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Mitigated clinical disease in water buffaloes experimentally infected with *Babesia bovis*

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ABSTRACT

Water buffaloes (*Bubalus bubalis*) are raised in tropical and subtropical regions of the world, and act as hosts of *Babesia bovis* parasites and the tick vector *Rhipicephalus microplus*. As no clinical cases of *B. bovis*-infection have been reported, we hypothesized that, unlike bovines, water buffaloes respond asymptomatically to an acute infection. To test this hypothesis, we inoculated two groups of 24-month-old Mediterranean breed water buffaloes with 10^8 erythrocytes infected with two Argentine *B. bovis* isolates: BboM2P (n = 5) or BboS2P (n = 5). These strains displayed mild (BboM2P) or high (BboS2P) pathogenicity in *Bos taurus* calves of the same age (n = 5 and n = 1, respectively), when tested in parallel. In water buffaloes, no changes in body temperature were observed with both strains, and no hematocrit changes were detected in BboM2P-inoculated animals. In contrast, in the BboS2P-inoculated water buffalo group significant but relatively minor reductions in haematocrit values were noted compared to the infected bovine. The parasitemia attained in water buffaloes was considerably lower than in bovines and could only be detected by nested PCR, or indirectly via serology, whereas in most bovines, it could also be detected in Giemsa-stained smears under the light microscope. Our results show that water buffaloes present no or significantly mitigated clinical symptoms to *B. bovis* infections and suggest that they are able to substantially reduce and/or eliminate *B. bovis* parasites from circulation by an efficient innate immune mechanism.

1. Introduction

Water buffaloes (*Bubalus bubalis*) are often bred in tropical and subtropical regions, many times sharing pastures with bovines (Romero-Salas et al., 2016). In addition to the utilization of their milk, meat, and leather, water buffaloes are useful for land ploughing and transportation, and have been an integral part of Asian agriculture since ancient times (Somparn et al., 2004). They have been also introduced to other regions, such as South America, the Middle and Near East, Africa and Europe because they are robust and adaptable in a great variety of conditions, including poor pastures and/or floodable lands. Water buffaloes have a moderate growth potential, yet their resistance to stress conditions, such as heat, seasonal fluctuations in both the quality and quantity of available feed and exposure to ecto- and endoparasites, gives them an adaptive advantage over cattle in tropical areas (Frisch and Vercoe, 1979). Although they usually remain healthy despite commonly being raised in poor sanitary conditions, they have a high range of disease susceptibility to different pathogens, which varies among bubaline breeds (Mingala et al., 2009; Yilmaz et al., 2012).

Several species of ixodid ticks can feed on water buffaloes, among which *Rhipicephalus microplus* has been reported as the most abundant in some regions of India, Brazil and Pakistan (Miranpuri, 1988; Corrêa Fdo et al., 2012; Rehman et al., 2017). Importantly, this tick can complete its whole life cycle feeding exclusively on bubaline blood (Benitez et al., 2012). Infections by the most pathogenic *R. microplus*-transmitted bovine piroplasmid, *Babesia bovis*, have been detected in water buffaloes by molecular and/or serological methods in several countries (Ferreri et al., 2008; Terkawi et al., 2011; Li et al., 2014; da Silva et al., 2014; Elsify et al., 2015; Mahmoud et al., 2015; Romero-Salas et al., 2016; Silveira et al., 2016). In cattle, *B. bovis* infections cause significant economic loss and limit livestock production in tropical and subtropical regions, where vector ticks thrive. High fever and a sharp decrease in hematocrit are the typical initial signs that appear in naïve *Bos taurus* bovines a few days after exposure to a pathogenic

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strain. This is often followed by severe disease manifestations, such as nervous signs, abortion and death (Florin-Christensen et al., 2014; Ganzinelli et al., 2018). In water buffaloes, *B. bovis* infections are generally assumed to be asymptomatic due to the absence of reported clinical cases. Yet, it is not known whether clinical signs are absent following infection, whether there are subclinical effects, or whether these ruminants recover rapidly from transient disease. The present work was designed to explore this aspect, through the experimental infection of non-splenectomized water buffaloes with two *B. bovis* strains that display different pathogenicity in cattle.

2. Materials and methods

2.1. Animals

Ten male water buffaloes (*Bubalus bubalis*), of the Mediterranean breed, and six male Holstein bovines (*Bos taurus*) were used. All animals were aged 18 months and weighed 220–250 kg. They were bred in tick-free regions of Argentina, and were free of *B. bovis* infections, as confirmed by PCR and ELISA (see 2.4. and 2.5). Animals were transported to the tick-free animal facilities of the Experimental Station of INTA at Mercedes, Corrientes, Argentina (EEA-Mercedes), kept in conditioned pens with *ad libitum* access to water and fed once a day.

2.2. Parasites

B. bovis strains BboS2P and BboM2P used in this study were isolated from clinical cases in bovines from the Argentine provinces of Salta and Corrientes, respectively, and kept in liquid nitrogen (Anziani et al., 1993; Vanzini, V.H., personal communication). BboM2P parasites were amplified in a splenectomized Holstein calf and blood was withdrawn at the peak of parasitemia. BboS2P was amplified in in vitro culture as described by Ristic and Levy (1980). In both cases, inoculation doses of 10^8 infected erythrocytes were calculated after the quantification of erythrocytes in a Neubauer chamber and the assessment of percentages of infected erythrocytes in Giemsa-stained smears.

In the case of BboM2P, though initially isolated from a clinical case that resulted in the death of the bovine donor, the strain was not as virulent as expected, and only moderate clinical signs were observed in the splenectomized calf. BboS2P, on the other hand, has not lost its pathogenicity after several processes of freezing and thawing, in vitro culture and amplification in splenectomized calves (Baravalle et al., 2012; Echaide, I., unpublished observations). The contrasting pathogenicity between both strains was utilized to compare the response of bovines with that of water buffaloes.

2.3. Experimental inoculation of B. bovis in bovines and water buffaloes

The study was divided into two separate experiments. In the first, five water buffaloes and five bovines were inoculated intramuscularly with 10^8 erythrocytes infected with the BboM2P strain; and, in the second, five water buffaloes and one bovine were inoculated intravenously through the jugular vein with 10^8 erythrocytes infected with BboS2P. The following parameters were recorded daily at 1–19 days post inoculation (dpi): rectal temperature, presence of clinical signs, parasitemia in Giemsa-stained smears prepared from the tail vein, and hematocrit using heparinized blood withdrawn from the jugular vein. Rectal temperature and hematocrit varied randomly in the buffaloes during the first three days of the experiment, likely due to handling-connected stress, and returned to basal levels at the fourth day. Thus, the values obtained at 4 dpi were used as basal levels for comparisons throughout the experiment. For molecular detection of parasites, citrated blood was removed from the jugular vein at day 14, and stored at −20 °C. For serological determinations, blood samples without anticoagulants were removed at 0, 7, 19, 30 and 60 dpi for both BboM2P-inoculated groups; at 0, 7, 11, 15, 18, 22, 24, 28, 36, 47 and 100 dpi for BboS2P-inoculated water buffaloes, and at 22, 36 and 47 dpi for the BboS2P-inoculated bovine. After separation of blood clots by centrifugation, serum samples were stored at −20 °C.

2.4. Molecular detection of B. bovis

DNA was extracted from citrated blood samples using a commercial kit (DNeasy® Kit for blood and tissues, Qiagen) and stored at −20 °C until used. *B. bovis* DNA was detected following the nested PCR method described by Figueroa et al. (1993). Positive and negative controls were included in each run. Products were analyzed by horizontal gel electrophoresis in the presence of ethidium bromide. The presence of a band of about 300 bp, as determined by comparison with 1 kb DNA ladder (Invitrogen), was indicative of *B. bovis* infection.

2.5. Detection of anti-B. bovis antibodies

An indirect ELISA that uses soluble antigens from in vitro cultured *B. bovis* merozoites was applied for the serological detection of anti-*B. bovis* antibodies (Echaide et al., 2004). A strong positive (C + +) and a negative control reference sera from water buffalo or bovine were included in each plate. Sera were diluted 1:10 and determinations were carried out in triplicates. Variations among A_{405} values of the triplicate determinations for each sample were lower than 10%. After subtracting the average value of the corresponding negative serum, positivity percentages (PP) were calculated according to the formula: PP = average A_{405} of the test serum × 100/average A_{405} of C + +. Samples were considered positive when the PP was equal or above the established cut-off value of 20%.

2.6. Statistical analysis

Student's *t*-test was applied to study significant differences between averages, and multiple analyses were carried out using Analysis of variance (ANOVA) and Tukeý́s test.

3. Results

3.1. Experimental inoculation of bovines and water buffaloes with BboM2P

In a first experiment, five bovines and five water buffaloes were inoculated intramuscularly with 10^8 BboM2P-infected erythrocytes. *B. bovis* parasites could be confirmed in four out of five experimentally infected bovines by direct observation of Giemsa-stained smears. Additionally, all bovines tested positive by nested PCR. In contrast, parasites could not be detected microscopically in water buffaloes, whereas three out of five animals tested positive using nested PCR (Table 1). A consistent but transient antibody response was observed in the bovine group, as determined by iELISA. Positivity percentages were above 40% for all animals at day 19, but later decreased approaching basal levels (Fig. 1). In the water buffalo group, only a single animal surpassed the 20% cut-off value at 60 dpi. Surprisingly, this animal had tested negative by nested PCR at day 14. In conclusion, four out of five water buffaloes and five out of five bovines were considered to be *B.*

Table 1
Detection of *B. bovis*-infection in bovines and water buffaloes inoculated with the *B. bovis* strains BboM2P and BboS2P.

Strain	BboM2P		BboS2P	
Animals	Bovines (n = 5)	Buffaloes (n = 5)	Bovines (n = 1)	Buffaloes (n = 5)
Microscopy	4	0	1	0
nPCR	5	3	ND	0
iELISA	5	1	1	5

ND: not determined.