Ticks and Tick-borne Diseases 9 (2018) 1358–1363

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

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

Daniel Benitez[a], Maria Mesplet[b], Ignacio Echaide[c], Susana Torioni de Echaide[c], Leonhard Schnittger[b,d], Monica Florin-Christensen[b,d,*]

[a] Estacion Experimental Agropecuaria (EEA)-Mercedes, Instituto Nacional de Tecnologia Agropecuaria (INTA), W3470, Mercedes, Argentina
[b] Instituto de Patobiología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas, INTA, 1686, Hurlingham, Argentina
[c] EEA-Rafaela, INTA, S2300, Rafaela, Argentina
[d] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), C1033AAJ, Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Water buffalo
Babesia bovis
Bovine babesiosis
Hematocrit
Parasitemia
Clinical signs

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

* Corresponding author at: Instituto de Patobiologia, CICVyA INTA-Castelar Los Reseros y Nicolas Repetto, s/n 1686 Hurlingham, Provincia de Buenos Aires Argentina.
E-mail address: jacobsen.monica@inta.gob.ar (M. Florin-Christensen).

https://doi.org/10.1016/j.ttbdis.2018.04.012
Received 15 February 2018; Received in revised form 16 April 2018; Accepted 16 April 2018
Available online 20 April 2018
1877-959X/

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.

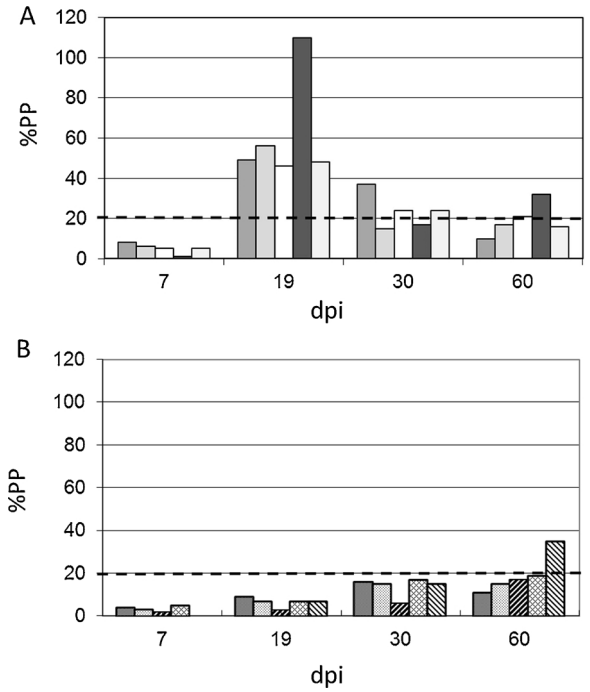

Fig. 1. Anti-*B. bovis* antibody response of bovines (A) and water buffaloes (B) inoculated with BboM2P. Serological diagnosis was carried out with an iELISA using cultured *B. bovis* merozoites. Bars correspond to positivity percentages (PP) obtained for each individual animal at 7, 19, 30 and 60 dpi. Bars with similar shades or patterns correspond to the same animals. Values above the established cut-off of 20% were considered positive.

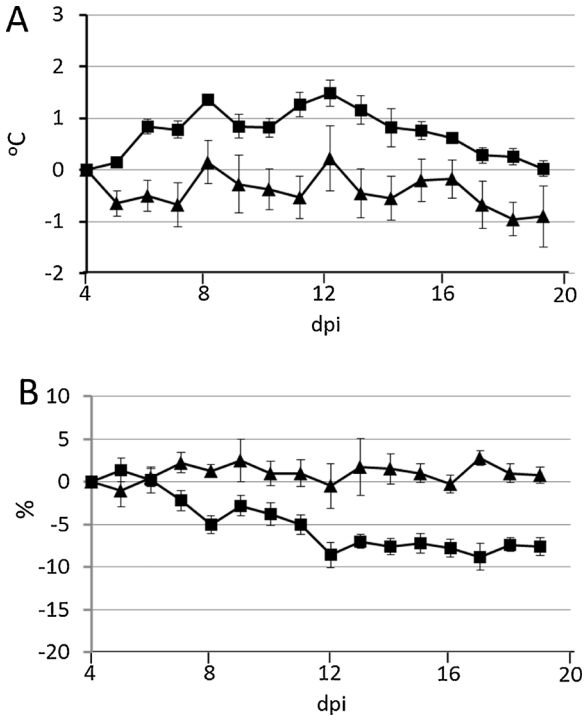

Fig. 2. Clinical parameters of bovines and water buffaloes inoculated with BboM2P. The graphs show the averages +/− SE of temperature (A) and hematocrit changes (B) with respect to basal levels in the water buffalo (triangles) and the bovine (squares) groups from day 4–19 pi.

bovis-positive by direct and/or indirect methods. The remaining water buffalo, that had tested negative by all methods applied in this study, was removed from the following analyses since a failure in the experimental inoculation could not be entirely ruled out.

Rectal temperature increases with respect to basal levels in the bovine and bubaline groups are compared in Fig. 2A. On average, moderate but significant temperature increases were registered from 6 dpi to 17 dpi ($p < 0.05$) in the bovine group. The maximal temperature increase registered in any one animal was 2.2 °C at 12 dpi. Water buffaloes, on the other hand, showed no temperature increases during this period. The difference between the average temperature increase of both groups of animals was highly significant ($p < 0.001$). Also, a moderate but significant average hematocrit decrease was registered in the bovine group from 7 dpi ($p < 0.003$) until the end of the experiment (Fig. 2B). The largest hematocrit decrease registered for one bovine was of 13% at 12 dpi, but the following day the animal increased its hematocrit, and percentage reductions with respect to its basal level were around 7% until the end of the experiment. By contrast, the water buffalo group showed no significant decreases in average hematocrit during the whole experiment. The difference in the average hematocrit decrease between both groups was again highly significant ($p < 0.001$). No bovine or water buffalo in this period presented clinical signs, and there was no need to apply babesicide drugs.

3.2. Experimental inoculation of bovines and water buffaloes with BboS2P

In a second experiment, five water buffaloes were inoculated intravenously with BboS2P. One bovine was inoculated in parallel as a positive control, to confirm the expected level of pathogenicity of this strain. Parasitemia (1%) was registered in Giemsa-stained smears in the inoculated bovine at days 6 and 7 pi. By contrast, in the water buffalo group, no parasitemia was detected in Giemsa-stained smears at any day of the experiment, or by nested PCR at 14 dpi (Table 1). Infection could be indirectly confirmed in the water buffaloes since a consistent antibody response was elicited by the inoculation. From 28 dpi until at least 100 dpi all water buffaloes were seropositive. The bovine also developed a clear antibody response (Fig. 3). In conclusion, effective infection with BboS2P could be confirmed by direct and/or indirect methods in all animals used in this study.

BboS2P pathogenicity was corroborated in the inoculated bovine. At 6 and 7 dpi, a body temperature increase of 2.5 °C and a hematocrit drop of 17% were recorded in this animal (Fig. 4A and B), together with general signs of weakness. For these reasons, a dose of imidocarb dipropionate (3.5 mg/kg) was administered to this bovine to prevent further progression of the infection. After drug treatment, temperature and hematocrit changes were attenuated, with a clear tendency to return to basal values, and the animal recovered well.

The water buffalo group experienced no significant changes in average body temperature during the course of the experiment (Fig. 4A). Hematocrit, however, significantly decreased in this group from 6 dpi on and remained low with respect to basal conditions until the end of the assay ($p < 0.05$) (Fig. 4B). Although individual decreases were in all cases less pronounced than the drop experienced by the BboS2P-inoculated bovine, two water buffaloes experienced a decrease of 12% at 10 dpi, and by the end of the observation period after 19 dpi, hematocrits still showed a 9 and 11% decrease. No other clinical signs were observed and no treatments were administered to the bubaline group.

4. Discussion

This work was aimed to analyze the pathogenic effects of *B. bovis* in the water buffalo through an experimental infection approach. Two *B. bovis* strains (BboM2P and BboS2P) were evaluated. These strains were isolated from two bovines from Argentina that displayed typical clinical signs of acute bovine babesiosis, including high fever and low hematocrit; in both cases, the infections had been fatal.

However, BboM2P seems less virulent than anticipated after long term storage, as evidenced by the moderate clinical signs elicited in

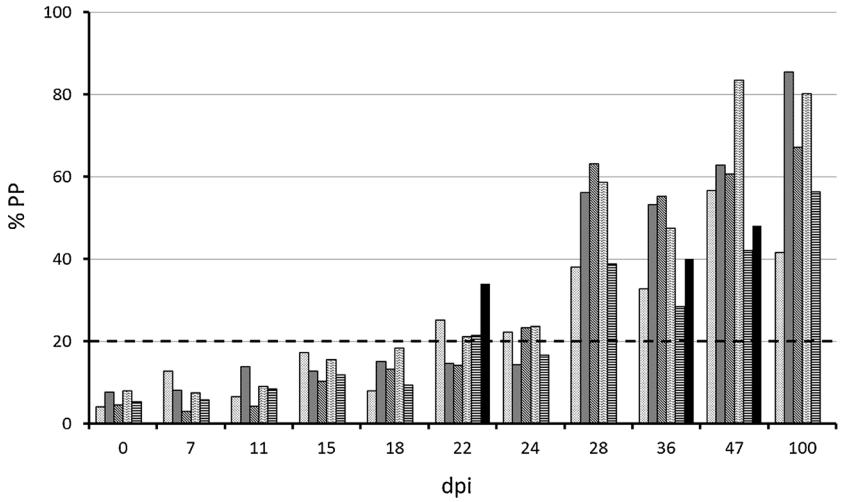

Fig. 3. Anti-*B. bovis* antibody response of water buffaloes and a bovine inoculated with BboS2P. Bars with different patterns correspond to positivity percentages obtained for each water buffalo in this group at different time points. Black bars correspond to the bovine, which was sampled only at 22, 36 and 47 dpi.

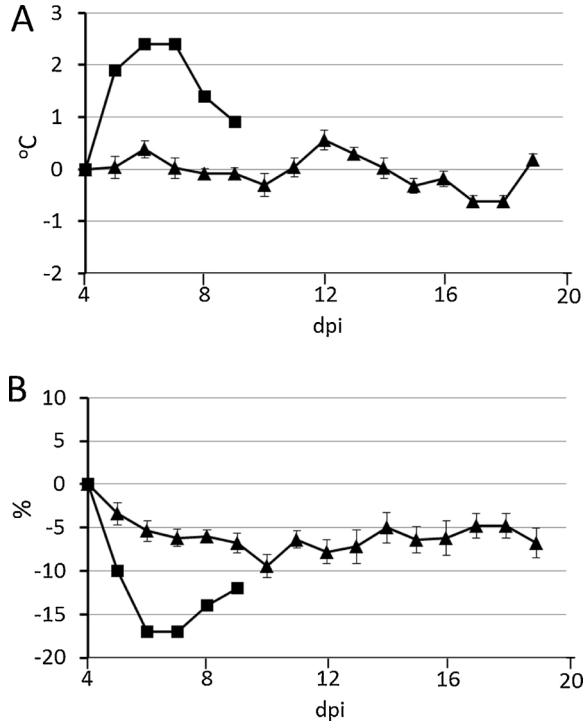

Fig. 4. Clinical parameters of bovines and water buffaloes inoculated with BboS2P. Averages +/− SE in body temperature (A) and hematocrit (B) changes with respect to basal values are shown for the buffalo group (triangles). Also, the corresponding values obtained for the bovine inoculated with this strain are shown. At 7 dpi, the bovine was treated with a babesicide agent.

cattle in the present study. This could have been due to an attenuation process experienced upon long-term storage. In addition to serial passages of parasitized blood through splenectomized calves, tick passage and in vitro cultivation have also been reported to occasionally result in attenuation of bovine *Babesia* parasites, while attenuation caused by freezing and thawing has not been previously described (Wright et al., 1982; Yunker et al., 1987; Florin-Christensen et al., 2014; Sondgeroth et al., 2014). On the other hand, no attenuation effects have been ever recorded for BboS2P even after numerous freezing and thawing events and long-term in vitro cultivation (Baravalle et al., 2012; Echaide, I., unpublished observations). The signals that elicit the attenuation process of *Babesia* spp. and the genes responsible for the attenuated/virulent phenotype are still unknown (Mesplet et al., 2011; Pedroni et al., 2013). Thus, more information is needed to understand the reason for the mild clinical signs elicited by BboM2P in this study.

The lack of virulence of BboM2P observed in this study was unexpected. However, it provided the advantage of analyzing the response of water buffaloes to two strains of a dissimilar pathogenic phenotype. Water buffaloes reacted clearly different than cattle to both strains. Notably, it was impossible to directly detect parasites in the blood of any water buffalo inoculated with either BboM2P or BboS2P by microscopy, and only some or none, respectively, showed positive parasite DNA detection by nested PCR. These results contrast with those obtained in bovines exposed to the same inocula, in which microscopic detection of parasites in blood smears was achieved in five out of six experimentally infected animals. Water buffaloes were apparently able to control infection in a much more efficient way than bovines. This is consistent with the observed lower prevalence of *B. bovis* infections in water buffaloes as compared to bovines raised in the same fields (Mahmoud et al., 2015; Romero-Salas et al., 2016). Although direct detection of parasites was not possible in BboS2P-inoculated water buffaloes with the applied methodologies, all animals mounted a specific humoral response, indicating the presence in the bloodstream of low numbers of parasites that stimulated the immune system.

The pronounced control of parasitemia achieved by water buffaloes corresponds with the absence of or significantly mitigated clinical signs observed in this study. Interestingly, infection with the highly virulent BboS2P strain of *B. bovis* elicited a strong immune response in the buffaloes and some reduction in hematocrit, although this reduction was much smaller than in the single bovine and no other clinical manifestations were observed. Likely, however, this effect after first exposure to the parasite is transient since studies of others have shown no differences in hematological parameters of infected *vs.* non-infected water buffaloes (Mahmoud et al., 2015).

An increased splenic function in water buffaloes as compared to *Bos taurus* bovines might be an explanation of the different parasitemia levels observed in our study for these two ruminant species. The protective immune response against *B. bovis* infection of cattle involves a type–1 T cell response dependent on IFN-γ, and the main source of this cytokine appears to be splenic natural killer (NK) cells. IFN-γ, synergistically acting with other cytokines, elicits a transient but effective parasiticide response mediated by nitric oxide (Goff et al., 2010; Rodriguez Schnittger et al., 2013). Supporting the assumption of a critical role for the water buffalo spleen in protection against *B. bovis*, it has been reported that splenectomized water buffaloes respond to an experimental infection with this parasite with strong clinical signs and

high parasitemia (Yao et al., 1997). The latter study, however, needs to be considered with caution, since no molecular confirmation of the identity of the inoculated parasite was presented. Thus, *B. orientalis*, a parasite often confused with *B. bovis* in the past and with strong pathogenic effects on water buffaloes, might have been used as inoculum (Liu et al., 2005).

Two studies support the involvement of innate immune mechanisms in the particular resistance that water buffaloes show to various pathogens. Transcriptional studies of cytokine genes in riverine and swamp buffaloes exposed to various pathogens suggested that an increased expression of INF-γ and TNF-α is connected with the higher resistance to infection of the riverine breed (Mingala et al., 2009). In addition, water buffaloes have been shown to own a richer repertoire of cathelicidins, a family of peptides with a wide antimicrobial spectrum, as compared to other mammals such as bovines. It has been proposed that these additional innate defense tools contribute to their higher resistance to a great number of infectious diseases as compared to cattle (Brahma et al., 2015).

Additionally, a slower rate of *B. bovis* invasion and/or growth in host bovine erythrocytes could account for the lower parasitemia levels found in water buffaloes, though more studies are needed to evaluate this possibility.

Finally, a different reaction of water buffaloes, as compared to bovines, to immunomodulatory agents present in tick saliva might also play a role in their increased resistance to *Babesia* sp. infections. Although there are no available studies that analyze this particular issue, previous work of our group has shown a strong skin inflammatory reaction in a water buffalo as response to an experimental infection with *R. microplus* ticks that was not observed in a *Bos taurus* bovine similarly treated, indicative of a higher immune reactivity of the buffalo to tick saliva allergenic components (Benitez et al., 2012). This, together with their habit of spending considerable time immersed in water or rolling in the mud, could limit tick infestation in water buffaloes. In agreement with this notion, a recent study showed a significantly lower tick burden in Pakistani water buffaloes as compared to *Bos taurus* cattle from the same areas (Rehman et al., 2017). On the other hand, reproductive parameters measured on *R. microplus* ticks engorged in a buffalo or a bovine were shown to be similar (Benitez et al., 2012), while it has not been established yet whether both types of ticks display comparable efficiencies for the transmission of *Babesia* parasites.

The resilience of water buffaloes to *B. bovis* infections might have an evolutionary explanation. Given enough time, parasites tend to develop survival strategies that involve the establishment of inapparent infections, causing little or no damage in the host, while being able to thrive, infect other individuals, and perpetuate the species (Schnittger and Florin-Christensen, 2018). Most likely, water buffaloes have been the ancestral hosts of *B. bovis*, as is generally assumed for the vector tick *R. microplus*, later crossing the species barrier to *Bos taurus* cattle. In the latter, not enough time in evolutionary terms has passed so far to allow such a co-evolutionary adaptation. The host-pathogen relationship between water buffaloes and *B. orientalis* might have started recently, which would explain the strong pathogenicity of this species on bubaline livestock. In a similar way, indicine cattle, which were domesticated in Asia in environments that were likely endemic for *R. microplus* ticks, show a stronger resistance to this tick species and the parasites it transmits than *Bos taurus* cattle, due at least partly to innate and adaptive immune responses (Parker et al., 1985; Jonsson et al., 2014). On the other hand, indicine cattle show a higher susceptibility to some African ticks than African taurine breeds, suggesting that each cattle genotype evolved protective responses to the ticks and tick-transmitted pathogens that abound in the areas where they were domesticated (Jonsson et al., 2014).

Infected and apparently healthy buffalos could be a source for transmission of *B. bovis* in endemic areas. So far, it has been shown that the presence of *Babesia* sp.-infected *Bos taurus* cattle influences the infection rate of water buffaloes reared on the same pastures (Romero-Salas et al., 2016). It would be relevant to find out if, conversely, the presence of *Babesia* sp.-infected water buffaloes constitutes a risk factor for *Bos taurus* infection, which would have important implications in the epidemiology of bovine babesiosis.

5. Conclusion

Infection of water buffaloes with *B. bovis* is a common finding in tick-infested areas. However, since there have been no reports of clinical babesiosis in these ruminants to date, it can be hypothesized that they are more resistant to a first exposure to the parasite than cattle. This hypothesis was verified in this work. Even in the case of intravenous infection with a highly virulent *B. bovis* strain, the buffalo response was greatly attenuated compared to a bovine response. Our results suggest that parasitemia was rapidly reduced in water buffaloes, and this could be due to an innate immune response. Unraveling the mechanisms by which water buffaloes are particularly resistant to *B. bovis* parasites might have interesting implications in the development of preventive measures against babesiosis in cattle.

Ethical statement

Animal procedures were according to the regulations for animal care and use for scientific purposes followed at the National Institute of Agricultural Technology (INTA), Argentina.

Declaration of interest

None.

Acknowledgments

Financial support for this work from the Ministry of Science and Technology, Argentina (PICT2010-00054), the European Commission (MEDLABAB, INCO0003691) and the National Institute of Agricultural Technology (INTA, CORRI-1243106) is gratefully acknowledged.

References

Anziani, O.S., Guglielmone, A.A., Abdala, A.A., Aguirre, D.H., Mangold, A.J., 1993. Acquired protection in Friesian steers with *Babesia bovis* attenuated vaccine. Rev. Med. Vet. 74, 47–49.

Baravalle, M.E., Thompson, C., Valentini, B., Ferreira, M., Torioni de Echaide, S., Florin-Christensen, M., Echaide, I., 2012. *Babesia bovis* biological clones and the inter-strain allelic diversity of the Bv80 gene support subpopulation selection as a mechanism involved in the attenuation of two virulent isolates. Vet. Parasitol. 190, 391–400.

Benitez, D., Cetra, B., Florin-Christensen, M., 2012. *Rhipicephalus (Boophilus) microplu* ticks can complete their life cycle on the water buffalo (*Bubalus bubalis*). J. Buffalo Sci. 1, 193–197.

Brahma, B., Patra, M.C., Karri, S., Chopra, M., Mishra, P., De, B.C., Kumar, S., Mahanty, S., Thakur, K., Poluri, K.M., Datta, T.K., De, S., 2015. Diversity, antimicrobial action and structure-activity relationship of buffalo cathelicidins. PLoS One 10, e0144741.

Corrêa Fdo, N., da Cunha, N.C., Rangel, C.P., da Fonseca, A.H., 2012. Ticks on buffaloes (*Bubalus bubalis*) in the state of Rio de Janeiro, Brazil. Rev. Bras. Parasitol. Vet. 21, 313–314.

da Silva, J.B., Dos Santos, P.N., de Santana Castro, G.N., da Fonseca, A.H., Barbosa, J.D., 2014. Prevalence survey of selected bovine pathogens in water buffaloes in the north region of Brazil. J. Parasitol. Res. 603484.

Echaide, I., Valentini, B., Torioni de Echaide, S., Mangold, A., Lugaresi, C., 2004. ELISA para el diagnóstico de babesiosis bovina causada por Babesia bovis. In: Proceedings of the XIX Panamerican Veterinary Congress. Buenos Aires, Argentina. 24-28/10/2004. http://www.panalimentos. org/panvet2004/index.htm.

Elsify, A., Sivakumar, T., Nayel, M., Salama, A., Elkhtam, A., Rizk, M., Mosaab, O., Sultan, K., Elsayed, S., Igarashi, I., Yokoyama, N., 2015. An epidemiological survey of bovine *Babesia* and *Theileria* parasites in cattle buffaloes, and sheep in Egypt. Parasitol. Int. 64, 79–85.

Ferreri, L., Benitez, D., Dominguez, M., Rodriguez, A., Asenzo, G., Mesplet, M., Florin-Christensen, M., Schnittger, L., 2008. Water buffalos as carriers of *Babesia bovis* in Argentina. Ann. N. Y. Acad. Sci. 1149, 149–151.

Figueroa, J.V., Chieves, L.P., Johnson, G.S., Buening, G.M., 1993. Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Vet. Parasitol. 50, 69–81.

Florin-Christensen, M., Suarez, C.E., Rodriguez, A.E., Flores, D.A., Schnittger, L., 2014. Vaccines against *bovine babesiosis*: bovine babesiosis where we are now and possible roads ahead. Parasitology 28, 1–30.

Frisch, J.E., Vercoe, J.E., 1979. Adaptive and productive features of cattle growth in the tropics: their relevance to buffalo production. Trop. Anim. Prod. 4, 214–222.

Ganzinelli, S., Rodriguez, A.E., Schnittger, L., Florin-Christensen, M., 2018. Babesiosis of domestic ruminants. In: Florin-Christensen, M., Schnittger, L. (Eds.), Parasitic Protozoa of Farm Animals and Pets. Springer International Publishing (in press ISBN 978-3-319-70132-5).

Goff, W.L., Bastos, R.G., Brown, W.C., Johnson, W.C., Schneider, D.A., 2010. The bovine spleen: interactions among splenic cell populations in the innate immunologic control of hemoparasitic infections. Vet. Immunol. Immunopathol. 138, 1–14.

Jonsson, N.N., Piper, E.K., Constantinoiu, C.C., 2014. Host resistance in cattle to infestation with the cattle tick *Rhipicephalus microplus*. Parasite Immunol. 36, 553–559.

Li, Y., Luo, Y., Cao, S., Terkawi, M.A., Lan, D.T., Long, P.T., Yu, L., Zhou, M., Gong, H., Zhang, H., Zhou, J., Yokoyama, N., Suzuki, H., Xuan, X., 2014. Molecular and seroepidemiological survey of *Babesia bovis* and *Babesia bigemina* infections in cattle and water buffaloes in the central region of Vietnam. Trop. Biomed. 31, 406–413.

Liu, Q., Zhao, J.L., Zhou, Y.Q., Liu, E.Y., Yao, B.A., Fu, Y., 2005. Study on some molecular characterization of *Babesia orientalis*. Vet. Parasitol. 130, 191–198.

Mahmoud, M.S., Kandil, O.M., Nasr, S.M., Hendawy, S.H., Habeeb, S.M., Mabrouk, D.M., Silva, M.G., Suarez, C.E., 2015. Serological and molecular diagnostic surveys combined with examining hematological profiles suggests increased levels of infection and hematological response of cattle to babesiosis infections compared to native buffaloes in Egypt. Parasit Vectors 8, 319.

Mesplet, M., Palmer, G.H., Pedroni, M.J., Echaide, I., Florin-Christensen, M., Schnittger, L., Lau, A.O., 2011. Genome-wide analysis of peptidase content and expression in a virulent and attenuated *Babesia bovis* strain pair. Mol. Biochem. Parasitol. 179, 111–113.

Mingala, C.N., Konnai, S., Cruz, L.C., Onuma, M., Ohashi, K., 2009. Comparative molecular-immunological analysis of swamp- and riverine-type water buffaloes responses. Cytokine 46, 273–282.

Miranpuri, G.S., 1988. Ticks parasitising the Indian buffalo (*Bubalus bubalis*) and their possible role in disease transmission. Vet. Parasitol. 27, 357–362.

Parker, R.J., Shepherd, R.K., Trueman, K.F., Jones, G.W., Kent, A.S., Polkinghorne, I.G., 1985. Susceptibility of *Bos indicus* and *Bos taurus* to *Anaplasma marginale* and *Babesia bigemina* infections. Vet. Parasitol. 17, 205–213.

Pedroni, M.J., Sondgeroth, K.S., Gallego-Lopez, G.M., Echaide, I., Lau, A.O., 2013. Comparative transcriptome analysis of geographically distinct virulent and attenuated *Babesia bovis* strains reveals similar gene expression changes through attenuation. BMC Genomics 14, 763.

Rehman, A., Nijhof, A.M., Sauter-Louis, C., Schauer, B., Staubach, C., Conraths, F.J., 2017. Distribution of ticks infesting ruminants and risk factors associated with high tick prevalence in livestock farms in the semi-arid and arid agro-ecological zones of Pakistan. Parasit Vectors 10, 190.

Ristic, M., Levy, M.G., 1980. *Babesia bovis*: continuous cultivation in a microaerophilous stationary phase culture. Science 207, 1218–1220.

Rodriguez Schnittger, A.E.L., Tomazic, M.L., Florin-Christensen, M., 2013. Current and prospective tools for the control of cattle-infecting *Babesia* parasites. In: Castillo, V., Harris, R. (Eds.), Protozoa: Biology, Classification and Role in Disease. Nova Publishers, Hauppauge, New York, USA, pp. 1–44 (ISBN-13: 9781624170720).

Romero-Salas, D., Mira, A., Mosqueda, J., García-Vázquez, Z., Hidalgo-Ruiz, M., Vela, N.A., de León, A.A., Florin-Christensen, M., Schnittger, L., 2016. Molecular and serological detection of *Babesia bovis*- and *Babesia bigemina*-infection in bovines and water buffaloes raised jointly in an endemic field. Vet. Parasitol. 217, 101–107.

Schnittger, L., Florin-Christensen, M., 2018. Introduction into parasitic protozoa. In: Florin-Christensen, M., Schnittger, L. (Eds.), Parasitic Protozoa of Farm Animals and Pets. Springer International Publishing, pp. 1–10 Chapter 1 (ISBN 978-3-319-70132-5).

Silveira, J.A.G., de Oliveira, C.H.S., Silvestre, B.T., Albernaz, T.T., Leite, R.C., Barbosa, J.D., Oliveira, C.M.C., Ribeiro, M.F.B., 2012. Molecular assays reveal the presence of *Theileria* spp. and *Babesia* spp. in Asian water buffaloes (*Bubalus bubalis*, Linnaeus, 1758) in the Amazon region of Brazil. Ticks Tick Borne Dis. 7, 1017–1023.

Somparn, P., Gibb, M.J., Markvichitr, K., Chaiyabutr, N., Thummabood, S., Vajrabukka, C., 2004. Analysis of climatic risk for cattle and buffalo production in northeast Thailand. Int. J. Biometeorol. 49, 59–64.

Sondgeroth, K.S., McElwain, T.F., Ueti, M.W., Scoles, G.A., Reif, K.E., Lau, A.O., 2014. Tick passage results in enhanced attenuation of *Babesia bovis*. Infect. Immun. 82, 4426–4434.

Terkawi, M.A., Huyen, N.X., Shinuo, C., Inpankaew, T., Maklon, K., Aboulaila, M., Ueno, A., Goo, Y.K., Yokoyama, N., Jittapalapong, S., Xuan, X., Igarashi, I., 2011. Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the northeast region of Thailand. Vet. Parasitol. 178, 201–207.

Wright, I.G., Mahoney, D.F., Mirre, G.B., Goodger, B.V., Kerr, J.D., 1982. The irradiation of *Babesia bovis*, II: the immunogenicity of irradiated blood parasites for intact cattle and splenectomised calves. Vet. Immunol. Immunopathol. 3, 591–601.

Yao, B., Zhao, J., Ma, L., Liu, Z., 1997. Studies on the pathogenicity of *Babesia bovis* in water buffaloes after cryopreservation and resuscitation. Trop. Anim. Health Prod. 29, 40S–42S.

Yilmaz, O., Ertugrul, M., Wilson, R.T., 2012. Domestic livestock resources of Turkey: water buffalo. Trop. Anim. Health Prod. 44, 707–714.

Yunker, C.E., Kuttler, K.L., Johnson, L.W., 1987. Attenuation of *Babesia bovis* by in vitro cultivation. Vet. Parasitol. 24, 7–13.