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Flow Cytometric Evaluation of Different Monoclonal Antibody Clones against Rabbit Leukocytes

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ABSTRACT

Many techniques are described to correctly identify rabbit leukocytes. Both automatic and manual counts are used for this purpose of identifying different subsets of rabbit white blood cells. However, other techniques such as Flow Cytometry are used in veterinary medicine to identify different surface and intracellular antigens of cells in a short period of time and with high accuracy. Antibodies are needed when a flow cytometric analysis takes place and it is mandatory that these antibodies react with the cells of the species of interest. Antibodies should always be chosen carefully in order to have the best performance when their use is predicted, especially when taking into consideration their species specificity. However, antibodies reacting to a specific species can react also to other species. To prove this statement, antibodies have to be tested in order to define and confirm their reactivity to other species. In this study thirty-three peripheral blood samples from healthy rabbits were collected and tested for the following antibodies: CD21 (Clone: LT21), CD5 (Clone: YKIX322.3), CD4 (Clone: YKIX302.9), CD8 (Clone: YCATE55.9), CD11b (Clone: M1/70). All antibodies report different species reactivity from rabbits. Results showed that only CD11b reacts with rabbit myeloid cells showing satisfactory results. The clone used in this study showed higher performance on monocytes compared to granulocytes. However, further studies with a higher caseload are warranted to confirm the results.

Key words: Flow Cytometry, Rabbits, Antibodies, Leukocytes

INTRODUCTION

The complete blood count is considered the most common required laboratory test and the first diagnostic step in veterinary medicine clinical pathology in order to accurately identify different cell populations in the peripheral blood (Harvey 2012; Vudriko et al. 2021). A broad range of certified techniques is used in animals for this aim. Nowadays rabbits represent a very valuable species for different purposes such as livestock, companion and laboratory animals (Mapara et al. 2012; Welch et al. 2017; Esteves et al. 2018; Magalhães et al. 2022; Cremonesi et al. 2022; Agradi et al. 2022). Considering the importance of rabbit species all over the world, especially in Europe, it is necessary to have accurate data regarding cell populations in the peripheral blood. It has been showed that automatic and manual counts can be used with a high accuracy in rabbits. Actually, Oikonomidis and colleagues have showed how the automatic system ADVIA 2120, commonly used in human medicine, can be an important tool when analyzing rabbit leukocytes. Moreover, they have described a high correlation between manual and automatic methods regarding heterophils, lymphocytes, and basophiles, and a moderate to low correlation for monocytes and eosinophils, respectively (Oikonomidis et al. 2021).

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Flow cytometry is commonly used in veterinary medicine to identify and stage different neoplastic disorders such as lymphomas, leukemias, and mast cell tumors, especially in dogs (Sulçe et al. 2022). Moreover, flow cytometry can be used for a great variety of indices such as apoptosis, minimal residual disease, prognostic indices, Ki67, etc. (Marconato et al. 2019; Chalfon et al. 2019; Riondato et al. 2021). The use of different antibodies to correctly identify different cell populations or antigens is the key to this category of flow cytometric analysis (Grandoni et al. 2020; Riondato et al. 2022). Specific antibodies for flow cytometric analysis that have species reactivity with rabbit leukocytes are available on the market.

Other antibodies that are not species-specific for rabbits can be used to differentiate rabbit leukocytes if their reactivity is proven. Finding new antibodies that are other species-specific except rabbits can lead to a wider range of reagents that can be used for this species by scientists and researchers. The principal aim of this study is the evaluation of different antibody reactivity that are not previously reported to react with rabbit leukocytes.

MATERIALS AND METHODS

Ethical approval

The experiment was run with the permission of the National Authority of Veterinary and Plants protection under the Ministry of Agriculture and Rural Development (prot. 824/2021) of Albania.

Sample collection

Two milliliters of rabbit peripheral blood samples were collected from the marginal ear vein and placed in Ethylenediaminetetraacetate tubes. All rabbits (33 female New Zealand white breed) were purchased by a licensed factory at the age of 120 days and were grown till five months at the enclosure of the Faculty of Veterinary Medicine at the Agricultural University of Tirana. Moreover, room temperature and humidity (<50%) and light cycle (16 hours of light and 8 hours of darkness) were controlled. Available commercial food (Agrotek ALB, Rruga e Vjetër Nacionale, Vorë, 1032, Albania) with a specific recapture, containing 15.74% crude protein was used to feed the rabbits. The health status of all rabbits was checked every two weeks in order to exclude any rabbit with pathological health conditions from the caseload. All efforts were made to minimize animal distress and to use only the number of animals necessary to produce reliable results.

Flow cytometric evaluation

All Flow Cytometric evaluations were performed within 24h from rabbit peripheral blood collection which was maintained refrigerated at 4°C till analysis. Cell concentration of samples was assessed with an Attune NxT flow cytometer (Thermo Fisher) and further all analyses were performed with the same cytometer. Fifty microliters of samples (1 x 10^6 white blood cells/tube) and antibodies were placed in incubation at 4°C for 30min the dark. The quantity of antibodies used for each sample was decided following the manufacturer's recommendations (quantity/cell). Red blood cell lysis was performed using 2mL of ammonium chloride lysis buffer for each tube for 5min. After these steps samples were centrifuged at 1200rpm for 5min and the supernatant was discarded and further washed with phosphate buffer saline obtaining finally the white blood cell pellet. This pellet passed through the flow cytometer to be analyzed. A gating strategy was adopted to exclude all debris from the gate of analysis (Sulçe et al. 2022).

Lymphocytes were used as a negative control population when myeloid (CD11b) markers were used while the same was applied with myeloid cells when lymphocytic markers were used (CD21, CD5, CD3, CD4, and CD8). In order to make the correct evaluation of the antibody functionality, the stain index (SI) was calculated for all samples and all antibodies using the following formula:

MFI of Positive – MFI of Negative/2 * SD of Negative
Where: MFI=Median fluorescence intensity
SD=CV * Mean Negative/100
SD=Standard deviation and is calculated as follows: (Median Positive * Median Negative)/100.

Antibodies

Different antibodies were used to evaluate their possible reactivity with rabbit leukocytes. None of the antibodies was declared to react with rabbit leukocytes from the manufacturer. All antibodies used in the study are listed in Table 1 containing also technical specifications.

Statistical analysis

Minimum, maximum and mean data regarding stain index for CD11b were calculated to observe the efficiency of the antibody toward myeloid cell populations. Box plots were created to better compare stain index data between Granulocytes and Monocytes. Statistical analyses were run with SPSS v.25 (IBM). Statistical analysis was not performed for CD5, CD21, CD4, and CD8 since no reactivity was detected for these antibodies.

RESULTS

In total 33 cases were included in this study. Lymphocytes showed no reactivity to CD5, CD21, CD4, CD8 in any of the cases. Myeloid cells including monocytes and neutrophiles showed a good reactivity to CD11b in all cases collected. Based on the stain index

Table 1: List of antibodies used to evaluate their possible reactivity with rabbit leukocytes

Antibody	Clone	Conjugation	Target Cells	Species Reactivity	Producer
CD21	LT21	FITC	Mature B-Cells	Bovine, Dog, Human, Pig	Thermo Fisher Scientific
CD5	YKIX 322.3	FITC	Mature T-Cells	Dog	Thermo Fisher Scientific
CD4	YKIX 302.9	FITC	T-Helper Cells	Dog	Thermo Fisher Scientific
CD8	YCATE 55.9	PE	T-Cytotoxic Cells	Dog	Thermo Fisher Scientific
CD11b	M1/70	PECy5	Myeloid Cells	Mouse	Thermo Fisher Scientific

data, clone M1/70 (CD11b) had a different performance on monocytes and granulocytes. Stain index for each population; granulocytes and monocytes was calculated. Indeed, based on the stain index, granulocytes showed satisfactory reactivity to CD11b (minimum 5.97, maximum 57.21 and mean 18.11). While monocytes showed a higher stain index (minimum 38.42, maximum 139.22 and mean 75.99). Stain index results are presented in Table 2.

Distinct fluorescence intensity was observed for each myeloid population. Monocytes showed a higher fluorescence compared to granulocytes making it easier for this population to be identified. In Fig. 1 the gating strategy and the most representative cases are showed. Differences in stain index between granulocytes and monocytes for CD11b are presented in Fig. 2.

DISCUSSION

Despite the fact that rabbit species has a major importance in many fields, studies focusing on discovering market-available antibodies that can react with rabbit leukocytes in flow cytometry are missing. This study demonstrated the reactivity of non-specific antibodies (CD5, CD21, CD4, CD8, and CD11b) to rabbit leukocytes in order to test their effectiveness in this species. Results from this investigation show that only CD11b could react with rabbit myeloid cells in all cases with a satisfactory outcome. A significant difference was observed between granulocytes and monocytes in terms of fluorescence intensity to CD11b. Levels of CD11b antigen expression can alter during many medical conditions in humans (Horvath et al. 2013; Kim et al. 2017; Yildiz et al. 2022) and in animals (Duan et al. 2016; Režić-Mužinić et al. 2018). However, the difference between these cell populations can simply be due to different surface levels of the specific antigen that binds to the CD11b clone used in this study. The CD11b was successfully used previously to characterize myeloid cell populations in dogs (Sulce et al. 2018). However, this study has several limitations. First, even though CD11b was positive in all cases for myeloid populations, the number of cases is low, and further studies are needed to confirm these results. Another limitation of the study is the lack of isotype control for CD11b even though lymphocytes were used as an internal negative control. Finally, the use of CD11b clones that are confirmed to react with rabbit leukocytes would be of great importance as a further confirmation of the aim.

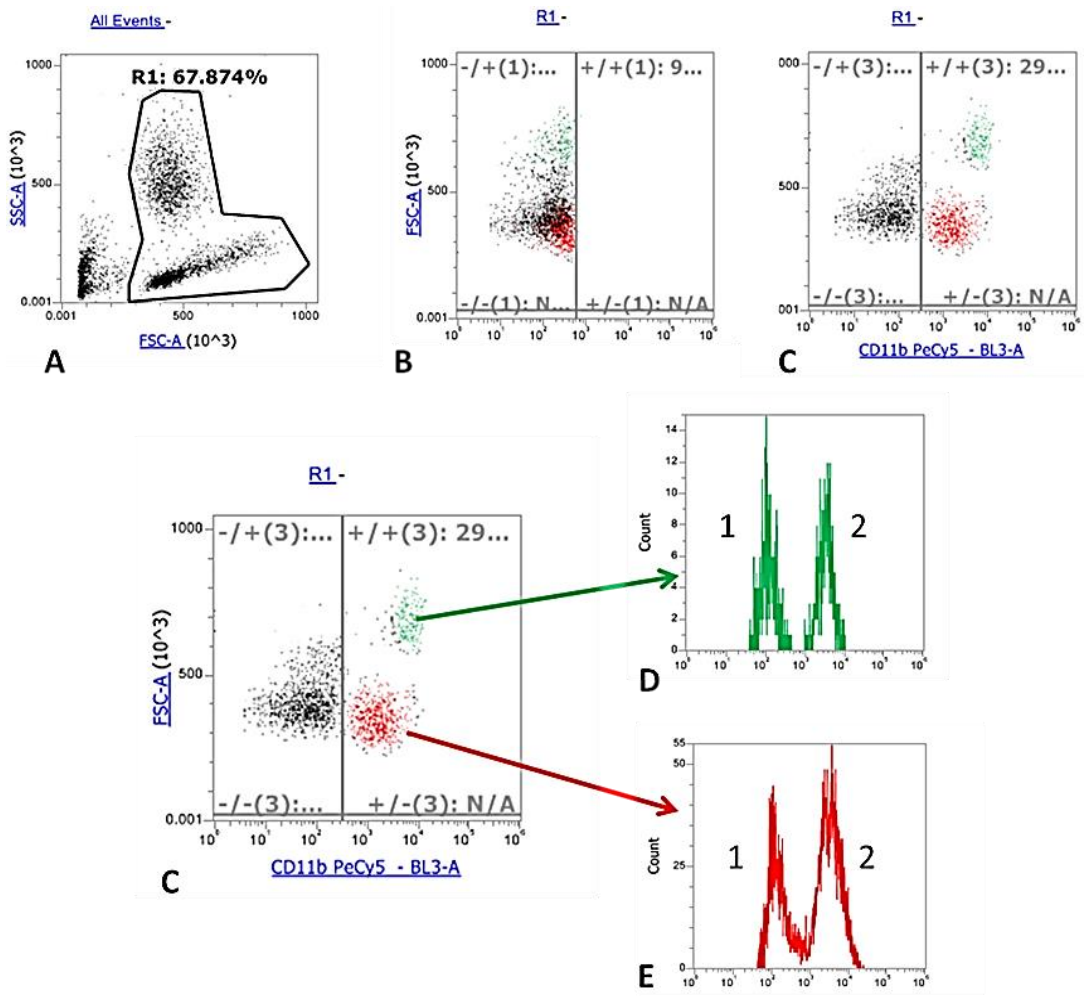

Fig. 1: Reactivity of myeloid populations to CD11b in rabbits: A) Gating strategy adapted to exclude debris from the gate of analysis, B) Cells prior to labeling (line set to their autofluorescence), C) Positive cells to CD11b where granulocytes are represented by red dots while monocytes by green ones, D) Histogram of negative control population (Lymphocytes 1) and positive cells (Monocytes 2), and E) Histogram of negative control population (Lymphocytes 1) and positive cells (Granulocytes 2).

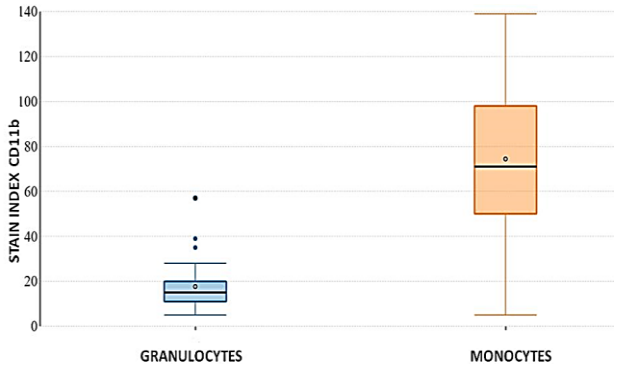

Fig. 2: Differences between granulocytes and monocytes toward reactivity to CD11b expressed in stain index.

Table 2: Stain index of Antibody/Clone (CD11b/M1/70) in granulocytes and monocytes

Cases	Granulocytes Stain Index	Monocytes Stain Index
1	27.98	108.84
2	57.21	137.37
3	6.32	49.14
4	17.75	43.92
5	8.42	38.42
6	17.28	92.70
7	13.20	44.41
8	26.97	76.97
9	21.45	100.67
10	15.04	84.61
11	16.40	100.25
12	14.47	65.76
13	11.42	50.04
14	24.29	46.51
15	14.78	63.43
16	16.20	56.45
17	26.41	139.22
18	14.42	110.23
19	35.37	98.23
20	6.98	47.33
21	19.78	79.78
22	15.01	57.83
23	10.70	61.14
24	18.00	54.92
25	17.80	88.48
26	10.76	94.77
27	15.16	113.56
28	9.75	71.29
29	19.32	110.44
30	39.50	73.06
31	5.97	40.27
32	11.25	55.73
33	12.33	51.94
Mean±SD	18.11±10.44	75.99±28.31

Conclusion

To the best of the author's knowledge, this is the first study where the chosen antibody clones were used to evaluate their reactivity to rabbit leukocytes. We identified CD11b clone M1/70 as a reliable marker for rabbit myeloid cell populations. The results showed that this clone can be routinely used to identify rabbit myeloid cells in flow cytometry. Moreover, monocytes showed a higher stain index compared with granulocytes. However further studies with a higher caseload are warranted to confirm the findings of this study.

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