Due to the small sample size, nonparametric statistical methods were used (GraphPad Prism, v.9, USA). The RBC assay data were analyzed by isotype for each timepoint by Friedman's Test. The effect of storage time was considered significant if $P < 0.05$. The platelet assay data per isotype were analyzed by two-way ANOVA to account for both time and temperature followed by Šidák's multiple comparisons test. The effects of storage time and temperature were considered significant if $P < 0.05$.

Results

Demographics

Ten clinically healthy horses from the Kansas State College of Veterinary Medicine teaching population were included in this study. Horses ranged from 5 to 26 years of age (mean 14.9 years) and included 4 quarter horse mares, 1 mare of unspecified breed, 1 quarter horse stallion, 1 quarter horse gelding, 1 warmblood gelding, 1 thoroughbred gelding, and 1 Tennessee Walker gelding. All horses were found to have adequate red blood cell and platelet densities based on CBC and blood smear evaluation using previously established reference intervals.

Assay precision

The samples were analyzed in 5 consecutive runs to determine the intra-assay precision. The coefficient of variation for each assay at the 0 hr time point, was 18.1% for the red blood cell assay and 14% for the platelet assay. The precision of both assays ($CV < 20\%$) is considered acceptable for flow cytometry-based methods.¹⁹

Determination of clinical decision threshold for healthy horses

The mean of each consecutive run was used to calculate the average percentage of Ig-positive red blood cells and platelets for each storage timepoint and temperature. This calculated mean plus two standard deviations (SD) determined the threshold to distinguish between positive and negative samples. Decision thresholds for RBC-Ig are summarized in Table 2.

Table 2: Decision thresholds for RBC-Ig stored at 4°C

Timepoint (hours)	IgG (%)		IgM (%)		IgA (%)	
	Mean $\pm$ 2SD	Decision threshold	Mean $\pm$ 2SD	Decision threshold	Mean $\pm$ 2SD	Decision threshold
0	0.5 $\pm$ 1.6	2.1	0.1 $\pm$ 0.5	0.6	0.3 $\pm$ 0.8	1.0
4	0.6 $\pm$ 1.8	2.4	0.0 $\pm$ 0.0	0.0	0.2 $\pm$ 0.9	1.1
24	0.4 $\pm$ 1.7	2.1	0.0 $\pm$ 0.0	0.0	0.2 $\pm$ 0.9	1.1
48	0.5 $\pm$ 1.7	2.1	0.0 $\pm$ 0.1	0.1	0.1 $\pm$ 0.4	0.6
72	0.5 $\pm$ 2.0	2.5	0.0 $\pm$ 0.0	0.0	0.2 $\pm$ 0.6	0.8

Recommended decision thresholds for RBC-Ig at 0 hours are <3% for IgG, <1% for IgM, and <2% for IgA.

For platelets, decision thresholds were calculated at all timepoints for both storage temperatures and are summarized in Tables 3 and 4.

Table 3: Decision thresholds for PSA-Ig stored at RT

	IgG (%)		IgM (%)		IgA (%)	
Timepoint (hours)	Mean $\pm$ 2SD	Decision threshold	Mean $\pm$ 2SD	Decision threshold	Mean $\pm$ 2SD	Decision threshold
0	1.6 $\pm$ 4.9	6.5	3.3 $\pm$ 5.9	9.2	5.7 $\pm$ 16.0	21.7
4	3.0 $\pm$ 8.2	11.3	4.3 $\pm$ 7.4	11.7	5.2 $\pm$ 7.6	12.9
24	5.0 $\pm$ 16.0	21.0	6.4 $\pm$ 6.8	13.2	8.5 $\pm$ 11.6	20.1
48	4.2 $\pm$ 13.4	17.6	8.2 $\pm$ 9.1	17.4	9.6 $\pm$ 11.6	21.2
72	2.7 $\pm$ 8.5	11.2	7.2 $\pm$ 9.4	16.6	12.5 $\pm$ 16.6	29.1

Table 4: Decision thresholds for PSA-Ig stored at 4°C

	IgG (%)		IgM (%)		IgA (%)	
Timepoint (hours)	Mean $\pm$ 2SD	Decision threshold	Mean $\pm$ 2SD	Decision threshold	Mean $\pm$ 2SD	Decision threshold
0	1.6 $\pm$ 4.9	6.5	3.3 $\pm$ 5.9	9.2	5.7 $\pm$ 16.0	21.7
4	2.1 $\pm$ 7.4	9.5	2.7 $\pm$ 3.8	6.5	4.7 $\pm$ 5.8	10.5
24	3.0 $\pm$ 11.9	14.9	1.8 $\pm$ 2.4	4.2	4.7 $\pm$ 6.3	11.0
48	2.2 $\pm$ 8.2	10.3	1.4 $\pm$ 2.3	3.7	7.4 $\pm$ 10.6	18.0
72	0.8 $\pm$ 2.6	3.4	1.6 $\pm$ 3.5	5.1	9.0 $\pm$ 15.9	24.9

Unlike RBC-Ig, a consistent decision threshold could not be determined per isotype for the PSA-Ig assay.

Effects of storage on RBC-Ig concentration

There was no statistically significant difference ($P < 0.05$) in the percentage of red blood cells that were positive for surface bound immunoglobulin, including IgG, IgM, and IgA when comparing ranked values of the five consecutive runs at each time point (Figure 5A-C). The percentage of IgG, IgM, or IgA positive red blood cells remained stable and below the isotype-specific thresholds for each horse at each storage time point indicating that any minor changes in RBC-Ig percentage due to storage are not clinically significant. These findings demonstrate that detection of RBC-Ig is stable in whole blood samples stored up to 72 hours at 4°C.

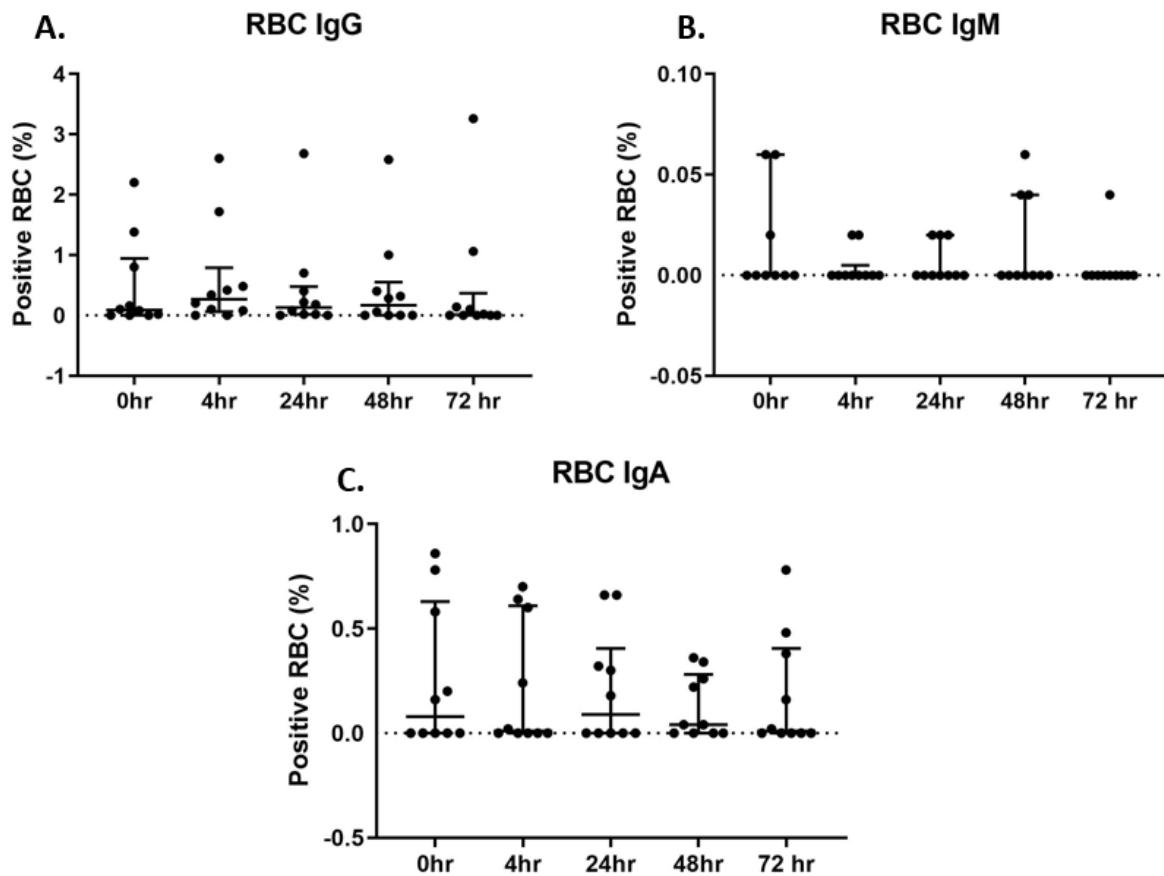


Figure 5: Percentage of red blood cells positive for surface-associated IgG (A), IgM (B), and IgA (C) via flow cytometry in fresh samples (0 hr) and four additional timepoints (4 hr, 24 hr, 48 hr, and 72 hr) in healthy horses. Each data point represents the mean of 5 replicates per one horse. Error bars = median and interquartile range.

Effects of storage on PSA-Ig concentrations

The percentage of platelets that were positive for surface bound immunoglobulin IgG, IgM, and IgA did not significantly change between timepoints when stored at 4°C ($P < 0.05$) although more variability is evident in the quantification of PSA-Ig versus RBC-Ig (Fig 5, 6). As seen in Table 2,3 and 4: decision thresholds for PSA-Ig are higher in general than RBC-Ig and increase steadily through 24 hours then become quite variable at the 48- and 72-hour timepoint suggesting instability in the assay. Decision thresholds are consistently higher in PSA-Ig stored at RT versus 4°C despite there only being clinically significant differences for IgM at timepoints greater than 24 hours (Figure 6A-C).

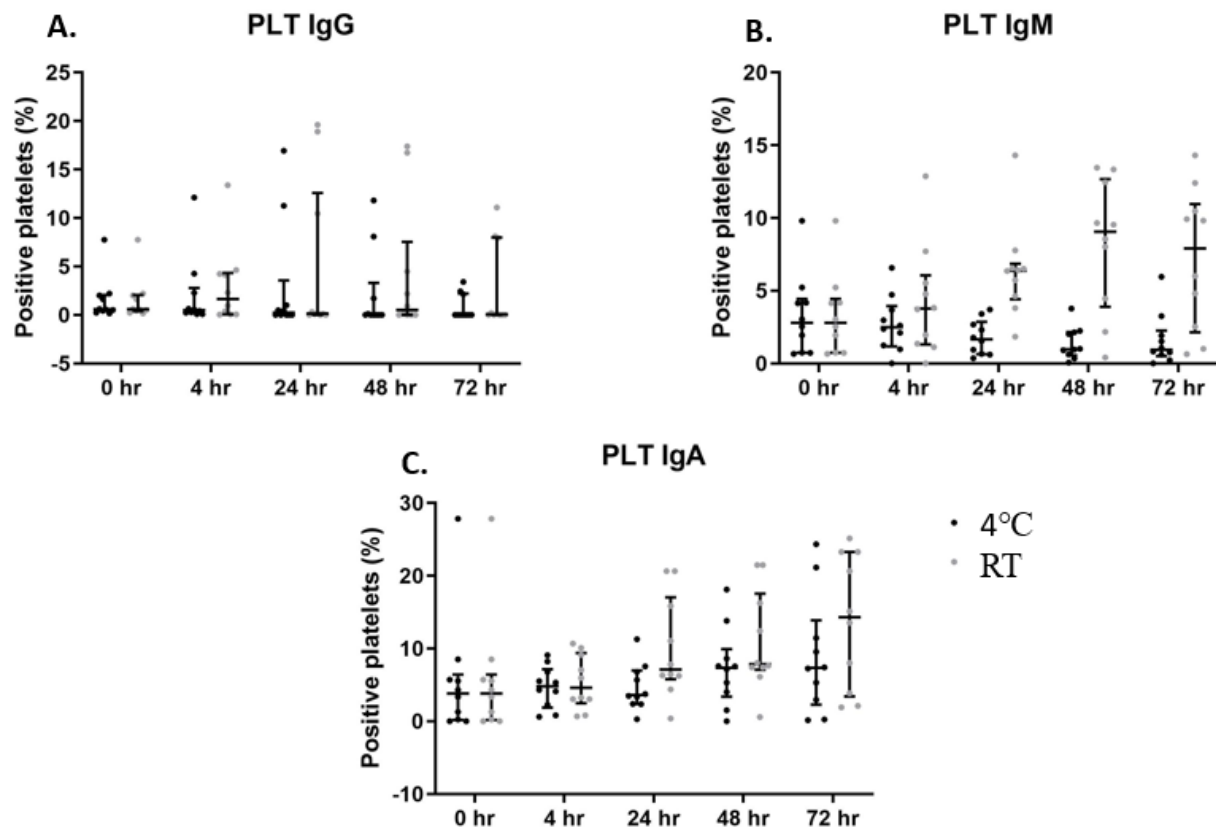


Figure 6: Percentage of platelets positive for surface-associated IgG (A), IgM (B), and IgA (C) via flow cytometry in fresh samples (0 hr) and four additional timepoints (4 hr, 24 hr, 48 hr, and 72 hr) stored at 4°C and room temperature in healthy horses. Each data point represents the mean of 5 replicates per one horse. Error bars = median and interquartile range.

Discussion

The results of our study show that whole blood from horses can be stored for up to 72 hours at 4°C without statistically or clinically significant alterations in red blood cell surface-bound IgG, IgM, or IgA concentrations when measured via flow cytometry. Statistical analysis of PSA-Ig also indicated no differences between analysis timepoints. However, although these changes were not statistically significant, when assessing the clinical decision threshold generated for each timepoint relative to 0 hours, changes in results due to sample storage would alter interpretation and therefore are clinically relevant. To minimize potential for unreliable results, our study indicates that ideally, samples should be processed within 4 hours of collection, although analysis within 24 hours may be acceptable when using storage-time specific decision thresholds. Platelet surface-bound IgA was inconsistent, with high percentages of positive platelets detected in healthy animals. This suggests that PSA-IgA cannot reliably be measured by flow cytometry in fresh or stored samples and should not be used to assess for ITP. Currently, it is not known how often IgA contributes to ITP in veterinary species, if at all. Published literature in both veterinary and human medicine support the involvement of IgG and/or IgM, with little to no evidence of IgA contribution. A human study including one hundred and one patients with immune thrombocytopenia measured IgG, IgM, IgA, and complement (C3) discovering many of those patients to have increases in all isotypes concurrently, including 44% with increased IgA. However, isolated elevation of IgA was found in only one patient suggesting the vast majority of cases can be detected by measuring IgG and IgM.²⁰

The variation in the percent positive platelets from one time point to the next was most evident when stored at room temperature, specifically after the 24-hour time point. There was no statistically significant difference between the time points in samples stored at 4°C, suggesting

more sample stability and reliability of results when stored at 4°C when compared to room temperature. Although not statistically significant, the differences were clinically significant enough to make a difference in a positive versus negative result based on the baseline clinical decision thresholds. These findings are consistent with a previous study in dogs that found PSA-Ig values increased 3 to 7-fold in samples that were stored for 24-72 hours. An increase in detectable PSA-Ig with prolonged storage may be explained in animals with ITP due to constitutive expression of immunoglobulin receptors that could cause an increase in nonspecific antibody binding during storage³; however, increases in PSA-Ig via this mechanism in clinically healthy animals is unexpected. The greater variation in percent positive platelets between timepoints at room temperature versus 4°C suggests this storage-associated increase in nonspecific antibody binding is not potentiated by colder temperature. Wilkerson et al. (2001) established separate reference intervals for samples analyzed within 4 hours and those analyzed after 24 hours due to the clinically significant increase in PSA-Ig in blood from clinically healthy dogs. Another study concluded that direct flow cytometry for the evaluation of PSA-Ig could be performed reliably on canine whole-blood samples stored up to 72 hours at 4°C.⁷ This study established a reference cut off by serial evaluation of a healthy dog. They found the average to be 1.5% positive platelets, but up to 7% positive platelets in a freshly collected sample. Therefore, a cut off value of 10% was chosen for the duration of the study and used to evaluate healthy and thrombocytopenic dogs. No cause for the increased percentage in the healthy dog was determined.⁷ Wilkerson et al. also included dogs with ITP and found that all samples that were analyzed within 24 hours had an increased percentage of platelets with surface-associated immunoglobulin, regardless of platelet concentration.¹³ This suggests that the platelet concentration does not solely explain the increase in percent positive platelets with increased

storage times. Platelet concentrations in the current study remained stable and within reference interval in all horses across all storage timepoints, further supporting that platelet count alone does not correlate with concentration of PSA-Ig detected.

Additional efforts in optimizing sample handling, storage conditions, and establishing lab-specific clinically relevant cut off values are important for the appropriate use of this assay and reliable interpretation of results. This study suggests a need for specific decision thresholds based on time from sample collection to analysis, as well as sample storage temperature. While the potential for time and temperature alterations in samples during transit presents a major challenge in the utility of this assay, determining these specific decision thresholds could improve the impact of storage conditions on clinical decision making.

Chapter 3 - Proposals for additional research and future directions

Reaching a definitive diagnosis of immune-mediated hematological disease in veterinary species has proven challenging, warranting a need for established diagnostic criteria and standardization of diagnostic approach. Distinguishing an immune-mediated process from other causes of anemia or thrombocytopenia is vital due to the differences in treatment and prognosis in these patients. Recently, a consensus statement, as well as a survey targeting small animal general practitioners has thoroughly reviewed the current diagnostic criteria and available assays in the diagnosis of IMHA in small animals.^{2,6} These criteria include a decreased packed cell volume (PCV), at least one of the defined abnormalities indicating hemolysis (e.g., ghost cells, hyperbilirubinemia, bilirubinuria, icterus, hemoglobinemia, or hemoglobinuria), and a positive saline agglutination test that persists with washing the erythrocytes. If a positive saline agglutination test does not persist with washing of the erythrocytes, then at least two of the following must be true: a positive saline agglutination test without washing, greater than 5 spherocytes per 1000x field, and/or detection of anti-erythrocyte antibodies by a Coombs test or flow cytometry.⁶ Similar detailed criteria are needed to guide diagnosis of IMHA in large animal patients and ITP in all veterinary species. Exploring and fine-tuning new or alternative diagnostic techniques will hopefully aid in establishing reliable and standardized diagnostic criteria. As previously discussed, detecting red blood cell and platelet surface associated immunoglobulin via flow cytometry has shown an increased sensitivity and high specificity in people, as well as dogs, cats, and horses and therefore could improve our current diagnostic capabilities in the characterization of veterinary immune-mediated hematologic disease. Additional studies are warranted to address the current challenges of this assay and to fully elucidate the potential of its utility in veterinary medicine.

One consideration when utilizing flow cytometric assays is the importance of appropriate sampling handling and the limitations of sample stability. This study, however, suggests the changes in percentage of RBC-Ig positive red blood cells observed in samples stored up to 72 hours, may not make a clinically significant difference. This decreases overnight shipping costs for submitting veterinarians and maintains confidence in results in the event that samples cannot be analyzed same day. This study also highlights the need for a more cautionary approach when handling samples assessing for PSA-Ig. In this case, ensuring overnight shipping at cold temperatures (4°C) is recommended in order to decrease the potential for erroneous results.

One concern, however, which is a limitation of the current study, is the use of only clinically healthy animals with no CBC abnormalities since it is not known if surface bound immunoglobulins are more likely to be affected by storage conditions in certain disease states. Additional studies measuring surface-associated immunoglobulin concentrations in anemic and thrombocytopenic horses, as well as those with known primary and secondary immune-mediated diseases are warranted to confirm the diagnostic utility of these assays. One prospective study with 292 dogs used flow cytometry to measure the concentration of RBC-Ig in anemic and nonanemic dogs of various disease states.¹⁶ This included dogs with infectious diseases, cancer, thrombocytopenia, and surgical and other noninfectious medical conditions. They found that anemic dogs were significantly more likely to have RBC-Ig than nonanemic dogs and dogs with IMHA had significantly higher RBC-Ig than any of the other groups. This study also concluded that the relative percentage of IgG positive red blood cells was significantly associated with the disease state and supports the use of this assay in the work up of patients with unexplained anemia.¹⁶ While this is assumed to also be true in equine patients, a similar high volume prospective study involving anemic and nonanemic horses has not been conducted.

Another challenge of this assay involves the lack of a standardization. The variability in equipment and reagent availability across laboratories makes a unified and consistent approach challenging. This includes the lack of readily available positive control material which is crucial for laboratories to optimize their assay conditions and protocols but is limited by sample viability requirements. One study prepared positive control material for dog IgG by incubating DEA 1.1 positive cells with anti-DEA 1 blood-typing serum.²¹ Unfortunately, the time and resources needed to prepare this positive control material is likely to be a limitation in most veterinary diagnostic laboratories.

There are few veterinary studies that demonstrate the utility and explore the potential benefits of this assay in supporting the clinical diagnosis and management of immune-mediated hematologic disease. Important questions that warrant future investigation include the effects of blood transfusions and/or immunosuppressive therapy on assay results, and if this assay could be used to monitor disease progression or response to therapy. Previous studies have reported variable findings on the effects of blood transfusions. Overmann et al. found 1 of 7 dogs with a recent blood transfusion to have a false positive Coombs test; however, they concluded no clear associations were identified.²² Further evaluation of the effects of previous blood transfusions on RBC-Ig concentrations could provide valuable clinical information, as many IMHA patients require blood transfusions. This could be assessed by enrolling anemic patients with known IMHA, defined as >3% cells positive for RBC-Ig by flow cytometry. The concentration of RBC-Ig could be reassessed by the same flow cytometric assay and protocol over the next three weeks to assess for any trends in the percentage of positive red blood cells. This study could potentially be complicated by the fact that many of these animals are given immunosuppressive medications, which could also have an uncertain effect on this assay. Accounting for this

variable could involve careful case selection with two separate groups, one with administration or recent history of immunosuppressive drugs and one without. The limited data available suggests immunosuppression does not immediately result in a negative DAT, but there is considerable variability between patients and the timing between initiation of therapy and testing.^{2,17} Few studies found anemic dogs treated with immunosuppression had DAT+ results that remained positive for days to weeks following treatment and suggested a negative Coombs test is expected with normalization of PCV and resolution of hemolysis.^{17,23} In one case series, 13 dogs with IMHA were serially monitored after initiation of immunosuppressive therapy. One dog developed negative red cell immunoglobulin status seven days after starting treatment whereas one dog did not have negative status until day 200. Data from all 13 dogs is not presented in that article, so a causal relationship between steroid use and negative RBC-Ig was not conclusively established.²⁴ Only rare, anecdotal evidence is available on the effects of immunosuppressive therapy on detection of RBC-Ig via flow cytometry in dogs, reporting there were rapid decreases in percent RBC-Ig after initiation of treatment.¹⁶ Additional investigation may provide evidence supporting the potential benefits of this flow cytometric assay in monitoring the patient's response to therapy. Evaluating the effects of immunosuppressive medications on detection of RBC-Ig via flow cytometry could be set up similarly to these previous studies that have utilized DAT, but with serial monitoring via flow cytometry. Serial monitoring with flow cytometry is inexpensive and the majority of patients with IMHA will receive immunosuppressive medications as part of clinical treatment. Therefore, collecting blood samples from both dogs and horses at the time of presentation could easily be compared to samples collected after the initiation of therapy. Based on the few studies previously mentioned, RBC-Ig concentrations would be expected to decrease with therapy, resolution of clinical signs,

and normalization of PCV. This could allow a quantitative way of helping clinicians decide when to taper or alter dosing of immunosuppressive drugs, which could have a significant impact on patient care and prognosis.

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