Flow Cytometry to Evaluate *Anaplasma marginale* Parasitemia Using a Fluorescent Nucleic Acid Stain

Rosalía Moretta,[a] Paula Ruybal,[a] María Mesplet,[a] Romina Petrigh,[a] Pablo Nuñez,[a] Gonzalo Gil,[a] Silvina Wilkowsky,[a] Graciela Garbossa,[b] and Marisa Farber[a]

[a]CICVyA–INTA Castelar, Buenos Aires, Argentina

[b]Facultad de Ciencias Exactas y Naturales, UBA, Buenos Aires, Argentina

In this work we describe a flow cytometry–based method using SYTO16 (a DNA intercalating agent) to quantify *Anaplasma marginale*–infected erythrocytes in blood from bovine animals. The linearity and reproducibility of the results obtained with SYTO16 labeling followed by flow cytometry analysis make it a suitable approach for measurement of parasitemia in *A. marginale* infections.

***Key words:* *Anaplasma marginale*; flow cytometry; parasitemia; SYTO16**

Introduction

Bovine anaplasmosis is a tick-borne infection caused by *Anaplasma marginale*, an obligate intracellular Gram-negative bacterium of the family *Anaplasmataceae*. Erythrocytes are the main site of infection of *A. marginale* in cattle and within these cells these bacteria reside and replicate in membrane-bound inclusions (also called inclusion bodies) that usually contain four to eight bacteria.[1] Even though light microscopy is the most widely used technique for estimation of parasitemia, this method relies on the expertise of the technician, leading to less-accurate and -reproducible results. Flow cytometry has been used to test the efficacy of antimicrobial agents against *A. marginale* in short-term erythrocyte cultures using the vital dye, hydroethidine.[2] In addition, the fluorochrome SYTO16, which is able to penetrate intact cells and bind strongly to nucleic acids, was employed to determine *Theileria sergenti* parasitemia in bovine whole blood.[3] In the present study we evaluated the SYTO16 staining method for measuring parasitemia in blood from bovines infected with *A. marginale*.

Materials and Methods

Cell staining for fluorescence-activated cell-sorting (FACS) was performed according to the method of Yagi and collaborators.[3] In brief, 3 μL of citrated blood were labeled with 50 μM SYTO16 (Molecular Probe, Eugene, OR) in a final volume of 1 mL for 30 min at 37°C in the dark. Cells were washed twice in PBS and resuspended in 1 mL of FACS flow buffer for flow cytometry analysis. The suspensions were analyzed by argon-ion laser fluorescence excitation at 488 nm and emission at 535 nm in log FL1 data mode using a Becton–Dickinson FACSCalibur and CellQuest computer software (BD Biosciences, San Jose, CA). An inclusion gate was set based on the forward- and

Address for correspondence: Rosalía Moretta, Instituto de Biotecnología, CICVyA, INTA, Los Reseros y Las Cabañas, CP 1712, Castelar, Buenos Aires, Argentina. Voice: +54-11-4621-1278. rmoretta@cnia.inta.gov.ar

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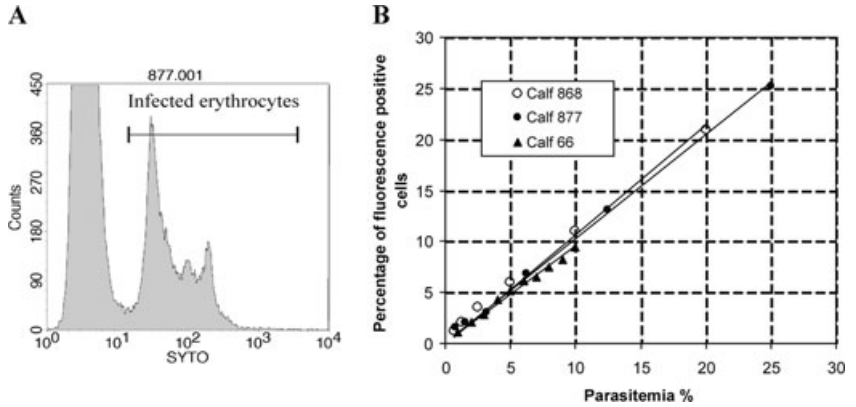

Figure 1. (**A**) Fluorescence histogram of stained *A. marginale*–infected erythrocytes. The *x*-axis shows SYTO16 fluorescence intensity (FL1 535 nm). Parasitized cells appeared as a second population with a log shift in mean fluorescent intensity as compared with uninfected cells. (**B**) Correlation between expected parasitemia in serial dilutions versus percentage of fluorescence-positive cells. Calf 877, $R^2 = 0.997$; Calf 868, $R^2 = 0.991$; Calf 66, $R^2 = 0.988$.

side-scatter characteristics of the control (SYTO16-treated uninfected erythrocyte). The percentage of infected erythrocytes was expressed as the percentage of fluorescence-positive cells (PFPCs). Parasitemia was determined by light microscopy in Giemsa-stained thin smears, counting the number of erythrocytes containing inclusion bodies per 1000 erythrocytes and expressed as the percentage of infected cells.

Results and Conclusions

Infected erythrocytes were clearly differentiated from noninfected erythrocytes (Fig. 1A). Parasitized erythrocytes appeared as a second population with a log-increase in mean fluorescent intensity. Infected cells showed a fluorescence resolution into discrete peaks in most samples. This fact might reflect that the number of bacteria contained in each erythrocyte could be variable but not random. Sensitivity and linearity of the SYTO16 staining protocol was evaluated using serially diluted infected-blood samples of known parasitemia. A very good correlation between the PFPCs and the expected parasitemia (initial parasitemia × dilution factor) was observed in three independent curves (Pearson's coefficient of correlation, $R^2 = 0.997$, 0.991, and 0.988; Fig. 1B). Reproducibility of the technique was assessed by performing duplicate labeling of each sample. The standard deviation of these independent measurements was always less than $\pm$ 1.0%.

The performance of the new staining method was evaluated during the time course of an experimental infection with *A. marginale*. The PFPC values during the acute phase resembled the parasitemia estimated by blood thin-smear examination. However, values obtained by light microscopy were always below PFPC values, showing an underestimation in the number of infected erythrocytes by the former method (data not shown). These results could be due to a greater sensibility of flow cytometry to detect infected erythrocytes in early stages of invasion (incipient inclusion bodies) that are not yet visualized by light microscopy.

In conclusion, this method accurately determined parasitemia in the range of 1–50% and allowed monitoring the magnitude of infected erythrocytes during the acute phase of the disease in a objective and rapid way.

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Conflicts of Interest

The authors declare no conflicts of interest.

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