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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

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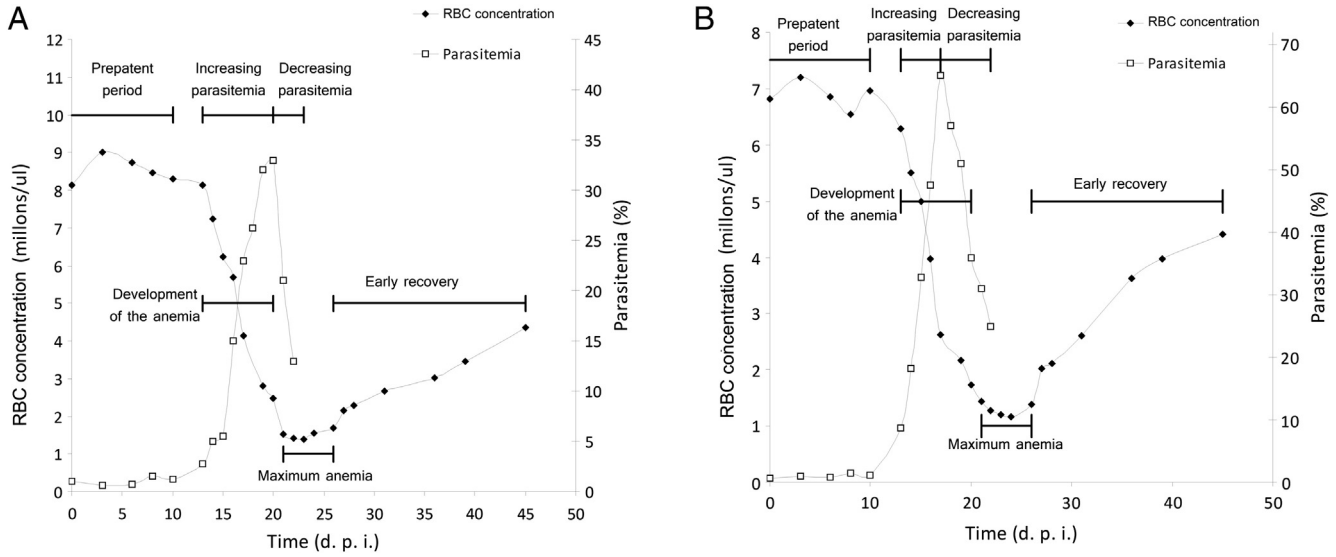

Fig. 1. General features of the infection. Evolution of RBC concentration and parasitemia during the infection in animals B524 (A) and B640 (B). The different phases of the infection and the disease are indicated.

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 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 populations was normalized to uninfected red cell MFI of the same sample and designated as NMFI.

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".

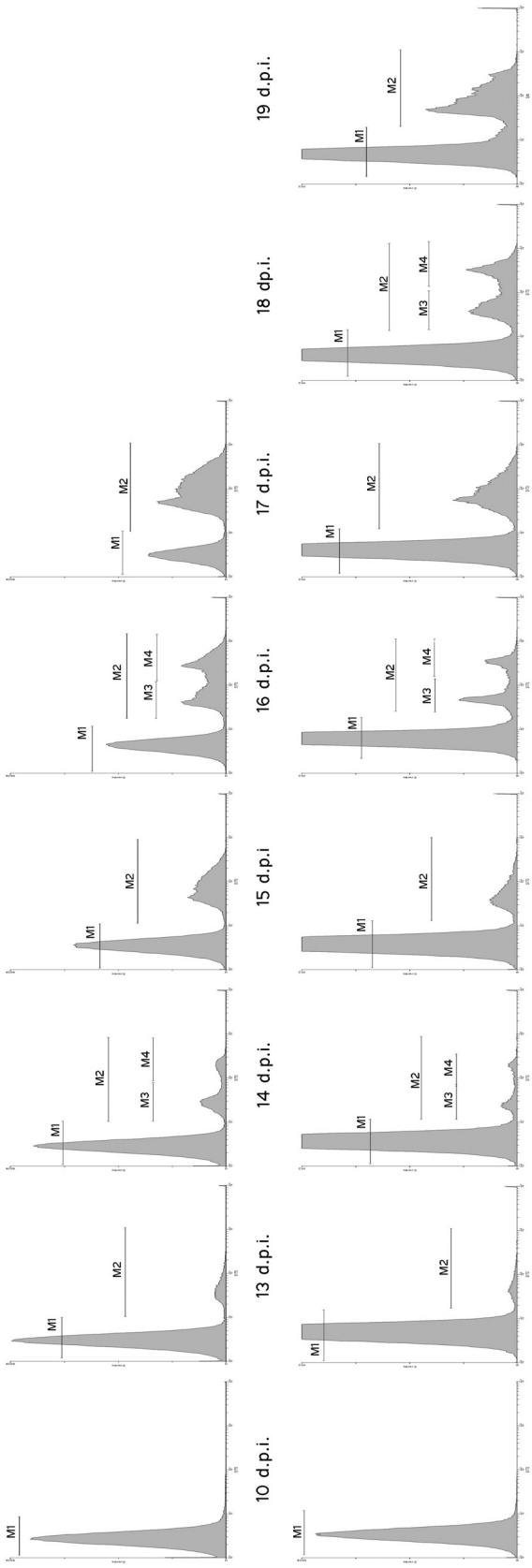

Fig. 2. Analysis of infected erythrocyte population during the period of increasing parasitemia. Flow cytometry histograms for animals B640 (upper panel) and B524 (lower panel). M1: uninfected erythrocytes; M2: infected erythrocytes; and M3 and M4: subpopulations of infected erythrocytes. The histograms showed for day 10 p.i. are representative of those observed during the prepatent period.

Arithmetic means are reported. In addition, minimum and maximum values observed for each parameter are indicated. Correlation coefficients are reported when least squares linear regressions were fitted to the data.

3. Results

3.1. General features of the infection

In order to study the dynamics of erythrocyte infection during bovine anaplasmosis two animals were inoculated with *A. marginale* and followed for 45 days. Fig. 1 shows RBC concentration and parasitemia during the experiment in animals B524 (A) and B640 (B). The three phases of the infection are indicated in this figure: prepatent, increasing and decreasing parasitemia. Likewise, the evolution of the disease was divided into: development of anemia, maximum anemia and early recovery.

3.2. Prepatent period

Erythrocytes containing *A. marginale* inclusions were first visualized in blood thin smears at day 10 p.i. in a low proportion of the total count (parasitemia < 0.05%). During the prepatent period, hematological parameters remained within normal limits for age-matched animals with the exception of MCV, which was slightly below the low end of the range (Table 1). Representative flow cytometry histograms of erythrocyte population throughout prepatency are shown in Fig. 2 (10 d.p.i.). MFI of uninfected erythrocytes had a mean value of 4.1 (min = 3.3; max = 4.8) and 3.4 (min = 2.9; max = 3.6) for B640 and B524 respectively.

3.3. Increasing parasitemia and development of the anemia

SYTO16 positive cells were detected by flow cytometry after day 13 p.i. Analysis of the histograms revealed the cyclic alternation of two distribution patterns of infected cells (Fig. 2). The first consisted in a single population skewed to the left in the fluorescence scale with a NMFI of 18 (min = 14; max = 23). The second was composed of two distinguishable subpopulations with NMFIs of 9.6 (min = 7; max = 12) and 58 (min = 44; max = 70) (Fig. 2, marked as M3 and M4).

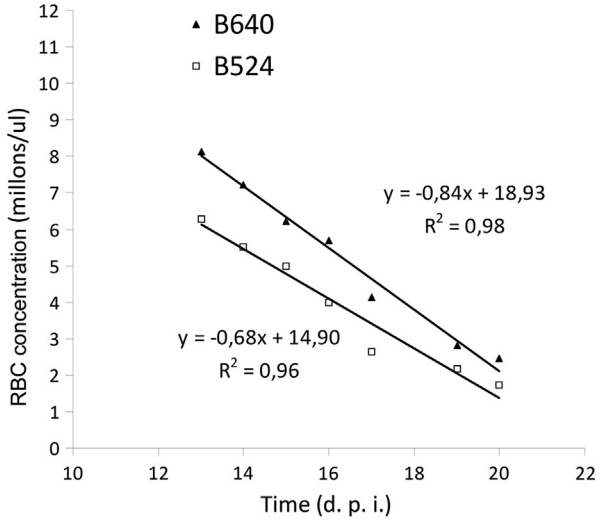

Fig. 3. Development of the anemia. Erythrocyte concentration values between days 13 and 20 p.i. were fit by a linear regression. Slopes: B524 = −0.68 RBC/day, r^2 = 0.96; B640 = −0.84 RBC/day, r^2 = 0.98.

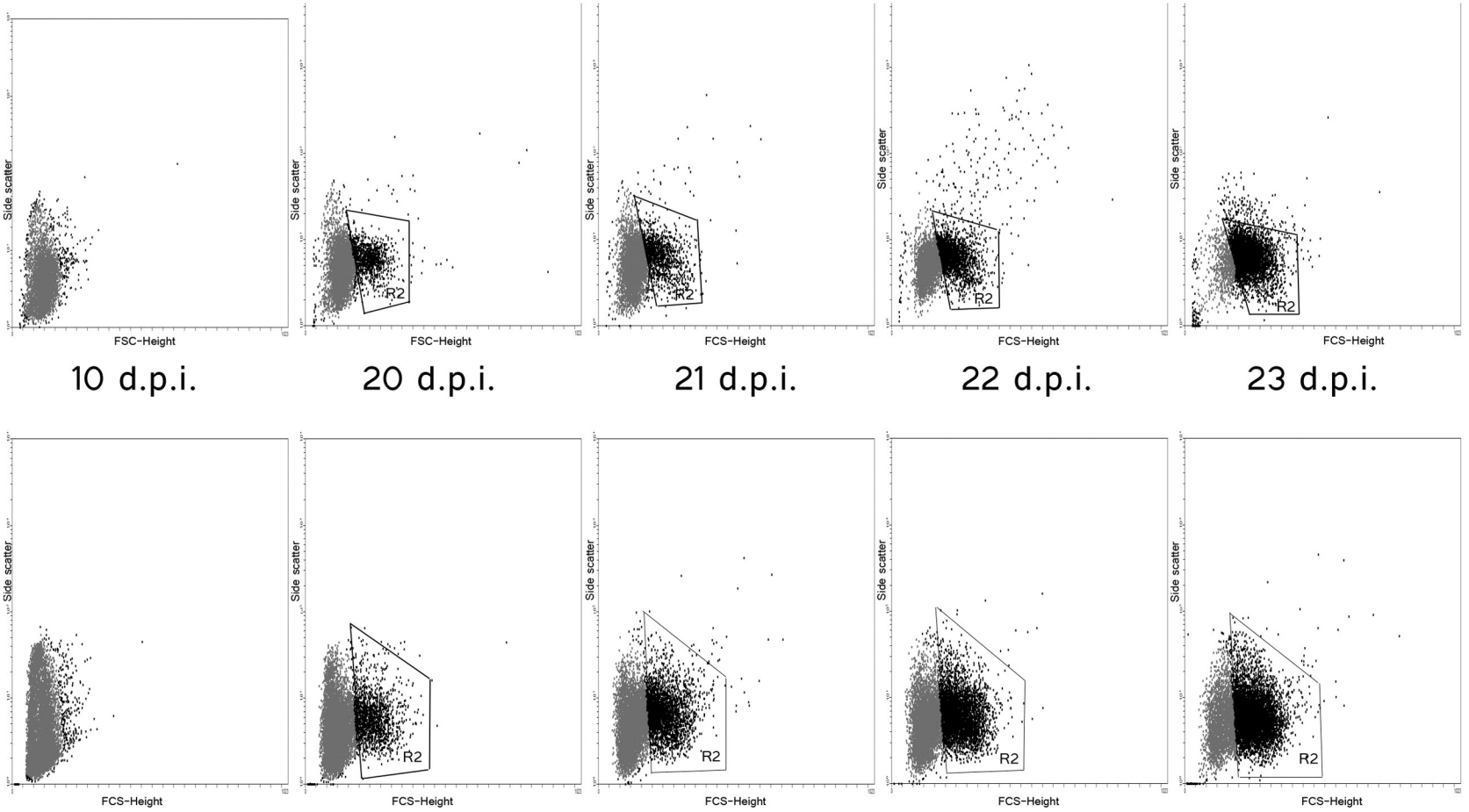

Fig. 4. Flow cytometry analysis of erythrocyte population during the period of maximum anemia. Normal erythrocyte population (referred to in the text as R1) was gated using the sample from day 10 p.i. (shown in gray). Upper panel: B524; lower panel: B640.

The development of anemia was coincident with the period of increasing parasitemia. Erythrocyte count diminished at a constant rate from days 13 to 20 p.i. (Fig. 3). In both animals the slope represents 10%/day of initial RBCc (Fig. 3, Table 1).

3.4. Maximum anemia

3.4.1. Flow cytometry analysis of RBC heterogeneity

The period of maximum anemia was characterized by a stable RBCc and a progressive increment in MCV and MCH (at constant MCHC) concurrent with the emergence of a population of cells larger than normal erythrocytes in FSC/SSC dot plots (Fig. 4). The percentage of these cells (defined by R1 and R2, Fig. 4) displayed a linear correlation with MCV (Fig. 5A). Therefore, considering two red cell populations with volumes V1 and V2 which are represented in a mixed sample according to the percentages P for V2 and (100 − P) for V1, MCV can be expressed as:

$$MCV = PV2 + (100-P)V1$$

or equivalently:

$$MCV = P((V2-V1)/100) + V1$$

where (V2 − V1) / 100 and V1 are, respectively, the slope and the intercept of the regression line. The volume of R2 population was calculated for each animal as:

$$V2 = \text{slope} * 100 + \text{intercept}$$

and had a value of 110 and 116 for B640 and B524 respectively. The ratio V2/V1 was 2.5 for B524 and 3.6 for B640.

MCH also displayed a linear relation to the percentage of larger cells (Fig. 5B). In this case the slopes can be interpreted as the difference in volume (V2 − V1) between the two populations at constant MCHC:

$$MCH = P((V2-V1)/100)(MCHC/100) + MCH1$$

where the intercept is the initial value of MCH. In this case V2 is:

$$V2 = \text{slope} * 10^4/MCHC + V1$$

and presented values of 103 and 90 which correspond to V2/V1 rates of 2.3 and 2.9 for B524 and B640 respectively. According to this analysis, the cell volume is augmented approximately three times in R2 population when compared with R1, which is in agreement with the variation in the erythrocyte size observed in thin blood smears (data not shown).

The evidence given above indicates that cells enclosed in R2 are most likely newly released macrocytes. The quantitative analysis of this population revealed the two components of RBCc stabilization. As it can be observed in Fig. 6A, the reduction in the number of erythrocytes in R1 is counteracted by the increment in R2 population.

3.4.2. Detection of SYTO16 fluorescence in R2 population

Larger cells presented a MFI of 11.3 (min = 9; max = 14), which was three times greater than MFI of erythrocyte population during the prepatent period and rising parasitemia (4 and 3.5 for B640 and B524 respectively). We reasoned that background fluorescence due to non-specific dye retention could be dependent on cell volume. In accordance with this assumption, FCS/FL1 dot plots show that larger cells extend from normal erythrocyte population towards higher fluorescence intensity values over the diagonal in a size-dependent manner (Fig. 6B).

Most of SYTO16 positive cells were restricted to R1 population (Fig. 6B). A subset of cells in R2 presented fluorescence intensities in the range of infected erythrocytes (Fig. 6B, R3). Since these cells represented a constant percentage of R2 population irrespective of parasitemia evolution, they were not considered infected (Fig. 6C).

3.5. Decreasing parasitemia

After reaching maximum parasitemia, flow cytometry analysis showed that the cyclic pattern of distribution alternation described in Section 3.3 continued in animal B640 and was interrupted by the treatment with oxytetracycline in animal B524 (Fig. 7). Interestingly, in B524 a four peak distribution was observed within the population of infected erythrocytes (Fig. 7, upper panel: marked as M3, M4, M5 and M6). We therefore tested if the increment in NMFI in these subpopulations was proportional to the increment in bacterial number. For this purpose the NMFI of the peaks was determined in four samples (Fig. 7, upper panel: 19 to 22 d.p.i.) and compared to bacterial number, considering that *A. marginale* replicates in the inclusion body as 2^n, with n = 0, 1, 2, 3. Fig. 8 shows the linear regression of the data ($r^2 = 0.98$), where

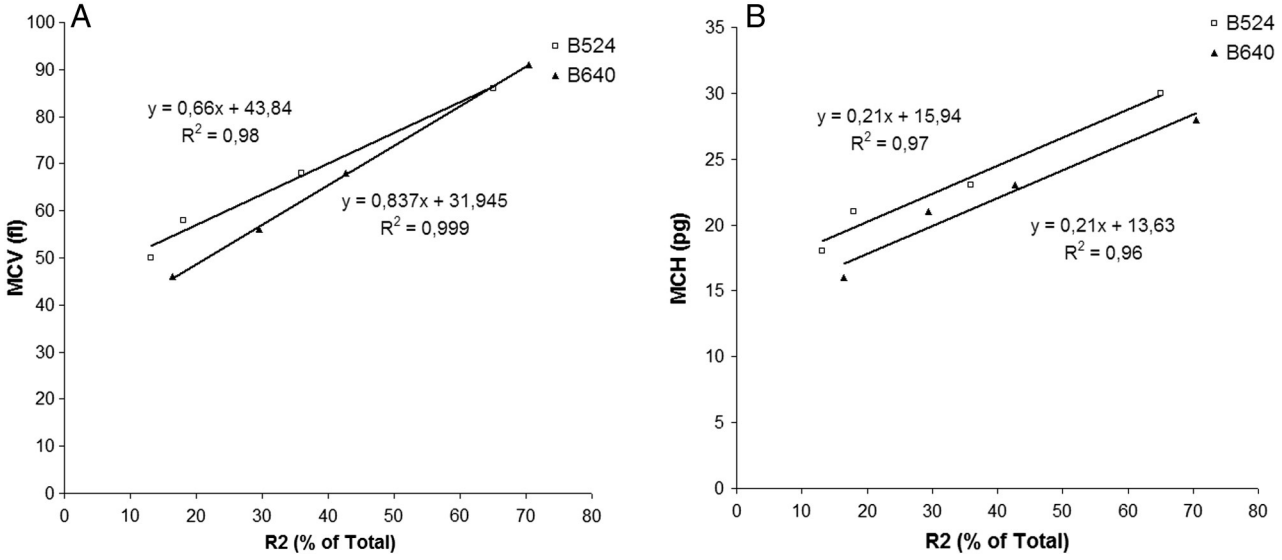

Fig. 5. Correlation between the percentage of cells in R2 and (A) MCV or (B) MCH. Data from days 20 to 23 p.i. were fit by linear regressions. (A) B524: 0.66 + 43.84, $r^2 = 0.98$; B640: 0.84 + 31.9, $r^2 = 0.999$; (B) B524: 0.21 + 15.94, $r^2 = 0.97$; B640: 0.21 + 13.6, $r^2 = 0.96$.

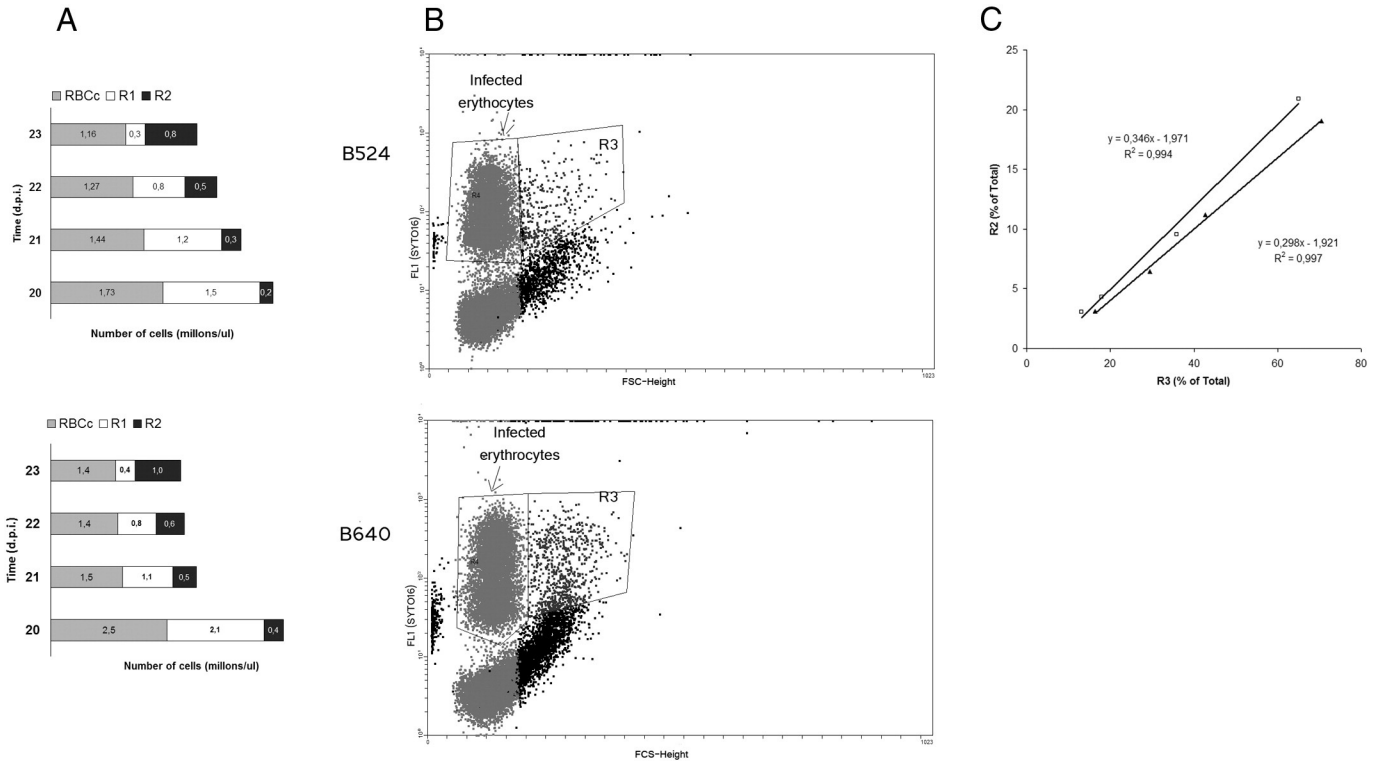

Fig. 6. Dynamics of erythrocyte population during maximum anemia. (A) Absolute values (millions/μL) for RBC total count, cells in R1 and cells in R2. (B) Representative FCS height/FL1 dot plots showing SYTO16 positive cells within R2 (R3), and infected erythrocytes within R1 (shown in gray). (C) Linear correlation between R3 and R2 percentages showing that a constant proportion of R2 cells was SYTO16 positive.

the slope indicates that erythrocytes infected with one microorganism have an average MFI that is 9.8 times greater than the MFI of uninfected red cells.

3.6. Early recovery

Parasitized erythrocytes were not observed by flow cytometry or in thin blood smears after day 22 p.i. Elevated MCV values persisted until the end of the experiment which caused PCV to be restored before 45 d.p.i. even though erythrocyte count was still below the range determined in the prepatent period (data not shown). The hematological profile during the early recovery phase was very similar in both animals and apparently independent of treatment. The evolution of RBCc is depicted in Fig. 1.

4. Discussion

In the present work, we have used the fluorescent dye SYTO16 to study the evolution of erythrocyte infection in bovine anaplasmosis. In accordance with our previous work infected erythrocyte population extended over a wide range of fluorescence intensity (Moretta et al., 2008). This is consistent with a non-synchronized infection and can be attributed to a variable number of microorganisms in the inclusion bodies.

The statistical power of flow cytometry allowed us to describe a non-random arrangement of bacterial number within infected cells during the development of the disease. FACS analysis at 24 h periods revealed that the increase in parasitemia was characterized by the alternate presence of two distinctive patterns of fluorescence distribution. The reiteration of these outcomes over time and in both animals is peculiar, considering that it is a consequence of at least three different processes with potentially different rates of progression (invasion, replication and exit). Additionally, erythrocyte removal could constitute a fourth variable if a specific subpopulation of infected cells is being taken out of circulation. The complexity of this scenario precludes us from obtaining a direct interpretation of the apparently cyclic increment in the proportion of cells that harbor a larger number of bacteria.

The rate of decrease in RBCc during the development of the anemia reflects the dynamic association between the removal and replacement of red blood cells. The release of red cells and erythroid precursors to the circulation is delayed in anemic bovines and occurs in response to severe erythrocyte loss (Doxey, 1977). Therefore, the replacement rate is not expected to vary until changes in MCV and MCH were recorded. Hence, the steady percentage of variation in RBCc (10%/day) can be considered a rate of erythrocyte removal. The fact that it was similar in both animals and independent of parasitemia is in agreement with previous reports for severe infections. As stated by Baker et al. (1961) it may actually represent “the size and potential of reticuloendothelial system functioning at a maximal rate in its catabolism of RBC”.

It has been shown for other hemotropic parasites that infection is restricted to particular age classes of erythrocytes (McQueen and McKenzie, 2004). In the case of *A. marginale*, Williams and Jones (1968) avoid recrudescence in infected cattle during patency with transfusions of blood from newborn calves containing numerous immature red cells. Based on this, the authors propose that *A. marginale* can only infect mature erythrocytes. In our study, newly released macrocytes were identified by their size during the period of maximum anemia (R2 population). We determined that a fixed proportion of these cells was SYTO16 positive. That proportion was also found to be very similar between the two animals. Thus, even though infection in R2 population couldn't be ruled out, the appearance of SYTO16 positive cells seems to be more related to the hematological profile (*i.e.* bone marrow activation and release of erythroid precursors) than with the evolution of the infection. In addition, the increment in NMFI in R2 population could be related to a greater RNA content in macrocytes. Nonetheless it has been shown that SYTO16 stains DNA with approximately twice the fluorescence with which it stains RNA (Yagi et al., 2000). Infected cells in R1 population were identified clearly during the time

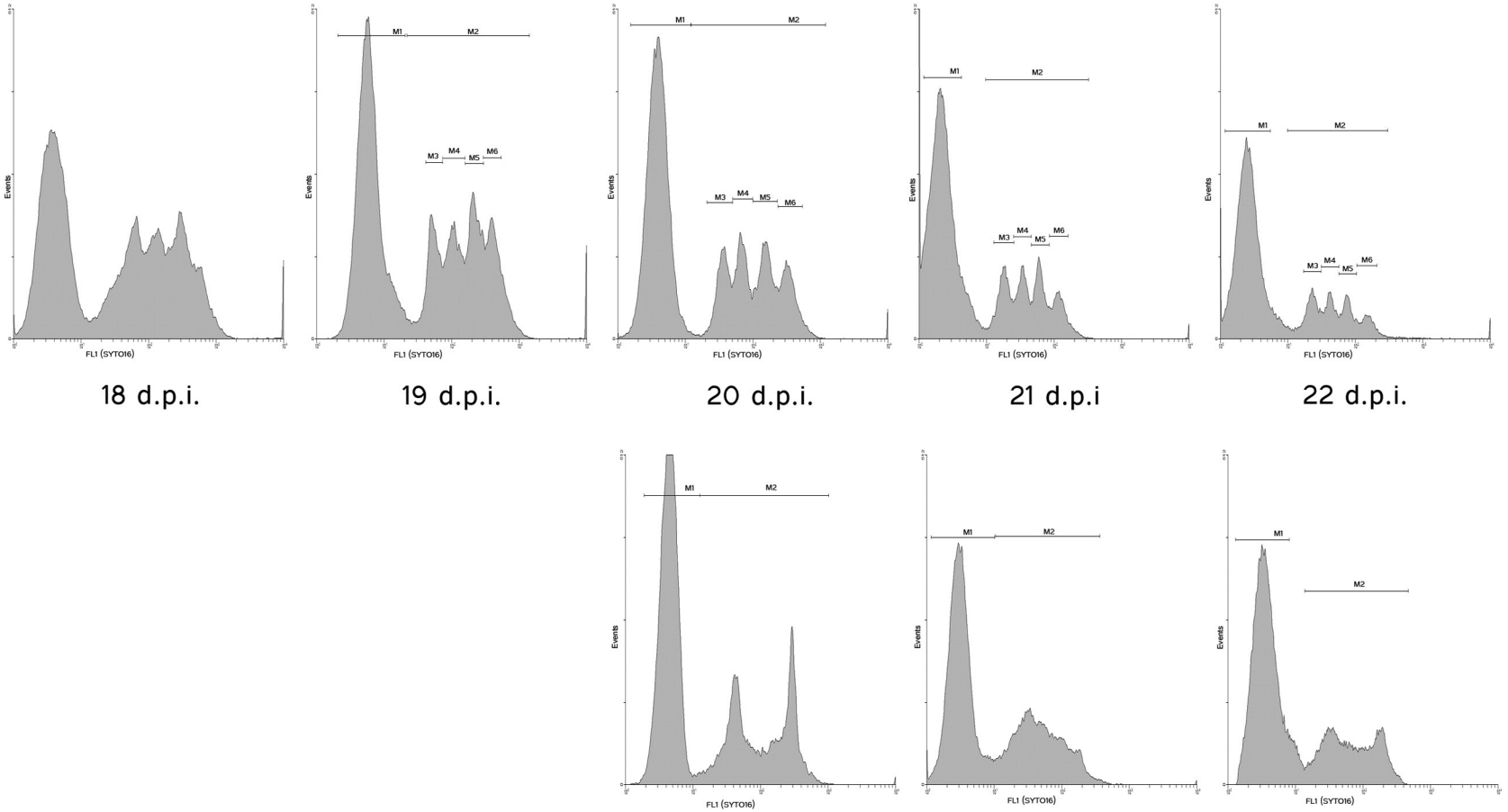

Fig. 7. Flow cytometry histograms during the period of decreasing parasitemia. Upper panel: B524; lower panel: B640. M1: uninfected erythrocytes; M2: infected erythrocytes. M3, M4, M5, M6: subpopulations of infected erythrocytes.

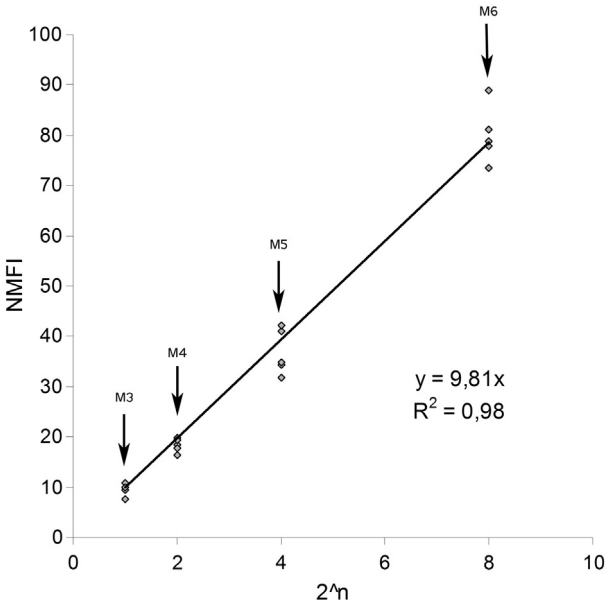

Fig. 8. Comparison between increasing NMFI and a theoretical number of bacteria given by 2^n.

of decreasing parasitemia and presented characteristic patterns of distribution with respect to fluorescence intensity.

In the present work, we observed four peaks at different fluorescence intensities in samples from animal B524 after treatment. The NMFI of these peaks displayed a remarkable correlation to the function 2^n, which was the mathematical approach we used to model the increase in bacterial number within the red blood cell. Our evidence supports that erythrocytes harboring 1, 2, 4 and 8 microorganisms can be distinguished by flow cytometry. Cell sorting and TEM analysis of these populations could confirm this result. Regretfully, these methodologies were out of our reach and will be addressed in future work.

The four peak arrangement was only observed after oxytetracycline administration. This antibiotic has been shown to cause a series of degenerations in the initial bodies that interfere with *A. marginale* normal cycle (Simpson, 1975). Thus, the pronunciation of the peaks after the treatment could be due to a decrease in the number of bacteria in intermediate stages of DNA synthesis during replication. The pattern of distribution conformed by two populations during raising parasitemia can also be defined by the low representation of cells infected with an intermediate number of bacteria (2 and 4). In this regard, we think that sampling with a period of the order of hours combined with SYTO16-flow cytometry analysis could shed some light into *A. marginale*'s duplication time and cycle of invasion.

5. Conclusion

The flow cytometry analysis reported in this work shows novel aspects of the infection with *A. marginale* that can lead to the advancement in the knowledge of the biology of this microorganism along with a better understanding of the pathogenesis of the disease.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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