NOTE *Clinical Pathology*

Reticulated Platelet Levels in Whole Blood and Platelet-Rich Plasma of Dogs with Various Platelet Counts Measured by Flow Cytometry

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ABSTRACT. Reticulated platelets (RP) are young platelets that contain residual RNA, and measurement of RP has been to assess thrombopoiesis. In the present study, flow cytometric counts of RP were compared using paired specimens elicited from dogs with various platelet counts by different RP collection procedures, the whole blood method (WBM) and platelet rich plasma method (PRPM). The flow cytometric counts of RP for the specimens collected by WBM showed good and stable agreement with those taken by PRPM from the same canine subjects. The result revealed that WBM, as well as PRPM, can be used clinically to determine RP levels in dogs with abnormal platelet counts.

KEY WORDS: canine, flow cytometry, platelet-rich plasma, reticulated platelets, whole blood.

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Reticulated platelets (RP) are young platelets that contain residual RNA, and measurement of RP has been to assess thrombopoiesis [2]. RP counting by flow cytometry is often adopted either to assess the marrow function of platelet productivity or to diagnose thrombocytopenic disorders, particularly idiopathic thrombocytopenic purpura in human [5, 6, 9].

Flow cytometric analysis of RP based on the thiazole orange staining of its RNA is increasingly employed for diagnostic classification of disease. RP measurements have been compared by analyzing the RNA contents of platelet specimens collected either from whole blood (WB) or platelet-rich plasma (PRP) prepared by different methods, which are referred to as whole blood method (WBM) and PRP method (PRPM), respectively [10].

Whereas the utility of PRPM has been confirmed in dogs with thrombocytopenia [3, 4, 7, 13], PRP is obtained from WB by centrifugation, requiring a relatively large volume of blood and a cumbersome technique. Conversely, WBM is comparatively simple, using a small volume of blood without a centrifuging process. Smith and Thomas had already tried WBM for dogs with normal platelet counts [11]; however, the adequacy of WBM is still unknown in dogs with abnormal platelet counts.

The present study attempted to clarify the availability of WBM for flow cytometric analysis of RP with regard to canine thrombocytopenia or thrombocytosis.

Nine clinically healthy beagle dogs and an equal number of affected dogs (all affected dogs were selected from clinical cases at the Animal Medical Center of Nihon University or The Veterinary Medical Center of The University of Tokyo) were assigned to the healthy and patients groups, respectively. Each case in the patient group is summarized in Table 1.

One milliliter of WB was collected once from each healthy individual with EDTA pretreated vessels. WB was also drawn from patients in the same manner as for healthy dogs with the exception of a chronic hepatitis case, in which blood was collected twice, before and during DIC. Thus, a total of 19 specimens were collected. These EDTA-treated blood samples were also used for measuring platelet counts by automated blood cell counter. Sample preparation was performed within 4 hr of blood collection by PRPM or WBM because the expression of CD61 on the platelet surface might begin to decrease at more than 4 hr after blood collection [12].

For the PRPM, PRP was obtained by centrifuging 1 m*l* of WB at 50 × g for 10 min, as described previously [3]. Identical pairs of 15 μl of PRP from the same subject were mixed with either 1 m*l* of thiazole orange (Becton Dickinson, U.S.A.) for RP stain or HEPES buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES; 0.22 μm filtrated) for determination of background, respectively. All specimens were then stored in the dark at room temperature (R.T.) for 90 min to allow for staining. Each 200 μl of stained and unstained pairs were subjected to ten times (1:9) dilution with HEPES buffer to produce a final volume of 2 m*l*.

For the WBM, either commercial VI-PL2 anti-human CD61 monoclonal antibody (MAb; PE-conjugated, Becton-Dickinson), which recognizes a GP IIIa portion of the GPIIb/IIIa complex expressed on the platelet surface, or 679.1Mc7 anti-mouse IgG1 isotype MAb (PE-conjugated, Beckman Coulter, U.S.A.) was used for the platelet-specific or non-specific control, respectively. Identical pairs 15 μl each of WB from the same subject were mixed with 10 μl of

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Table 1. Results of platelet analyses on each blood samples from affected dogs

Dog No.	Diagnosis	Breed	Age (yrs)	Sex	Platelet counts ($\times 10^3/\mu l$)	RP			
						WBM		PRPM	
						%	$\times 10^3/\mu l$	%	$\times 10^3/\mu l$
1	Hyperestrogenism with testicular tumor	Shetland Sheepdog	7	M	2	7.3	0.1	10.7	0.2
2	Babesiosis	Toy Poodle	1	M	50	22.7	11.3	21.0	10.5
3	Acute pancreatitis with DIC	Golden Retriever	9	MN	174	8.1	14.0	7.4	12.8
4	Chronic hepatitis Without DIC	Wire Fox Terrier	11	FS	178	5.2	9.2	4.2	7.4
	With DIC				92	23.6	21.6	16.4	15.1
5	Gastrointestinal bleeding	Shiba	8	M	190	27.0	51.3	25.1	47.7
6	Myelofibrosis treated with prednisolone	Shih Tzu	4	FS	260	12.3	31.9	16.4	42.7
7	IMHA	Miniature Dachshund	6	M	302	26.4	79.8	24.6	74.2
8	ITP treated with predonisolon	Shetland Sheepdog	9	M	343	33.7	115.4	31.6	108.5
9	Non-regenerative hemolytic anemia	Miniature Dachshund	11	MN	699	6.3	44.1	5.3	37.2

either VI-PL2 or 697.1Mc7, respectively, and incubated at R.T. for 20 min. After addition of 1 m*l* of either thiazole orange for RP or HEPES buffer for the control, the specimen pairs were stored in the dark simultaneously at R.T. for 90 min. Then 200 μl of each specimen was diluted to produce a final volume of 2 m*l* in the same manner as for PRPM sample preparation described above.

FACSCalibur (Becton Dikinson), a three-color analytical argon-laser system, was used for flow cytometric counting of RP. The parametric FACS's conditions employed in the present study were as follow. The logarithmic voltage settings were forward scatter (FSC)=E00, side scatter (SSC)=350, green fluorescence (FL1)=650 and orange fluorescence (FL2)=570. The spectral compensation setting were FL1-%FL2=6.0, FL2-%FL1=17.0 for CD61-PE, FL1-%FL2=6.0 and FL2-%FL1=17.0 for thiazole orange. Platelet gates were configured based on the 5,000 plotted data of FSC versus SSC for PRPM [3] and based on the combination of CD61 expression and the light-scattering properties in 5,000 events for WBM [11]. A histogram plot was constructed with log green fluorescence intensity on the x-axis and cell numbers on the y-axis. Using samples without thiazole orange, a gate was set to detect cells with increased fluorescence intensity so that <1% of unstained platelets were included in the gate. The percentages of platelets with increased fluorescence intensity were measured.

Absolute counts of RP were elicited by multiplying the RP ratio by the platelet counts of WB. The corresponding RP counts from specimens prepared by either PRPM or WBM elicited from the same subject were compared with Spearman's rank correlation coefficient analysis. A p value of less than 0.05 was considered to indicate a significant difference in the present study.

The mean platelet counts were $432.5 \times 10^3 \pm 71.8 \times 10^3/\mu l$ and $229.0 \times 10^3 \pm 197.1 \times 10^3/\mu l$ the in healthy and patient groups, respectively. Of the 19 specimens (9 specimens from the healthy group, 10 from the patient group, respectively), 10 specimens were within normal platelet ranges ($200 \times 10^3 - 500 \times 10^3/\mu l$), 6 specimens showed decreased platelet numbers ($< 200 \times 10^3/\mu l$) and 3 specimens had increased numbers of platelets ($>500 \times 10^3/\mu l$).

Table 2. Results of platelet analyses obtatined from healthy and affected dogs

Parameters	Mean ± SD	
	WBM	PRPM
Platelet counts ($\times 10^3/\mu l$)	325.4 ± 189.6	N.D.
RP (%)	11.9 ± 9.4	11.9 ± 8.2
RP ($\times 10^3/\mu l$)	32.4 ± 26.9	33.3 ± 25.3

N. D.: Not Determined.

The RP counts of each affected dogs are listed in Table 1. The mean RP counts obtained from the 19 specimens are summarized in Table 2. The correlations of RP counts in specimens prepared from the same subject either by PRPM or WBM were evaluated on the rank correlation profiles among mutual data. The percentage of RP counts from the reciprocal preparation method showed positive correlation in the Spearman's rank test ($r=0.80$, $p<0.001$; Fig. 1).

The diagnostic merits of flow cytometry analysis of RP are already known in relation to canine disease such as thrombocytopenia. Whereas PRPM has been used as the sole preparation method of RP specimens in previous surveys [3, 4, 7, 13], the adequacy WBM in flow cytometry analysis of the RP count has been unclear in relation to canine thrombopoiesis diseases. In present study, we compared the RP counts in paired specimens prepared by PRPM and by WBM from canine subjects with/without abnormal platelet counts for the purpose of clarifying the diagnostic applicability of WBM as a proper preparative method for RP specimens used in flow cytometric diagnosis in the dog, and we found that there was a significant correlation

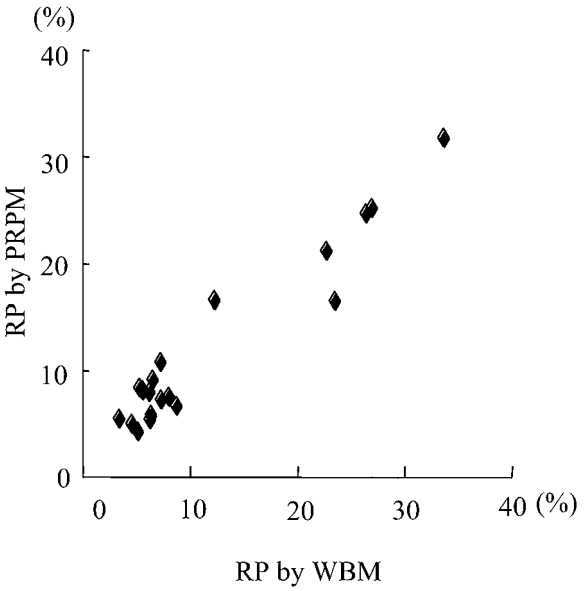

Fig. 1. Correlation between RP levels as measured by WBM and PRPM in 19 samples obtained from 9 healthy and 9 affected dogs.

between the RP counts measured by each method. The results of the present study indicate that WBM could be applied to flow cytometry as an alternative method for canine RP preparation instead of the conventional PRPM.

PRPM might exclude large and dense platelets since centrifugation is required to obtain PRP from WB [1]. The preparation of PRP reportedly leads to only 57% platelet recovery [8]. To obtain an adequate volume of PRP, a relatively large volume of WB is required. Conversely, no centrifugation is needed for sample preparation using WBM. Advantages of WBM include the ability to retain a larger amount of platelets and the fact that it requires a relatively small volume of blood. Therefore, WBM would be easier to use for detection of RP in dogs, particularly small breeds.

In conclusion, the present revealed that WBM, as well as PRPM, can be used clinically to determine the RP levels in dogs with abnormal platelet counts.

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