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A rapid multiple immunofluorescence method for the simultaneous detection of CD20 and CD3 in canine and feline cytological samples

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ABSTRACT. CD20 and CD3 are considered reliable markers for B and T cells, respectively. This study aimed to develop a rapid multiple immunofluorescence (RMIF) method for the detection of CD20 and CD3 on a single cytology slide. Air-dried smears were prepared using samples collected from dogs (n=26) and cats (n=6). Immunosignal detection using the newly developed method required 60 min. Clear immunosignals for CD20 and CD3 were detected in 24 of 26 samples in dogs and in all 6 cats. As the RMIF (CD20/CD3) method can detect markers of both B and T cells simultaneously on a single cytology smear, it would be an efficient tool for the immunophenotyping of canine and feline lymphoma samples.

KEY WORDS: CD20, CD3, immunocytochemistry, immunophenotyping, lymphoma

Cytology is a powerful tool for the clinical diagnosis of lymphoproliferative diseases. Immunophenotyping of lymphocytes provides critical diagnostic information to clinicians and clinical pathologists. We previously developed a rapid multiple immunofluorescence (RMIF) method for the detection of CD79α (B-cell marker) and CD3 (T-cell marker) on a single smear slide [14]. This method is useful for the rapid clinicopathological diagnosis of lymphoma in dogs and cats, but the detection of other markers, in addition to CD79α/CD3, is also useful for definitive diagnosis by cytology. CD20 is a well-known B-cell marker, and its expression is noted in the pre-B-cell stage up to the activated B-cell stage [2]. Furthermore, it has been widely used as a reliable marker for immunohistochemical and immunocytochemical diagnosis of canine and feline B-cell lymphoma [4, 8, 10–12]. However, whether it could be used for diagnosis with the RMIF method has not yet been evaluated. This study aimed to develop an RMIF method for the simultaneous detection of CD20 and CD3 in a single slide in immunophenotyping experiments, as well as evaluate its clinical utility.

The study was performed in accordance with the Guidelines for Animal Experimentation of Kagoshima University, Japan (No. VM15040). Cytological smears were prepared from samples collected from dogs (n=26) and cats (n=6) presented at the Veterinary Teaching Hospital of Kagoshima University, Japan. Cytopathological evaluations were performed prior to the experiments, and samples from cases in which diagnosis could be unambiguously made by cytology were used for this study. Cytological diagnosis was made according to morphological criteria, and an algorithm of diagnostic cytology [1]. Briefly, lymphoma was diagnosed if immature, large lymphocytes comprised more than 50% of the cell population. Cases with large lymphocytes comprising >30% but <50% of the cell population were considered suspected cases, and excluded from the present study. Cases with low-quality cytological preparations, such as those with high levels of blood contamination, few nuclear cells, and numerous crushed cells, were also excluded. Leftover untreated slides not used for clinical diagnosis were thoroughly air-dried, and stored at −30°C until used in the present study. The RMIF was performed as described previously, with a minor modification [13], and the procedure is presented in Table 1. Five-minutes fixation with formalin was set according to preliminary experiments and our previous report for detection of CD79α and CD3 [14]. For the simultaneous detection of CD20 and CD3 using the RMIF (CD20/CD3) method, an anti-CD20 rabbit polyclonal antibody (PA5-16701, 1:400; Thermo Fisher Scientific, Cheshire, UK) and an anti-CD3 mouse monoclonal antibody (clone F7.2.38, 1:50; DakoCytomation, Glostrup, Germany) were used as the primary antibodies. Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:500; Life Technologies, Paisley, UK) and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:500; Life Technologies) were used as the secondary antibodies. As a control, the antibodies used for the simultaneous detection of CD79α and CD3 in the previously reported

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RMIF (CD79α/CD3) method were used [14], that is, anti-CD79α mouse monoclonal antibody (Clone HM57, 1:100; Bio-Rad, Hercules, CA, USA) and anti-CD3 rabbit polyclonal antibody (IR50361, ready-to-use; DakoCytomation) as primary antibodies, and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500; Life Technologies, Paisley, UK) and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500; Life Technologies) as secondary antibodies. All antibody preparations were diluted with a blocking solution containing 0.25% casein in 10 mM phosphate-buffered saline. Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI; Dojinkagaku, Tokyo, Japan). For negative control experiments, a blocking solution, normal mouse IgG (DakoCytomation), and normal rabbit IgG (Thermo Fisher Scientific) were used instead of the primary antibodies. We also attempted to sequentially apply May-Grunwald's (MG)-Giemsa staining followed by RMIF staining on the same smear. The stained slides were imaged using a fluorescence microscope (BX-53; Olympus, Tokyo, Japan) equipped with a cooled charge-coupled device camera (DP-73; Olympus). The captured images were processed using imaging software (cellSens Standard; Olympus).

It took 60 min to complete the detection process using the developed RMIF (CD20/CD3) method. The immunosignals for both CD20 and CD3 were clear and strong, and the T and B cells could be clearly distinguished among the various lymphocytes smeared on a single slide (Fig. 1). The results of the examined cases are summarized in Table 2. Both RIMF (CD20/CD3) and RIMF (CD79α/CD3) methods worked suitably in 24 of the 26 canine samples and in all 6 feline samples (Table 2). The immunophenotypes for the 30 cases with clear signals determined using the RIMF (CD20/CD3) and (CD79α/CD3) methods matched completely (Table 2).

In six canine samples and three feline samples, the CD20 signals appeared to be more specific than the CD79α signals. CD20 signals were observed in the cell margins, and the distinct fluorescent outline simplified the process of identifying B cells among the various cells in the smears. In contrast, CD79α signals were observed in the cytoplasm in multiple lymphocytes, and weak yet apparent nonspecific CD79α signals were observed for the nine samples that displayed stronger, highly specific CD20 signals (Fig. 2). Sample quality was assessed by MG-Giemsa staining after staining by the RMIF method. MG-Giemsa staining quality was satisfactory even when performed after RMIF method staining, and cytomorphological quality was confirmed to be well preserved in all samples, including samples with low specific CD79α signals.

CD20 has been shown to be a reliable marker for B cells in the immunohistochemical diagnosis of canine and feline lymphoma [8, 12], and the present study demonstrated its compatibility with B-cell detection in commonly used air-dried cytology samples. The RMIF method offers several advantages over the conventional enzyme-based immunocytological methods, including greater sensitivity, specificity, and rapidness [13], and the present RMIF (CD20/CD3) method could be a useful tool for the diagnosis of feline and canine lymphoma.

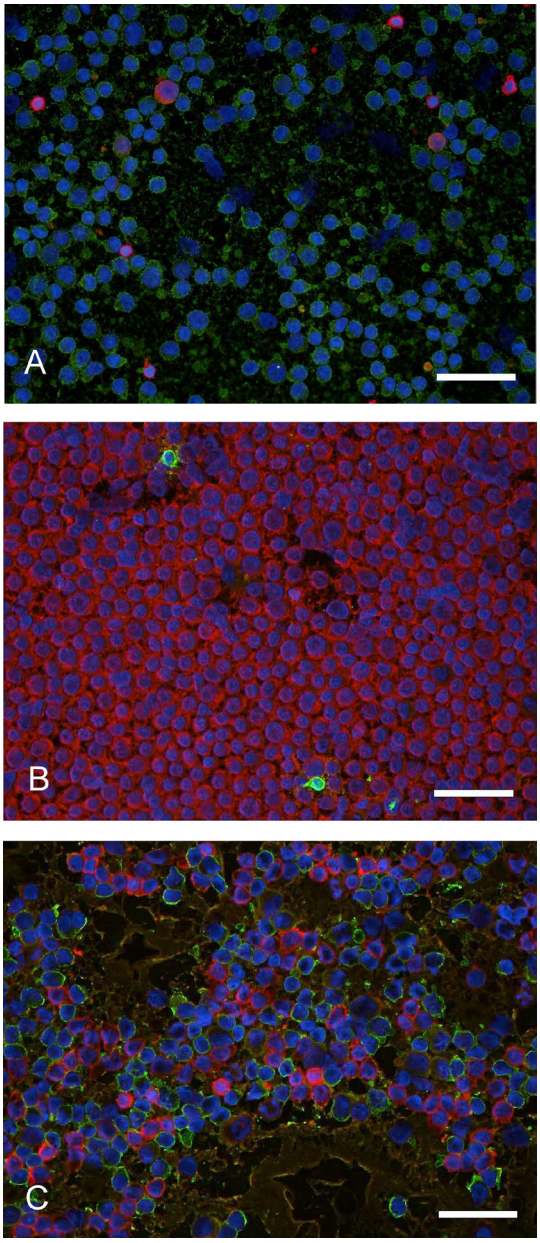

Fig. 1. Lymph node samples stained using the rapid multiple immunofluorescence (RMIF) CD20/CD3 method. A: B-cell lymphoma in a dog. B: T-cell lymphoma in a dog. C: Reactive lymphoid hyperplasia in a dog. Green signal; CD20-Alexa 488, red signal; CD3-Alexa 594, blue signal; DAPI. Samples were prepared using fine needle biopsy. Scale bars=20 μm.

CD20 is the target molecule of the biological drug rituximab (an anti-CD20 monoclonal antibody), a molecular-targeted drug for the treatment of B-cell lymphomas in humans [9]. The detection of CD20 in lymphocytes is crucial for determining the appropriate treatment strategy for a patient. In veterinary medicine, studies on anti-CD20 therapy have also been performed in canine B-cell lymphoma [5, 7]. Since the RMIF (CD20/CD3) method in the present study could rapidly and clearly detect CD20 in canine B cells in conventional cytological smears, this test is likely to become an important tool for diagnosis, as well as for determining the treatment strategy for canine lymphoma.

In addition to CD20, several markers, such as CD79α and paired box gene 5 (Pax5), have been used as markers for B cells. Although CD79α is reliably used as a B cell marker, CD79α is widely expressed in cells ranging from precursor B cells to plasma cells [2]. Consequently, interpretation of CD79α-positivity requires adequate caution if a large number of CD79α-positive plasma cells appear in a reactive hyperplastic lymph node with chronic inflammation. Pax5 is also expressed in a range of cells, from pro-B cells to mature B cells, and has recently been used as a more specific pan-B cell marker [6], and it has also been used for

Table 1. Rapid multiple immunofluorescence CD20/CD3 staining procedure

1	Air-dry	Thoroughness*
2	Incubate with 10% neutral buffered formalin	5 min
3	Wash with PBS	10 sec**
4	Incubate with 0.25% casein/PBS	5–10 min
5	Incubate with primary antibodies	15 min***
6	Wash with PBS	10 sec**
7	Incubate with secondary antibodies and DAPI	15 min***
8	Wash with PBS	10 sec**
9	Mount	

*: with cold air, **: with a direct stream from a bottle, ***: at 37°C. PBS: phosphate buffered saline.

Table 2. Comparison of the performances of rapid multiple immunofluorescence (RMIF) CD20/CD3 and RMIF CD79α/CD3 for the testing of clinical samples

Case	Species	Sample	Clinicopathological diagnosis	Predicted immunophenotype	
				CD20/CD3	CD79α/CD3
1	Canine	Superficial LNs	Lymphoma	B	B
2	Canine	Superficial LNs	Lymphoma	B	B
3	Canine	Superficial LNs	Lymphoma	B	B
4	Canine	Superficial LNs	Lymphoma	B	B
5	Canine	Superficial LNs	Lymphoma	B	B
6	Canine	Superficial LNs	Lymphoma	B	B
7	Canine	Superficial LNs	Lymphoma	B	B
8	Canine	Superficial LNs	Lymphoma	B	B
9	Canine	Superficial LNs	Lymphoma	B	B
10	Canine	Superficial LNs	Lymphoma	B	B
11	Canine	Superficial LNs	Lymphoma	B	B
12	Canine	Superficial LNs	Lymphoma	B	B
13	Canine	Superficial LNs	Lymphoma	T	T
14	Canine	Superficial LNs	Lymphoma	T	T
15	Canine	Superficial LNs	Lymphoma	T	T
16	Canine	Superficial LNs	Lymphoma	T	T
17	Canine	Superficial LNs	Lymphoma	T	T
18	Canine	Superficial LNs	Lymphoma	T	T
19	Canine	Superficial LNs	Lymphoma	ND	ND
20	Canine	Superficial LNs	Reactive	Mixture	Mixture
21	Canine	Superficial LNs	Reactive	Mixture	Mixture
22	Canine	Superficial LNs	Reactive	ND	ND
23	Canine	Pleural effusion	Chylous	Mixture	Mixture
24	Canine	Pleural effusion	Chylous	Mixture	Mixture
25	Canine	Pleural effusion	Chylous	Mixture	Mixture
26	Canine	Urinary sediment	Lymphoma	B	B
27	Feline	Superficial LNs	Lymphoma	B	B
28	Feline	Kidney	Lymphoma	B	B
29	Feline	Anterior mediastinal mass	Lymphoma	T	T
30	Feline	Pleural effusion	Lymphoma	B	B
31	Feline	Pleural effusion	Chylous	Mixture	Mixture
32	Feline	Pleural effusion	Chylous	Mixture	Mixture

LNs: lymph nodes, ND: not determined due to high background staining.

diagnostic purposes in canine and feline diseases [3, 15]. However, the pathological and clinical significance of Pax5 expression in canine and feline lymphoma has not been fully elucidated, and the use of Pax5 alone in immunocytochemical diagnosis is not yet recommended. As the number of smear slides is limited in cytology, unlike that in core-biopsy pathology, it makes sense to select CD20 as a B-cell marker. The present RMIF (CD20/CD3) method can detect markers of both B and T cells simultaneously in a single smear slide, and it would be useful as an advanced yet practical diagnostic technique for canine and feline lymphoma.

Limitations of the present study include lack of phenotyping data obtained by immunohistochemistry (IHC) of core-biopsy

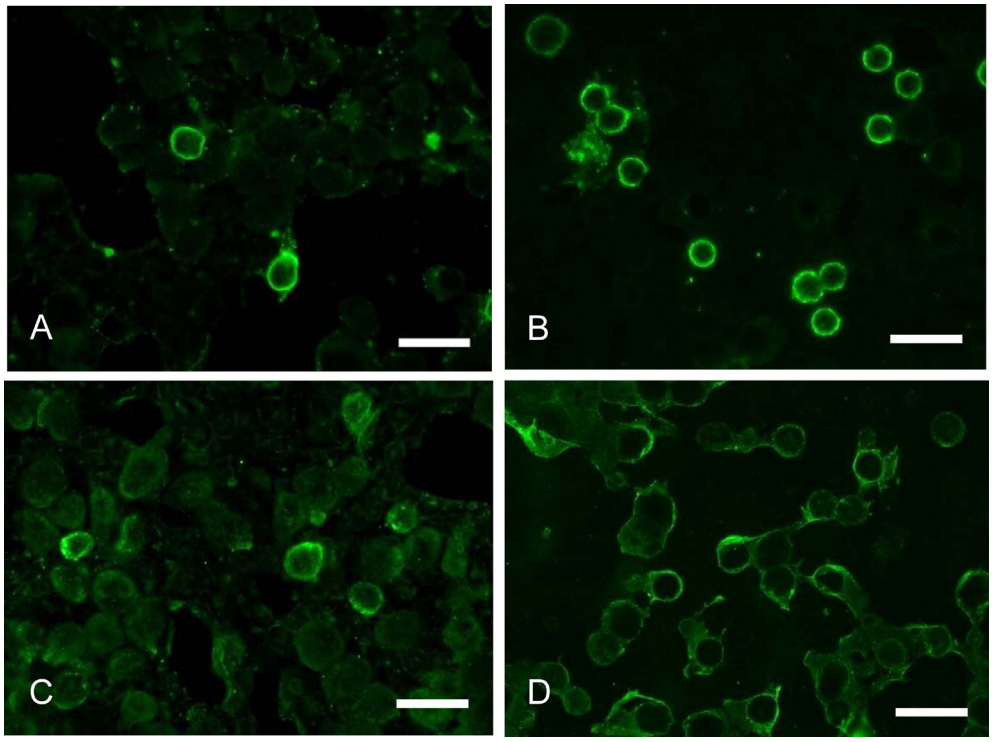

Fig. 2. Comparison of the rapid multiple immunofluorescence (RMIF) CD20/CD3 and RMIF CD79α/CD3 methods. A & B: Detection of CD20. C & D: Detection of CD79α. A & C: Fine needle biopsy samples from the lymph node of a dog with T-cell lymphoma. B & D: Sediment smear from the chylous effusion of a cat. In the RMIF CD20/CD3 samples, green signals for CD20 were observed in the margins of certain lymphocytes, and negative reactions were clearly observable in most lymphocytes. In the RIMF CD79α/CD3 samples, the weak, nonspecific green signals were detected in multiple lymphocytes. In all panels, a fluorescence filter adapted to Alexa 488 was used. Scale bars=10 µm.

specimens and/or genotyping data obtained by PCR for antigen receptor rearrangement (PAPR). In future analyses, the specificity and sensitivity of the RMIF (CD20/CD3) method should be evaluated by comparing its results with ICH and PAPR data.

In conclusion, the RMIF (CD20/CD3) method yielded satisfactory results with respect to the simultaneous detection of CD20 and CD3 in a single cytological preparation. The procedure is rapid and simple, and re-staining with Romanowsky-type stain after RMIF method staining is also satisfactory for checking sample cytomorphology. The RMIF (CD20/CD3) method can be used effectively for cytological immunophenotyping of canine and feline lymphoma, particularly when standard immunohistochemistry of core-biopsy specimens is not feasible.

POTENTIAL CONFLICTS OF INTEREST. The authors have no conflicts of interest to disclose.

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