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Research paper

Validation of a clinically applicable flow cytometric assay for the detection of immunoglobulin associated platelets in dogs

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ABSTRACT

Thrombocytopenia is commonly encountered in veterinary practice when evaluating canine patients. It can occur in infectious, neoplastic, inflammatory, toxic, and immune-mediated conditions. Elucidating the underlying cause for thrombocytopenia can therefore represent a challenge to veterinary practitioners. Additionally, determination of whether an immune process could be contributing to a patient's thrombocytopenia is important for refining differentials and enhancing understanding of a particular disease process. A possible candidate test for the development of a clinically applicable assay in dogs is flow cytometry. Therefore, the purpose of this study was to develop a clinically applicable direct and indirect flow cytometric assay for the detection of canine immunoglobulin associated platelets. Direct and indirect flow cytometry was performed in nine healthy beagles and twelve client-owned thrombocytopenic dogs at four time points: fresh and after 24, 48, and 72 h of storage at 4 °C. For healthy dogs, there was no significant difference between fresh and 24 and 48 h samples but there was a significant difference between fresh and 72 h samples. There was no significant difference between fresh and 24, 48, or 72 h samples in the thrombocytopenic dogs. A cut-off value of ≤ 10% antibody binding was defined to differentiate negative and positive classifications and was determined by serial direct flow evaluations in a healthy dog. Based on this cut-off value, healthy and thrombocytopenic dogs were consistently categorized at every time point. The average intra-assay coefficient of variation for the thrombocytopenic dogs was 4.32%. The indirect flow cytometric methods evaluated herein did not provide reliable or repeatable results in healthy or thrombocytopenic dogs. Direct flow cytometry represents a potentially clinically useful test for the detection of immunoglobulin associated platelets in dogs that can be processed and evaluated within a realistic amount of time which would allow for testing in a larger number of patients. Based on the findings from this study using our protocols, indirect flow cytometry was not clinically applicable in dogs.

1. Introduction

Thrombocytopenia is a common blood work abnormality observed in dogs that can be related to artefactual pseudothrombocytopenia (e.g. sample handling, anti-coagulant induced) but can also be due to immune and non-immune processes on platelets. It is also the most common acquired hematologic abnormality observed in dogs (Brooks and Catalfamo, 2010). The major mechanisms of thrombocytopenia include decreased or lack of production, increased destruction or consumptive processes, abnormal loss, and sequestration (Neel et al., 2014). Thrombocytopenia can occur as a result of one of these mechanisms or a combination due to infectious, neoplastic, inflammatory, toxic, and immune-mediated conditions. Determining the underlying cause for thrombocytopenia in dogs can therefore be challenging. However, establishing if an immune process is contributing to the observed thrombocytopenia can help further characterize a patient's disease and guide future diagnostic testing. Specifically, for immune processes, immunoglobulin associated platelets or antibodies can form that are directed against platelets which can contribute to a shortened platelet life span and resultant abnormally low platelet number (Wilkerson et al., 2001; Brooks and Catalfamo, 2010). The detection of immunoglobulin associated platelets in dogs has been previously assessed by various techniques such as ELISAs, immunofluorescence, immunoradiometric methods, and flow cytometry but none of these tests are commonly used in veterinary practice (Kristensen et al., 1994a, 1994b, 1995b; Lewis et al., 1995a, 1995b; Scott et al., 2002).

The development of a clinically applicable test for detection of immunoglobulin associated platelets would be of great benefit to veterinary patients. In order to be clinically applicable and have a more widespread impact, the test must not only provide repeatable and

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reliable results but also must be viable after several days of storage. This requirement is a realistic expectation since many private practices and veterinary academic institutions would not have the capability to perform this type of test on-site. Previous studies have shown varying results regarding the stability of stored samples for flow cytometry; one study showed that samples could be reliably evaluated when stored appropriately up to 72 h after collection (Lewis et al., 1995a, 1995b) whereas another author reported that samples should not be evaluated after 24 h (Wilkerson et al., 2001; Wilkerson and Shuman, 2001; Wilkerson, 2012). If blood samples must be evaluated within 24 h of collection, this would increase costs of shipping to owners and veterinary practitioners in addition to greatly restricting when samples could be received. However, if a blood sample could be evaluated after up to 72 h of appropriate storage, this would allow patients to be evaluated from distant locations and would widen the acceptable test receiving timeframe.

A possible candidate test for the development of a clinically applicable assay in dogs is flow cytometry. Flow cytometry can be performed using direct or indirect methods and can provide the percentage of immunoglobulin associated platelets (percent IgG). The direct method entails using a patient's platelets and directly screening for antibodies whereas the indirect method involves using a patient's sera and a healthy dog's platelets to indirectly screen for antibodies (Kristensen et al., 1994b; Lewis and Meyers, 1996). Flow cytometry could therefore be a possible test used in practice to help determine if an immune process is contributing to a patient's thrombocytopenia but would not be able to distinguish between causes of thrombocytopenia. To date, there is not an assay that has been able to definitively differentiate the etiology of thrombocytopenia in a dog such as whether it is due to infectious, neoplastic, or primary immune-mediated causes (Bachman et al., 2015). However, regardless of the etiology, determining that an immune process is present can help narrow the differential list and lead to more directed diagnostics.

Therefore, the detection of immunoglobulin associated platelets in dogs is important and can enhance understanding of a patient's disease. The purpose of this study was to develop a clinically useful direct and indirect flow cytometric assay for the detection of immunoglobulin associated platelets in dogs. We hypothesized that the direct assay would provide reliable results out to 72 h when stored appropriately but that the indirect assay would not provide reliable results and was unlikely to be clinically applicable.

2. Materials and methods

2.1. Animals and samples

To provide negative samples, clinically healthy beagles were prospectively enrolled into the study which was approved by the Institutional Animal Care and Use Committee at Colorado State University prior to blood collection. All of the dogs were housed in the same conditions and were not receiving any medications. A total of 3 ml of blood were collected via jugular venipuncture and placed into an EDTA (BD Vacutainer®, USA) tube and inverted several times for appropriate mixing.

One of the healthy beagles was chosen in order to establish a cut-off value for serial flow assessments at our institution which was also approved by the Institutional Animal Care and Use Committee prior to blood collection. Samples were collected from this dog once a week for 10 weeks and were processed for direct flow cytometry within 4 h. For every blood collection, a total of 2 ml were collected via jugular venipuncture and placed into an EDTA (BD Vacutainer®, USA) tube and inverted several times for appropriate mixing.

To serve as positive samples, client-owned dogs presenting to the Colorado State University (CSU) Veterinary Teaching Hospital between September 2016 and September 2017 were prospectively enrolled into the study. The study was approved by the Institutional Clinical Review Board and all owners signed a client-consent form at the time of enrollment. To be eligible for the study, the platelet count had to be less than or equal to 75,000 platelets/μl (ADVIA 120 Hematology System, Germany) with few to no clumping observed and each dog had to have a final diagnosis of presumptive canine immune-mediated thrombocytopenia (IMTP). A total of 3 ml was collected via the cephalic vein or lateral saphenous vein and placed into an EDTA (BD Vacutainer®, USA) tube and inverted several times for appropriate mixing.

To provide negative sera for the indirect method, sera was used from healthy beagles not enrolled in the current study but that had been previously shown to have a normal platelet count and were considered negative via direct flow cytometry. To provide positive sera for the indirect method, sera was used from unhealthy dogs that had been previously documented to have thrombocytopenia, were considered positive via direct flow cytometry, and were diagnosed with IMTP. All sera was stored at −80 °C until processing.

2.2. Storage of EDTA anti-coagulated whole blood

In order to assess the direct flow cytometry assay at different storage time points, after each blood collection, the whole blood samples were stored at 4 °C before processing. Each sample was individually processed at four separate time points; fresh, 24 h, 48 h, and 72 h after blood collection. For the fresh sample, all of the samples were processed within 4 h of blood collection.

2.3. Direct flow cytometry for immunoglobulin associated platelets

The protocol used for this study was modified from other protocols previously described in the literature (Kristensen et al., 1994a, 1994b; Terrazzano et al., 2006). EDTA (BD Vacutainer®, USA) anticoagulated blood (500 μl) was mixed with an equal volume of sterile PBS. This mixture was centrifuged at 200 × *g* for 1 min 30 s at 20 °C to generate platelet-rich plasma (PRP). PRP was removed from the erythrocyte layer and placed into an Eppendorf tube (Light Labs SNAPLOCK Microcentrifuge Tubes, USA). Each PRP sample was adjusted to 2×10^6 cells/ml using a manual hemocytometer. Platelets were then pelleted by centrifugation at 1000 x g for 5 min at 20 °C. The platelets were resuspended and washed three times at the same speed in a solution containing 3 mM EDTA, 1% bovine serum albumin (BSA), and PBS. Each sample was incubated at room temperature protected from light with 50 μl of a 1:200 dilution of FITC-labeled rabbit anti-dog IgG (Jackson ImmunoResearch Labs, USA) for 30 min. After incubation, the platelets were washed three times with PBS-EDTA-BSA solution and resuspended in 200 μl of PBS-EDTA-BSA solution for flow analysis. The percentage of IgG positive cells (percent IgG) in duplicate was recorded from each sample. Gate settings used for this study were previously established with PE-labeled mouse anti-human CD61 (eBioscience, USA) using healthy dog samples. All samples were analyzed in duplicate using a Cyan ADP instrument (Beckman Coulter, USA) and the generated data was analyzed using commercial software (FlowJo, USA).

2.4. Indirect flow cytometry for immunoglobulin associated platelets

Several protocols were investigated for this study that were modified from other protocols previously described in the literature (Kristensen et al., 1994a, 1994b; Waner et al., 1995; Harrus et al., 1996; Terrazzano et al., 2006; Bachman et al., 2015). The platelet suspensions generating PRP were prepared as described above in Section 2.3 but several PRP suspensions (2×10^6 cells/ml, 3×10^6 cells/ml, and 4×10^8 cells/ml) were utilized for the various protocols. Briefly, various volumes (10 μl, 20 μl) and dilutions of sera from abnormal dogs (1:10, 1:50, 1:100, 1:200) were incubated with various volumes (10 μl, 20 μl) of platelet suspensions from healthy dogs at various temperatures (room temperature, 37 °C) for 30 or 60 min. Note: Fresh sera not previously frozen and aliquots of frozen sera stored at −80 °C were used.