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SUPPLEMENT ARTICLE

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Highly sensitive nested PCR and rapid immunochromatographic detection of *Babesia bovis* and *Babesia bigemina* infection in a cattle herd with acute clinical and fatal cases in Argentina

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Abstract

Bovine babesiosis is a tick-transmitted haemoparasitic disease caused by *Babesia bovis* and *B. bigemina* affecting cattle of tropical and subtropical regions around the world. Pathogens are transmitted by the tick vector *Rhipicephalus microplus* displaying a widespread distribution in northeastern Argentina. The disease is characterized by significant animal morbidity and mortality resulting in considerable economic loss. In this study, *B. bovis* and *B. bigemina* infection was investigated in a cattle herd of 150 adult bovines of pure Braford breed raised in a tick-hyperendemic field using molecular and serum antibody tests. A highly sensitive nested polymerase chain reaction (nPCR) assay targeting a species-specific region of the apocytochrome b gene resulted in direct *B. bovis* and *B. bigemina* detection in 27.3% and 54.7% of bovines, respectively. A recently developed immunochromatographic strip test (ICT) based on recombinant forms of spherical body protein 4 and the C-terminal region of rhoptry-associated protein 1 showed that 71.3% and 89.3% of bovines were seropositive for *B. bovis* and *B. bigemina*, respectively. The mixed infection rate as observed by direct (19.3%) and indirect detection (65.3%) coincided with those expected, respectively. Importantly, four months after sampling, nine bovines of the studied herd showed clinical signs of bovine babesiosis of which six animals eventually died. Microscopic detection of infected erythrocytes in Giemsa-stained blood smears confirmed *B. bovis* infection. Our study demonstrates that although animals showed a relatively high and very high rate of immunity against infection with *B. bovis* (71.3%) and *B. bigemina* (89.3%) parasites, respectively, clinical cases and fatalities due to the infection with *B. bovis* were observed. It is proposed that the most adequate control measure in the studied epidemiological situation is to vaccinate animals to prevent losses and/or an outbreak of bovine babesiosis.

KEYWORDS

Babesia bigemina, *Babesia bovis*, bovine babesiosis, clinical cases, molecular detection, serological detection

1 | INTRODUCTION

Bovine babesiosis is a tick-transmitted haemoparasitic disease caused by *Babesia bovis* and *B. bigemina* affecting cattle of tropical and subtropical regions around the world. Both pathogens are transmitted by the tick vector *Rhipicephalus microplus* displaying a widespread distribution in northeastern Argentina. The disease is characterized by significant animal morbidity and mortality resulting in considerable economic loss in livestock industry (Späth, Guglielmone, Signorini, & Mangold, 1994; Bock, Jackson, Vos, & Jorgensen, 2004; Schnittger, Rodriguez, Florin-Christensen, & Morrison, 2012; Rodriguez, Schnittger, Tomazic, & Florin-Christensen, 2013; Ganzinelli, Rodriguez, Schnittger, & Florin-Christensen, 2018).

While infections of young animals are asymptomatic, primary exposure of adult cattle may lead to haemoglobinemia, haemoglobinuria, anorexia, fever, abortion and death in severe cases (Bock et al., 2004; Zintl, Gray, Skerrett, & Mulcahy, 2005). *B. bovis*-infected erythrocytes are sequestered in vital capillary beds, obstructing blood circulation resulting in the subsequent development of fatal clinical complications such as cerebral babesiosis (Gohil et al., 2013). In areas where calves are exposed to a high rate of pathogen transmission (>0.005) during the first year of life, protective immunity develops and acute clinical disease is absent in adult animals resulting in an epidemiological situation that is referred to as enzootic stability (Mahoney & Ross, 1972). Animals that recover from disease develop an asymptomatic chronic infection that is characterized by low blood parasitemia and referred to as carrier state ensuring ongoing parasite transmission (Mahoney, 1964, 1969; Calder et al., 1996; Florin-Christensen, Suarez, Rodriguez, Flores, & Schnittger, 2014). Herd immune prevalence has been reported as a useful indicator of enzootic stability. Mahoney (1974) reported that if at least 75% of calves have been exposed to babesiosis by 6 to 9 months of age no incidences of disease are observed and a state of natural enzootic stability exists, making control measures dispensable. Thus, it has been put forward that the determination of the degree of herd immunity allows predicting the rate of acute disease and informs on the necessity to implement appropriate control measures and strategies. However, Jonsson, Bock, Jorgensen, Morton, and Stear (2012) have questioned the usefulness of the concept of enzootic stability because it is a situation that can be influenced by various external and environmental factors such as climate, host genotypes and management strategies. Thus, it has been shown to be practically limited due to the inability to guarantee sufficient exposure of cattle/calves and the necessity of continuous monitoring of herd immunity, and can therefore not be relied upon as a disease control strategy (Jonsson et al., 2012).

Bovine babesiosis may be confirmed by direct and/or indirect detection. Direct parasite detection is commonly done by microscopic examination of Giemsa-stained smears of peripheral blood during the acute phase of disease when parasitemia is high. Additionally, PCR-based assays allow species-specific and highly sensitive direct detection of *Babesia* spp., however, even when nested PCR (nPCR) is applied the parasite may still escape detection (Figueroa, Chieves, Johnson, & Buening, 1993; Calder et al., 1996). Recently, improved highly sensitive nPCR assays for direct *B. bovis* and *B. bigemina* detection have been reported (Romero-Salas et al., 2016). These nPCR detection assays have shown a hundred and a thousand times lower detection limit for *B. bovis* and for *B. bigemina*, respectively, than the corresponding nPCR assays recommended by the OIE as gold-standard (Figueroa et al., 1993; OIE World Organization for Animal Health, 2018). Different formats of indirect serological tests have been developed and are applied. Indirect enzyme-linked immunosorbent assays (iELISA) and competitive ELISA (cELISA) assays using recombinant *B. bovis* and *B. bigemina* surface antigens have replaced the indirect fluorescence-antibody test (IFAT) for the detection of antibodies to *Babesia* spp. (Dominguez et al., 2004, 2012; OIE World Organization for Animal Health, 2018). Most recently, immunochromatographic tests (ICT) have been developed affording a rapid simple detection of specific antibodies as is preferred when epidemiological surveys under field conditions are carried out (Guswanto, Allamanda, Mariamah, Munkjargal, et al., 2017; Guswanto, Allamanda, Mariamah, Sodirun, et al., 2017). Apart from assessing vaccine efficacy and confirmation of diagnosis, ICT is also suitable to evaluate herd immune status. Indirect techniques reveal previous exposure to *B. bovis* and *B. bigemina*, which, for the former, has been shown to correlate with the ability to transmit the infection for at least 13 months after inoculation (Johnston & Tammemagi, 1969). In the case of *B. bigemina*, it has been shown that bovines may clear infection and antibody levels may decline below the negative threshold within months after infection (Goff et al., 2008). Furthermore, the presence of antibodies may not accord with the presence of the parasite during the acute stage of infection (Galuppi et al., 2011). Hence, the application of molecular direct and serological indirect detection affords complementary information on infection and immune status of an animal or herd, respectively. The study region has been declared an area of enzootic stability in which tick control measures are voluntary for farmers (SENASA, 2004; Ferreri et al., 2008). Furthermore, reports on the prevalence of bovine babesiosis are scarce (Paoletta et al., 2018; Jaramillo Ortiz et al., 2018).

The main purpose of this study was to investigate the molecular and serological prevalence of *B. bovis* and *B. bigemina* infection in a tick-hyperendemic field. Furthermore, a recently developed highly sensitive nPCR assay and simple rapid ICT were used to evaluate the efficacy and complementation that these methods provide. In addition, six fatal clinical cases of bovine babesiosis were observed four months after the sampling had been carried out and are discussed in the context of obtained epidemiological results.

2 | MATERIAL AND METHODS

2.1 | Bovine blood sample and DNA extraction

A total of 150 bovines of Braford breed (146 females and 4 males, >2.5 years) from Colonia Carlos Pellegrini, Corrientes, Argentina (Figure 1, 28°0.26´, 0.76´´S and 56°0.50´, 25.73´´O) were randomly sampled in February 2018. All animals of the herd were born in the

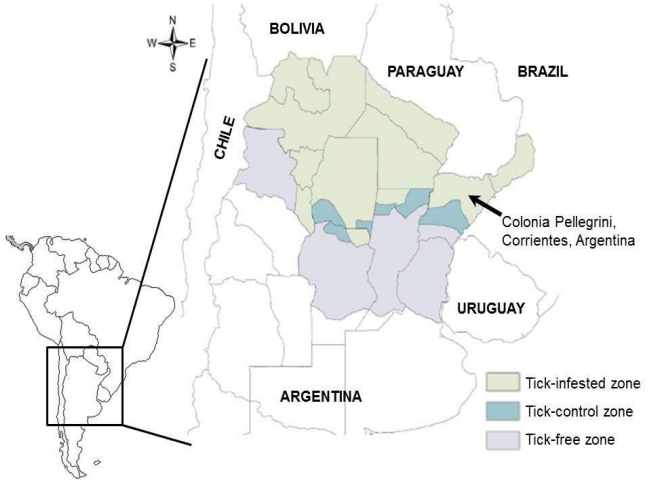

FIGURE 1 Map of northern Argentina showing the location of the field in which animals were sampled (arrow) [Colour figure can be viewed at wileyonlinelibrary.com]

same farm and have been raised in this field at a density of 1 animal per hectare. None of the animals were vaccinated and were therefore expected to present a natural immunity due to high tick endemicity. Furthermore, none of the animals showed signs of clinical disease at the moment of sampling, and bovines were subjected to tick control with ivermectin (3.15%, injectable) when needed. The procedures performed in this study were guided by the principles of animal welfare of Argentina. Blood was aseptically obtained by jugular vein puncture (Vacutainer™, Becton Dickinson, one sampling per animal) with or without anticoagulant. The former were used for the isolation of genomic DNA (gDNA) performed according to the manufacturer´s protocol (EasyPure Blood Genomic DNA Kit, TransGen Biotech, China) to obtain a minimum concentration of 50 ng/µl as quantified using a Nano Drop 1,000 (Thermo Fisher Scientific). The latter was allowed to clot at room temperature, and serum was then separated by centrifugation and stored at −20°C until further use.

2.2 | Nested PCR

Genomic DNA samples were analysed by nPCR for screening of *B. bovis* and *B. bigemina* as previously described by Romero-Salas et al., (2016). Used primer pairs are shown in Table 1. This assay has been shown to have an extremely high detection limit of 0.1 fg for *B. bovis* and *B. bigemina*. After amplification, electrophoresis in a 1.8%-agarose gel was carried out and generated fragments were stained with ethidium bromide and visualized under ultraviolet light (Figure 2). Genomic DNA of *B. bovis* T2Bo and *B. bigemina* S1A was used as positive and double distilled water as a negative control.

2.3 | Immunochromatographic test

Separate ICT strips containing recombinant spherical body protein 4 (SBP-4) of *B. bovis* (bovICT) and C-terminal-truncated rhoptry-associated protein 1 (rRAP1/CT17) of *B. bigemina* (bigICT) were prepared for the detection of BbSBP-4 and BbigrRAP1/CT17-specific antibodies, respectively (Kim et al., 2008; Terkawi et al., 2011). ICT was carried out as described in Guswanto, Allamanda, Mariamah, Munkjargal, et al., (2017); however, thirty microlitres of each serum sample was diluted in an equal amount of PBS and dropped onto the sample pad of the ICT strip. ICT strips were stored in 4–8°C, while the negative and positive control serum samples were stored in −20°C until further use.

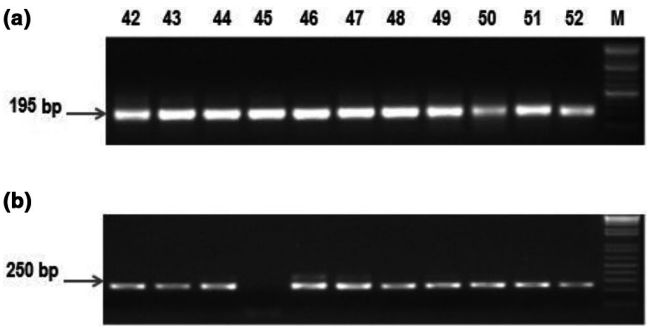

FIGURE 2 Detection of *Babesia bovis* and *Babesia bigemina* in bovine blood samples as analysed by nPCR. Agarose gel electrophoresis of amplification products of *B. bovis* (a) and *B. bigemina* (b). Lane 42–52, field samples; arrows indicate the expected amplicon size; M, molecular marker

TABLE 1 Primer sets used for the *Babesia bovis* and *Babesia bigemina* nPCR assay

Haemoparasite	Assay	Primer sequence	Amplicon size (bp)	Tm (°C)
B. bovis	PCR	TGAACAAAGCAGGTATCATAGG	260	60
		CCAAGGAGATTGTGATAATTCA		
	nPCR	TCCACGATCTGTGATACGTCA	195	62
		CAAATCCTTTGCAAACTCCAA		
B. bigemina	PCR	TCCAACACCAAATCCTCCTA	394	62
		CGTGGGTTTCGTTTTTGTAT		
	nPCR	AAGAGATACCATATCAGGGAACCA	250	62
		TTGGGCACTTCGTTATTTCC		

2.4 | Statistical analysis

A chi-squared test was done to evaluate the significance of observed vs. expected mixed infections. A p value of less than 0.05 ($p < .05$) was considered statistical significant.

3 | RESULTS

Cattle from a tick-hyperendemic field were analysed for the presence of *B. bovis*- and *B. bigemina*-specific antibodies by ICT and for the presence of *B. bovis* and *B. bigemina* parasite by nPCR. For *B. bovis*, 71.3% ($n = 107$) of serum samples tested positive, whereas 89.3% ($n = 134$) serum samples were found to test positive for *B. bigemina*. In contrast, direct parasite detection by nPCR showed a considerably lower positive rate for both species, since only 27.3% ($n = 41$) but 54.7% ($n = 82$) DNA samples were found positive for *B. bovis* and *B. bigemina*, respectively (Table 2). Regarding mixed infection, the presence of specific antibodies against both parasites was found in 65.3% ($n = 98$) of animals in the cattle herd, and by direct molecular detection both pathogens were detected in 19.3% ($n = 29$) of bovines. Interestingly, the calculated conditional probability of co-infection showed a high level of agreement for serological (expected: 63.7% vs. observed: 65.3%; $X^2 = 0.13$, $df = 1$, $p > .5$, non-significant) and also for molecular determinations (expected: 14.7% vs. observed: 19.3%; $X^2 = 2.13$, $df = 1$, $p > .1$, non-significant). Four months after sampling, nine bovines (>2.5 years of age) of the studied herd showed severe clinical signs of bovine babesiosis from which six animals eventually succumbed. Microscopic detection of infected erythrocytes in Giemsa-stained blood smears confirmed the infection with *B. bovis* (data not shown). Unfortunately, it was not possible to obtain serological data from these animals. Based on our results, it can be determined that 24.0% of animals within the study group presented exclusively *B. bigemina*- but no *B. bovis*-specific antibodies and 4.7% did not present specific antibodies against any of the two pathogens. Consequently, altogether 28.7% of studied animals can be considered unprotected against the infection with *B. bovis*.

TABLE 2 Serological and molecular detection of *Babesia* spp. infection in cattle from Corrientes, Argentina

		ICT	nPCR
	***Babesia* spp.**	**Per cent % ($n = 150$)**	**Per cent % ($n = 150$)**
Total infection	*B. bovis*	71.3 (107)	27.3 (41)
	B. bigemina	89.3 (134)	54.7 (82)
Single infection	*B. bovis*	6 (9)	8 (12)
	B. bigemina	24 (36)	35.3 (53)
Mixed infection	*B. bov + B. big*	65.3 (98)	19.3 (29)
Negative		4.7 (7)	37.3 (56)

4 | DISCUSSION

In this study, recently developed molecular and serological tools were used to detect *B. bovis* and *B. bigemina* infections in a cattle herd raised in a tick-hyperendemic field of Argentina. Direct parasite detection using nPCR was carried out by amplification of the apocytochrome b genes of *B. bovis* and *B. bigemina* (Romero-Salas et al., 2016). This test has recently been demonstrated to have a significantly higher sensitivity than hitherto reported nPCR assays including that of Figueroa et al. (1993). It follows that application of this nPCR assay considerably reduces or even dissolves false-negative results. The ICT applied in this study is based on the recombinant spherical body protein 4 and the C-terminal-truncated rhoptry-associated protein 1 for serological detection of *B. bovis* and *B. bigemina* infection, respectively (Guswanto, Allamanda, Mariamah, Munkjargal, et al., 2017). The selected proteins have shown a high antigenicity against *B. bovis* and *B. bigemina* strains from geographically distant areas of the world and a good agreement with other serological tests such as ELISA (Terkawi et al., 2011; Guswanto, Allamanda, Mariamah, Munkjargal, et al., 2017; Guswanto, Allamanda, Mariamah, Sodirun, et al., 2017). The ICT is easy to implement, can be carried out with little expertise, is rapid to perform as results are available after 15 min and is inexpensive making studies in the field feasible which prompted us to apply it in this study. A high specificity of 94% and 94% and sensitivity of 90% and 92% for the detection of specific antibodies against *B. bovis* and *B. bigemina*, respectively, has been previously established based on a panel of known positive and negative sera (Guswanto, Allamanda, Mariamah, Munkjargal, et al., 2017). When comparing the number of animals that tested positive by direct and indirect detection, direct detection of *Babesia* spp. in cattle using nPCR was distinctly lower (*B. bovis* 27% and *B. bigemina* 54.7%) than indirect detection of *Babesia* spp. specific antibodies using the serologic ICT assay (*B. bovis* 71.3% and. *B. bigemina* 89.3%).

The observed discrepancies in the results between both methods may be partially explained by the inverse time kinetics between the level of parasitemia and the generation of specific antibodies after infection. During the acute phase of infection, the parasitemia is high, whereas specific antibodies have not yet been generated. However, animals that have survived acute infection show for a prolonged time high levels of circulating parasite-specific antibodies whereas parasitemia drops low and may even escape the detection of molecular assays (Figueroa et al., 1993; Pipano et al., 2002; Romero-Salas et al., 2016). In the case of *B. bovis* infection, the ability of parasitized erythrocytes to cytoadhere to peripheral capillaries may further significantly reduce the parasitemia in the peripheral blood resulting in a lower detection level of *B. bovis* compared to that of *B. bigemina* (O'Connor, Long, & Allred, 1999; Uilenberg, 2006; Gohil et al., 2013).

The expected rate of mixed infection as determined by calculation of the conditional probability showed a high level of agreement with observed co-infection for serological and molecular assessments, respectively. Furthermore, mixed infections as determined

by indirect serological detection (65.3%) were found to be more frequent than those determined by direct molecular detection (19.3%). Both observations are in accordance with previous reports of *Babesia* spp. infected cattle populations from other enzootic area such as Mexico, Indonesia and Argentina (Romero-Salas et al., 2016; Guswanto, Allamanda, Mariamah, Munkjargal, et al., 2017; Guswanto, Allamanda, Mariamah, Sodirun, et al., 2017; Paoletta et al., 2018). These findings strongly suggests that each, the exposure of cattle to *B. bovis* and *B. bigemina* and the establishment of co-infection, are a stochastic independent processes, in which no positive or negative synergetic factors influence the infection of either species.

Herd immunity, determined as the rate of animals in which parasite-specific antibodies could be detected, has been used as an indicator of enzootic stability (Mahoney & Ross, 1972; Mahoney, 1974; Bock et al., 2004; Florin-Christensen et al., 2014). Importantly, it has been estimated that a tick transmission rate higher than 0.005 results in a herd immunity (>75%) that protects against clinical disease and enzootic stability is observed (Bock et al., 2004). However, endemic stability is a sensitive balance that can be easily brought out of equilibrium by variations of climate (favourable vs. non-favourable for tick development), cattle breed (more resistant *Bos indicus* × *Bos taurus* crosses vs. susceptible pure *B. taurus* cattle), fluctuation of the tick population (caused by seasonal changes etc.) and control management strategies (especially mode and time schedule of the application of acaricide vs. vaccination) (Mahoney & Ross, 1972; Mahoney, 1974; Florin-Christensen et al., 2014; Ganzinelli et al., 2018).

In the studied epidemiological situation, serological determinations showed that specific antibodies against *B. bigemina* (89.3%) were detected significantly more frequent than against *B. bovis* (71.3%). Thus, most animals have been protected against infection with *B. bigemina*, whereas at least 28.7% of animals must be considered unprotected against the infection with *B. bovis*. Although it was not possible to establish the immune status of the diseased individual animals in this study, the observation that succumbed animals showed infectious agents that were consistent with *B. bovis* morphology suggests that disease incidences and fatalities were due to *B. bovis* infection of non-immune animals. The studied field is situated in a region of high tick infestation that has been declared to be enzootically stable by sanitary authorities. However, protection of cattle against bovine babesiosis is not ensured in this region.

We propose that the most adequate control measure in this situation is to vaccinate animals to prevent an outbreak of bovine babesiosis. Our finding suggests that even when high herd immunities are reached animals cannot be considered protected. The ICT assay applied in this study is a diagnostic method that can be employed for routine immune herd surveillance.

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CONFLICT OF INTEREST

The authors declare no financial or personal relationships with other people or organizations that could inappropriately influence their work.

ETHICAL APPROVAL

The authors declare that ethical statement is not applicable because sample collection or questionnaires from animals has been gathered.

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