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The dynamics of erythrocyte infection in bovine anaplasmosis: A flow cytometry-based analysis

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ABSTRACT

Anaplasma marginale (*A. marginale*) is an obligate intracellular bacterium that infects bovine erythrocytes causing extravascular hemolysis and anemia. In the present work, we combine SYTO16 labeling of parasitized cells with the statistical power of flow cytometry to study the evolution of erythrocyte infection during bovine anaplasmosis.

1. Introduction

Bovine anaplasmosis is a tick-borne disease caused by infection with the rickettsial pathogen *Anaplasma marginale*. Common features of the disease include anemia, weight loss, decreased milk production, abortion and death, which cause important losses to livestock production worldwide (Kocan et al., 2010).

A. marginale is an obligate intracellular bacterium that infects erythrocytes (Amerault et al., 1973; Francis et al., 1979; Kocan et al., 2004). The course of the infection is divided into three main stages: the prepatent period, the acute phase and the persistent or chronic infection. The prepatent period is defined as the time elapsed between the inoculation and the observation of infected erythrocytes in thin blood smears. The acute phase is characterized by both, increasing parasitemia and the development of anemia, and corresponds to the period in which most of the clinical signs of the disease become noticeable (Lotze, 1947; Kreier et al., 1964). If the bovine succeeds in controlling the infection, decreasing parasitemia is accompanied by anemia remission. Finally, chronic infection is established and the animal continues to be parasitized for years showing cyclic subclinical peaks of rickettsemia with a 6 to 8 week period (Kieser et al., 1990).

Bacteria reside within a vacuole derived from the erythrocyte membrane where they replicate, generating inclusions that contain a variable number of microorganisms (Ristic and Watrach, 1963; Simpson et al., 1967; Francis et al., 1979). Up to date, many aspects of the invasion (attachment, entrance, replication and exit from the host cell) have remained elusive (Erp and Fahrney, 1975; Blouin et al., 1993). Remarkably, *A. marginale* is not found free in plasma even with a parasitemia greater than 60% (Ristic and Watrach, 1963). Moreover, *in vitro* assays to mimic the biological mechanism of spreading in red cells were unsuccessful (Kessler et al., 1979; Blouin et al., 1993).

The lack of knowledge on *A. marginale*'s cycle of invasion has caused the black box paradigm to prevail, especially with respect to the relationship between erythrocyte infection and removal. De et al. (2012) favor the damage on the host cell derived from *A. marginale*'s metabolism as a plausible link. Giardina et al. (1993) claim to have found bacterial proteins in the red cell surface. Finally, an autoimmune mechanism due to cross reactivity of antibodies against *A. marginale* with erythrocyte surface proteins has been theorized (Schroeder and Ristic, 1965). Kreier et al. (1964) show that both, infected and non-infected erythrocytes were phagocytosed in the bone marrow of cattle. However, given the small size of the bacterium Ristic and Watrach (1963) sustain that erythrocytes infected with just one microorganism are usually missed by light microscopy inspection, shedding doubts on the real status of phagocytosed cells (Melendez, 2005). In either case, the focus of the controversy lies in

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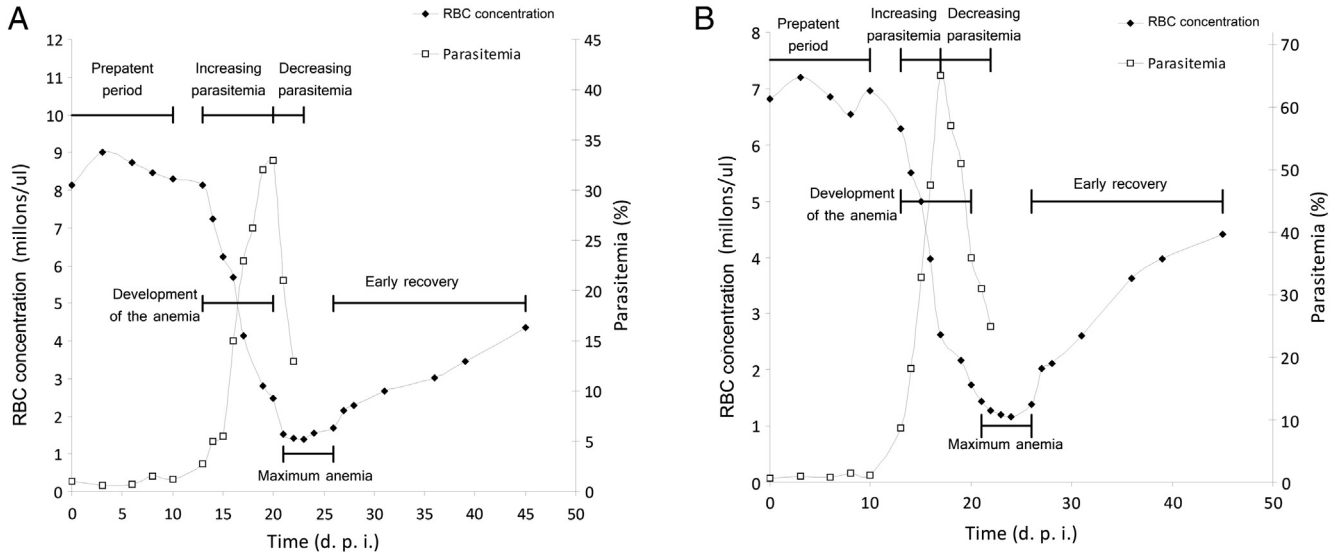

Fig. 1. General features of the infection. Evolution of RBC concentration and parasitemia during the infection in animals B524 (A) and B640 (B). The different phases of the infection and the disease are indicated.

whether erythrocyte removal rate can be attributed to the percentage of parasitized cells (accounted for as an indirect measurement of the progression of the infection).

We have previously described the use of SYTO16 to determine parasitemia by flow cytometry. This methodology has proven to enable the accurate identification of *A. marginale* infected erythrocytes (Moretta et al., 2008). Also, similar approaches have been used to determine parasitemia in hemotropic protozoan pathogens such as *Babesia gibson* and *Plasmodium* spp. (Yamasaki et al., 2008; Barkan et al., 2000). In the present work we use this strategy to study the dynamics of erythrocyte infection in the course of bovine anaplasmosis to give an initial step towards answering the fundamental question: What is in the box?

2. Materials and methods

2.1. Experimental infection with A. marginale

Two male Angus (B524, B640; 18 months old) bulls were injected intravenously with 4 mL of blood with 69% of infected erythrocytes (*A. marginale* SALTA1 strain) obtained from a splenectomized animal at peak parasitemia. Animals were fed with alfalfa pellets twice a day and water *ad libitum*. Clinical monitoring of the animals was performed by specialized veterinarians who determined the need for treatment. At day 17 post-inoculation (p.i.) B524 presented mild respiratory distress. A single dose of long-acting oxytetracycline (20 mg/kg) was sufficient to ameliorate the animal's condition. At the end of the experimental period, the animals were euthanized by the injection of 50 mg/kg of sodium thiopental followed by jugular exsanguination. The experiment was conducted following the Guide for the Care and Use of Animals—INTA (approved by resolution CICVyA No. 14/07).

2.2. Hematology

Blood samples collected by jugular vein puncture were anticoagulated with 0.13 M EDTA in a proportion of 50:1, and evaluated in an automated hematology analyzer (CELL-DYN 3500-LASER Abbott Laboratories, Illinois, U.S.A.). The report included: red blood cell concentration (RBCc, million cells/μL), hemoglobin concentration (g/dL), packed cell volume (%), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration (MCHC, g/dL).

2.3. Flow cytometry

Blood samples for flow cytometry were processed and labeled as described elsewhere (Moretta et al., 2008). Briefly, 3 μL of blood was labeled with 250 μM SYTO16 (Molecular Probe, Eugene, OR) 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 acquisition (FACSCalibur, BD Bioscience).

2.4. Data analysis

Flow cytometry data was analyzed using the software winMDI 2.9. Parasitemia was determined as the percentage of SYTO16 labeled cells with fluorescence intensity in the range of 10^1 to 10^3 (see M2 in Fig. 2) (Moretta et al., 2008).

Mean fluorescence intensity (MFI) was determined as the geometrical mean of the selected population (*i.e.* M1, M2, M3 and M4 showed in Fig. 2). Since SYTO16 is a cell-permeant dye, slight variation in incubation/washing time can give rise to variability in fluorescence intensity among samples. For comparison purposes, MFI of infected erythrocyte

Table 1
Values of hematological parameters recorded during the prepatent period, expressed as the mean of the values obtained at different times. The range of variation is indicated in brackets (min–max).

	PCV (%)	RCC (10^9/mL)	Hemoglobin (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Normal range[a]	24–46	5–10	8–15	40–60	11–17	30–36
B640	29 (28–31)	8.4 (8.1–9)	10.5 (10–11.1)	35	12.5 (12–13)	36 (35–36)
B524	27 (25–29)	6.8 (6.2–7.2)	9.5 (9–10.2)	39.8 (39–40)	14.5 (14–15)	36.5 (36–37)

[a] From "The Merck Veterinary Manual".