Veterinary Parasitology 304 (2022) 109686

Contents lists available at ScienceDirect

Veterinary Parasitology

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

International interlaboratory validation of a nested PCR for molecular detection of *Babesia bovis* and *Babesia bigemina,* causative agents of bovine babesiosis

Sabrina Ganzinelli [a,b], Charles Byaruhanga [c], María E. Primo [b,d], Zinathi Lukanji [c], Kgomotso Sibeko [c], Tshepo Matjila [c], Luis Neves [c], Daniel Benitez [e], Batmagnai Enkhbaatar [f,g], Arifin Budiman Nugraha [f,h], Ikuo Igarashi [f], Monica Florin-Christensen [a,b], Leonhard Schnittger [a,b,*]

[a] *Instituto de Patobiología Veterinaria, IP-IPVET, CICVyA, INTA-CONICET, Los Reseros y Nicolas Repetto s/n, Hurlingham (B1686WAA), Buenos Aires, Argentina*
[b] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*
[c] *Vectors and Vector-borne Diseases Research Programme, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa*
[d] *Instituto de Investigación de la Cadena Láctea, INTA-CONICET, Ruta 34 km 227, 2300 Rafaela, Santa Fe, Argentina*
[e] *Estación Experimental Agropecuaria INTA-Mercedes, Juan Pujol al Este s/n, W3470, Mercedes, Corrientes, Argentina*
[f] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2 Sen-11, Inada-cho, 080-8555, Obihiro, Hokkaido, Japan*
[g] *Laboratory of Molecular Genetics, Institute of Veterinary Medicine, Mongolian University of Life Sciences, Ulaanbaatar, Mongolia*
[h] *Department of Animal Infectious Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, IPB University, Jl. Agatis, Kampus IPB Dramaga, Bogor, Jawa Barat, 16680, Indonesia*

ARTICLE INFO

Keywords:
Bovine babesiosis
Babesia bovis
Babesia bigemina
Carrier animal
Cytochrome b
nPCR

ABSTRACT

Babesia bovis and *B. bigemina* are tick-transmitted parasites causing bovine babesiosis, characterized by significant morbidity and mortality leading to economic losses to the livestock industry in tropical and subtropical regions worldwide. Animals that recover from acute infection remain carriers with low parasitemia acting as a source of transmission, and often escape detection. An improved diagnosis of a *B. bovis* and/or *B. bigemina* infection of carrier animals is enabled by the availability of detection methods with high sensitivity. To this end, two nested PCR assays targeting the cytochrome b (*cytb*) genes of *B. bovis* and *B. bigemina* (*cytb*-nPCR), have been recently developed and an increased sensitivity with respect to reference protocols has been shown (Romero-Salas et al., 2016). In this study, the specificity against a panel of hemoparasites that potentially co-occur with *B. bovis* and *B. bigemina* was demonstrated to ensure applicability of the *cytb*-nPCR assays in a wide range of regions where bovine babesiosis is endemic. Furthermore, we compared both reported *cytb*-nPCR assays with reference nPCR and qPCR protocols for (i) their capability to detect carrier animals in the field, and (ii) their reproducibility when performed in different laboratories by independent operators. We show that, in a panel of bovine field samples (n = 100), the *cytb*-nPCR assays detected a considerably higher number of 25% *B. bovis* and 61% *B. bigemina*-positive animals compared to 7% and 20% *B. bovis* and 55% and 49% *B. bigemina*-positive animals when tested by reference nPCR and qPCR protocols, respectively. *Cytb*-nPCRs were also found superior in the detection of carrier animals when field samples from Africa were analyzed. In addition, both the *B. bovis* and *B. bigemina cytb*-nPCR assays were independently validated in a single blinded study in three laboratories. Importantly, no significant differences in the number/percentage of infected animals was observed using *cytb*-nPCR assays. In summary, the *cytb*-nPCR assays detected a considerably higher number of chronically infected *B. bovis* and *B. bigemina* carrier animals compared to reference nPCR and qPCR protocols, when applied in different epidemiological field situations. Furthermore, a high reproducibility between laboratories could be demonstrated.

* Corresponding author at: Instituto de Patobiología Veterinaria, IP-IPVET, CICVyA, INTA-CONICET, Los Reseros y Nicolas Repetto s/n, Hurlingham (B1686WAA), Buenos Aires, Argentina.
E-mail address: schnittger.leonhard@inta.gob.ar (L. Schnittger).

https://doi.org/10.1016/j.vetpar.2022.109686
Received 6 January 2022; Received in revised form 27 February 2022; Accepted 1 March 2022
Available online 3 March 2022

1. Introduction

Bovine babesiosis caused by the tick-transmitted intraerythrocytic apicomplexan hemoprotozoa *B. bovis* and *B. bigemina* has a huge impact in livestock cattle industries in tropical and subtropical areas around the world (Bock et al., 2004; Florin-Christensen et al., 2014). Decline in meat and milk production, treatment of clinical cases, abortions, and death, as well as trade restrictions on cattle lead to an economic burden associated with this disease (Wagner et al., 2002; Suarez and Noh, 2011).

Babesia bovis and *B. bigemina* are the most common species infecting cattle (Schnittger et al., 2012, 2022). Clinical cases present hyperthermia, hemoglobinuria, anemia, anorexia, abortion and are often fatal if untreated (Bock et al., 2004; Ganzinelli et al., 2018). Bovine babesiosis caused by *B. bovis* is more severe than that caused by *B. bigemina* since, following invasion of red blood cells, the parasite induces the formation of knobs on the surface of the parasitized erythrocyte mediating its adherence to endothelial cells. This provokes sequestration of infected red blood cells in capillary beds, leading to severe ischemia and respiratory and neurological signs (Hutchings et al., 2007; Suarez and Noh, 2011). Delayed treatment of bovine babesiosis often results in poor prognosis. Nevertheless, animals that recover from acute babesiosis become carriers of the hemoparasites and serve as a reservoir of infection allowing persistent parasite transmission, and the spread of the disease to naive animals (Mahoney, 1969).

A number of techniques have been developed for the diagnosis of bovine babesiosis. During the acute stage of the disease, laboratory diagnosis is traditionally done by microscopic detection of infected erythrocytes in stained blood films (Bock et al., 2004). This approach is time consuming and laborious, and inadequate to detect the extremely low parasitemia levels presented by chronically infected animals.

The development of highly sensitive and specific molecular diagnostic tools is essential for the identification of asymptomatic carrier animals. To this aim, several PCR-based detection methods targeting a variety of genes have been established. Nonetheless, it has been demonstrated that parasite infections may escape even highly sensitive PCR-based methods, prompting optimization of assay design in order to increase sensitivity of detection (Calder et al., 1996; Alvarez et al., 2019). Currently, the use of a variety of PCR-based protocols for which sensitivity levels have not been thoroughly established and/or determined prevents comparative analyses of different epidemiological scenarios.

In a previous study, we reported the development of nested PCR assays for the detection of *B. bovis* and *B. bigemina* using the *cytb* genes as targets (*cytb*-nPCR). These molecular assays have been applied to detect both parasites in bovines and water buffaloes in epidemiological studies from Mexico, and in bovines from Argentina (Romero-Salas et al., 2016; Ganzinelli et al., 2020). In the present study, *cytb*-nPCR assays to detect *B. bovis* and *B. bigemina* carrier animals in bovine field samples were compared to reference nPCR and qPCR protocols. In addition, the reproducibility of both *cytb*-nPCR assays was evaluated by an inter-laboratory validation.

2. Materials and methods

2.1. Parasite and bovine blood samples

Three different sample panels (I–III) were compiled. Panel I consisted of DNA samples (n = 150) from cattle of the province of Corrientes, Argentina, obtained from a previous study in February 2018 (Ganzinelli et al., 2020). Blood was aseptically collected by jugular vein puncture using Vacutainer™ tubes containing sodium citrate as anticoagulant (Becton Dickinson, USA). Genomic DNA was isolated using the EasyPure Blood Genomic DNA Kit (TransGen Biotech, China). The random sampled animals were born in the same farm and had been raised in this field at a density of one animal per hectare, in an area of high endemicity of *Rhipicephalus microplus* ticks. The animals were occasionally subjected to tick control with ivermectin (3.15%, injectable), by the farm veterinarians. None of the animals had been vaccinated against bovine babesiosis or showed signs of clinical disease during sampling. The procedures performed in this study were guided by the principles of animal welfare of Argentina.

Panel II consisted of bovine DNA samples (n = 28) obtained from previous studies conducted at the Department of Veterinary Tropical Diseases, University of Pretoria, from 2015 to 2017 (Choopa, 2015; Nyoni-Phili, 2017; Sili, 2018). Blood was collected from cattle from the Mnisi community, Bushbuckridge Municipal Area, Mpumalanga Province, South Africa (n = 10); Namitangurine and Botao villages in the Zambezia Province, Mozambique (n = 10), and Tchicala -Tcholoanga, Huambo Province, Angola (n = 8). Genomic DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). The studies were approved by the Animal Ethics Committee of the University of Pretoria, South Africa (reference nos. V047-12 and V060-17) and by the Department of Agriculture, Forestry and Fisheries (Pretoria, South Africa; No. 12/11/1/1/6 and 12/11/1/1/9). None of the animals from the three locations had been vaccinated against bovine babesiosis, and they were apparently healthy. Bovine babesiosis is endemic in the three areas, with the presence of both *B. bovis* and *B. bigemina* registered in South Africa and Mozambique, while exclusively *B. bigemina* is endemic in Angola. The tick vectors *R. microplus* and/or *R. decoloratus* (Choopa, 2015; Makgabo, 2019; Stoltsz et al., 2020; Sili et al., 2021) were recorded in the three countries, however in Angola, *R. microplus* is considered a recent introduction (Gomes and Neves, 2018).

Panel III consisted of positive DNA controls and DNA samples of microorganisms that potentially co-occur with *Babesia* spp. in the bovine blood (n = 6). Genomic DNA of hemoparasites *Trypanosoma brucei, T. congolense, T. vivax* (field blood samples, concentration 1 ng/µl each), *Theileria parva* (from blood of a naturally infected African buffalo, KNP102, parasitaemia 0.009%), *Anaplasma centrale* (live blood vaccine dilution of bacteremia 3.3%, Onderstepoort Biological Products, Pretoria, South Africa), *A. marginale* (bovine blood sample from University of Pretoria farm, 4.5 ng/µl), were obtained from the DNA collection of the Department of Veterinary Tropical Diseases, University of Pretoria, South Africa. Control genomic DNA was isolated from *B. bovis* strain T2Bo *in vitro* culture and from blood of a bovine experimentally infected with *B. bigemina* strain S1A, using the EasyPure Blood Genomic DNA Kit (TransGen Biotech, China). Samples were stored at − 20 °C until use.

2.2. cytb-nPCR assays

Two nested PCR assays that target the *B. bovis* and *B. bigemina cytb* gene resulting in final amplicon lengths of 195 and 250 bp, respectively, were used as previously described (Romero-Salas et al., 2016). Briefly, the first and second PCR amplification reactions were carried out in a final reaction volume of 12.5 µl containing: 2 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each forward and reverse primer (Supplementary Table 1), 0.3 U Dream Taq DNA Polymerase (Thermo Scientific, Waltham, MA, USA), and as template: 9 µl DNA in the first PCR reaction, and 2 µl of the generated amplicon in the second PCR amplification. Thermal cycling conditions started with an initial denaturation step at 95 °C for 3 min after which 30 cycles were carried out consisting of a denaturation step at 95 °C for 30 s, an annealing step according to the used primer pairs as indicated in Supplementary Table 1, and an extension reaction at 72 °C for 30 s. A subsequent final extension reaction was done at 72 °C for 7 min.

2.3. Reference nested PCR protocols

A nested PCR adapted from Figueroa et al. (1993) with modifications as registered in the Terrestrial Manual of the World Organisation for Animal Health (OIE, 2021) was applied for the amplification of a *B. bovis rap-1* gene fragment of 291 bp and a *B. bigemina speI-avaI* fragment of

170 bp. Briefly, the first and second PCRs were carried out in a final volume of 10 µl that included 200 µM of each dNTP, 0.5 µM of each forward and reverse primers (Supplementary Table 1), 0.5 U Dream Taq DNA Polymerase (Thermo Scientific, Waltham, MA, USA), and as template 1 µl DNA for the first PCR reaction, and 1 µl of the generated amplicon for the second PCR amplification. Thermal cycling conditions were as follows: an initial denaturation step at 95 °C for 5 min was followed by 35 cycles, each of them consisting of a denaturing step at 95 °C for 30 s, an annealing step at 55 °C for 1 min, and an extension step at 72 °C for 1 min. The final elongation step was at 72 °C for 10 min.

2.4. qPCR protocols

Real-time PCR assays to amplify 152 and 174 bp fragments of the18S rRNA genes of *B. bovis* and *B. bigemina*, respectively, were used as previously described Byaruhanga et al., unpublished; (Stoltsz et al., 2020; Byaruhanga et al., 2022). Each PCR reaction contained 0.8X TaqMan® Universal PCR Master Mix (Applied Biosystems, Life Technologies, Johannesburg, South Africa), 2 µl of template, 0.5 µM of each primer and 0.25 µM of a fluorescently labeled probe in a total reaction volume of 20 µl. Primers and probes are specified in Supplementary Table 1. Thermal cycling was done in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Johannesburg, South Africa) under the following conditions: uracil N-glycosylase digest at 50 °C for 2 min, followed by AmpliTaq Gold pre-activation at 95 °C for 10 min and then 45 cycles of thermal cycling at 95 °C for 20 s and 57 °C for 1 min. Amplification analyses were performed using StepOne™ Software version 2.3 for StepOne™ and StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Life Technologies, Johannesburg, South Africa).

2.5. Sample distribution and participating laboratories

Samples of panel I (n = 150) were used to independently validate the *cytb*-nPCR assays by different operators in three laboratories. The participating laboratories belong to the Instituto de Patobiología Veterinaria (IPVet), INTA-CONICET, Hurlingham, Argentina; the Instituto de Investigación de la Cadena Láctea, (IDICAL), INTA-CONICET, Rafaela, Argentina; and the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria (UP), Onderstepoort, South Africa. DNA was extracted from all tested samples by a single operator, aliquoted and "single blindly" delivered to the three participating laboratories. The operators applied exactly the same protocol and the reagents were purchased from the same provider. The single blinded trials performed by each laboratory were designated Lab1 (IPVet), Lab2 (IDICAL) and Lab3 (UP).

2.6. Statistical analysis

A chi-square ($X^{2)}$ test of homogeneity was performed to evaluate whether the null-hypothesis can be rejected (H_0: application of two molecular tests results in the same number of positive and negative samples); a *p* value of less than 0.05 ($p < 0.05$) was considered statistical significant. Chi-squarevalues and degrees of freedom (df) were calculated. Concordance among the three laboratories was established using Cohen's *kappa* (κ) values (idostatistics.com/cohen-kappa-free-calculator). Besides the κ value, also the test agreement was calculated. For κ value, the following descriptors were assigned: κ < 0.2 = poor agreement; 0.21–0.4 = fair agreement; 0.41–0.6 = moderate agreement; 0.61–0.8 = substantial agreement; 0.81–0.99 = very good agreement.

3. Results

3.1. Analytical specificity of the cytb-nPCR assay

The analytical specificity of the *cytb*-nPCR assays was evaluated using DNA samples of the *B. bovis* T2Bo and the *B. bigemina* S1A strains, as well as from a panel of potentially sympatric hemoparasites and hemobacteria (panel III, n = 6). The *B. bovis* and *B. bigemina cytb*-nPCR assays exclusively detected *B. bovis* and *B. bigemina*, respectively, and no cross-reactivity with any of the tested hemoparasites or hemobacteria was observed (data not shown). This demonstrates that both *cytb*-nPCR assays are applicable in most areas where *B. bovis* and *B. bigemina* are endemic.

3.2. Detection of carrier animals in bovine field samples by cytb-nPCR assays in comparison with reference nPCR and qPCR protocols

The diagnostic sensitivity of the *cytb*-nPCR assays was compared with the reference nPCR and qPCR protocols using genomic DNA samples collected from two areas endemic for *B. bovis* and *B. bigemina* (sample panel I, Argentina; and panel II, Africa). The *B. bovis cytb*-nPCR assay detected a highly significantly increased number of *B. bovis* carrier animals compared to the corresponding reference nPCR (panel I, n = 100; 25 *vs*. 7%, respectively; $X^2 = 12.05$, df = 1, $p < 0.001$). Furthermore, a higher number of *B. bovis* carrier animals was obtained for the *cytb*-nPCR assay compared to the qPCR protocol (25 *vs*. 20%, respectively), though this difference was not found to be significant ($X^2 = 0.72$, df = 1, p = 0.39) (Fig. 1A, Table 1). While 27 of the 100 samples tested were positive in at least one assay, 73 samples were negative in all three PCR-based assays. Importantly, of the 25 samples that scored positive by *B. bovis cytb*-nPCR, only 6 and 18 samples reacted positive by the reference nPCR and qPCR, respectively. On the other hand, exclusively two samples scored negative by the *cytb*-nPCR, but positive by both the reference nPCR and qPCR, or only by the qPCR protocol, respectively.

Comparable results were observed when field samples from South Africa and Mozambique were analyzed (panel II, n = 20), 75% samples (n = 15) tested positive by *B. bovis cytb*-nPCR, while only 25% (n = 5; $X^2 = 10.00$, df = 1, $p < 0.01$) and 60% samples (n = 12; $X^2 = 1.03$, df = 1, p = 0.31) scored positive by reference nPCR and qPCR, respectively (Table 1). For the statistical testing of field samples of panel II (n = 28), samples from Angola (n = 8) were excluded since this region is considered non-endemic for *B. bovis* (see Section 2.1).

When bovine field samples (panel I, n = 100) were tested with the *B. bigemina cytb*-nPCR and the reference nPCR and qPCR 61%, 55%, and 49% positive carrier animals were detected, respectively. *Babesia bigemina cytb*-nPCR allowed the detection of a higher number of carrier animals compared to the reference nPCR ($X^2 = 0.74$, df = 1, p = 0.39) and to the qPCR protocols ($X^2 = 2.91$, df = 1, p = 0.09; Fig. 1B, Table 1); however, the difference was not significant.

Of the 100 bovine field samples, 38 were negative by all three assays, while the remaining 62 samples scored positive by at least one of the molecular assays used. Furthermore, of the 61 samples that tested positive by the *B. bigemina cytb*-nPCR, 54 and 48 samples scored positive by the reference nPCR and qPCR, respectively. In contrast, only a single sample scored negative by *cytb*-nPCR but positive by the other two assays.

When a set of samples from South Africa, Mozambique and Angola (panel II, n = 28) was analyzed, of the 23 samples that tested positive by *B. bigemina cytb*-nPCR, 22 tested positive by the reference nPCR ($X^2 = 0.11$, df = 1, p = 0.74). However, a difference with respect to panel I was observed since a significantly lower number of 8 carrier animals were detected by qPCR ($X^2 = 16.26$, df = 1, $p > 0.001$).

3.3. Interlaboratory validation of the reproducibility of cytb-nPCR assays using bovine field samples

A set of 150 samples (panel I) from bovine fields samples was independently single blindly analyzed by *B. bovis* and *B. bigemina cytb*-nPCR in three laboratories (Supplementary Table 2).

B. bovis cytb-nPCR detected 27.3%, 28.0% and 33.3% of positive carrier animals when tested by Lab1, Lab2 and Lab3, respectively ($X^2 = 1.56$, df = 5, p = 0.46). Similarly, *B. bigemina cytb*-nPCR detected

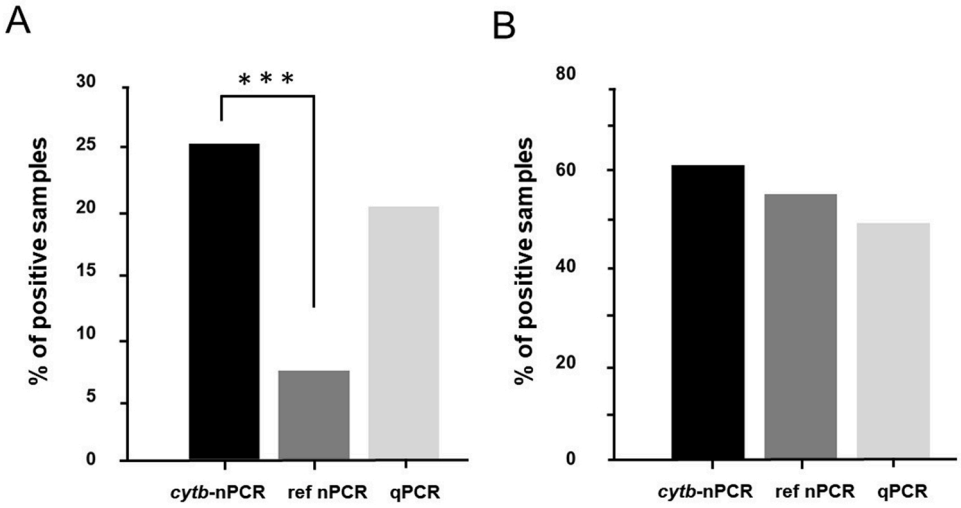

Fig. 1. Detection of *B. bovis* and *B. bigemina* by three PCR-based methods on cattle field samples. Percentage of *B. bovis* (A) and *B. bigemina* (B) positive samples from Corrientes, Argentina (panel I, n = 100) analyzed by *cytb*-nPCR, and a reference nPCR and qPCR. The asterisk shows significant differences ($X^2 = 12.05$, df = 1, $p < 0.001$) between two groups.

Table 1
Number of of *B. bovis* and *B. bigemina* positive carrier animals in bovine field samples by *cytb*-nPCR assays compared to reference nPCR and qPCR.

Babesia bovis			*Babesia bigemina*		
cytb-nPCR	nPCR	qPCR	*cytb*-nPCR	nPCR	qPCR
Bovine field samples from Argentina (panel I, n = 100)					
25 (25%)	7***(7%)	20^{ns} (20%)	61 (61%)	55^{ns} (55%)	49^{ns} (49%)
Bovine field samples from Africa (panel II, n = 20)			Bovine field samples from Africa (panel II, n = 28)		
15 (75%)	5** (25%)	12^{ns} (60%)	23 (82%)	22^{ns} (79%)	8*** (29%)

The level of significance of the chi-square test is given in reference to the *cytb*-nPCR assay: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ^{ns}, not significant.

54.7%, 49.3% and 52.7% of positive samples when tested by Lab1, Lab2 and Lab3, respectively ($X^2 = 0.87$, df = 5, p = 0.65). Thus, no significant differences in the number of parasite-infected animals were observed between the three laboratories.

To determine the concordance, the k value was estimated and shown to range from 0.65 to 0.72 for the *B. bovis cytb*-nPCR, and from 0.76 to 0.79 for the *B. bigemina cytb*-nPCR between any of the compared laboratory combinations. This corresponds to a test concordance between 85.3% and 88.7% for the detection of *B. bovis* and between 88% and 89.33% for the detection of *B. bigemina* demonstrating a substantial agreement and high reproducibility of both assays (Supplementary Table 2).

4. Discussion

The availability of a standardized, reliable and specific diagnostic method that can be used globally is crucial for efficient epidemiological surveillance of *B. bovis* and *B. bigemina* infections and the rational use of control measures. In this study, a recently developed molecular tool was validated for the detection of *B. bovis* and *B. bigemina* infections in bovines of different tick-endemic areas. Direct parasite detection using *cytb*-nPCR was carried out by amplification of a region of the *cytb* genes of *B. bovis* and *B. bigemina* (Romero-Salas et al., 2016; Ganzinelli et al., 2020).

After primary infection, parasites are relatively abundant for up to 10 days in the blood of *Babesia*-infected bovines (Bock et al., 2004). Serologic diagnosis is indirect and does not distinguish between past exposure and present infections. Although serological assays can be very useful in epidemiology studies, the only conclusive evidence for a confirmatory diagnosis of a current infection is the demonstration of the presence of the etiological agent (Rodriguez et al., 2013). Direct detection of bovine babesiosis can be confirmed by microscopic examination of Giemsa-stained blood smears and by molecular methods (Ganzinelli et al., 2018). PCR-based methods are highly sensitive and very early infections can be PCR-positive prior to the generation of detectable antibody levels (Goff et al., 2003, 2006). Nevertheless, in infected asymptomatic carrier bovines, parasites may escape direct detection by PCR-based molecular detection methods (Calder et al., 1996; Gubbels et al., 1999; Schnittger et al. 2004; Benitez et al., 2018). Hence, there is a need for highly sensitive and specific diagnostic assays able to detect *Babesia*-infected chronic carrier animals.

This study describes the performance of *cytb*-nPCR assays for the direct detection of *B. bovis* and *B. bigemina*. The analytical specificity of the *cytb*-nPCR assays was evaluated using a panel of genomic DNA samples of hemoparasites and hemobacteria that potentially co-occur in bovine blood in areas that are endemic for *B. bovis* and *B. bigemina*. As expected, results for both *B. bovis* and *B. bigemina cytb*-nPCR assays showed no cross-reactivity with any of the tested microorganisms, suggesting their applicability in most areas that are endemic for bovine babesiosis (Romero-Salas et al., 2016).

Recently, the analytical sensitivity of the *cytb*-nPCR to detect *B. bovis* and *B. bigemina* was estimated by comparison with the most widespread molecular detection assays for both pathogens (Figueroa et al., 1993; in a modified format included in OIE, 2021). The *B. bovis* and *B. bigemina cytb*-PCR assays were found to be considerably more sensitive that the reference nPCR format (Romero-Salas et al., 2016). The results obtained agree with other reports, which demonstrated a substantial increase in the sensitivity of molecular diagnostic methods when a mitochondrial-encoded gene is used as a target for standard or qPCR assays, since the mitochondrial genome is present in multiple copies (Buling et al., 2007).

Correspondingly, in the present study, the *cytb*-nPCR for *B. bovis* detected a significantly higher number of infections in field samples than the other two methods. A higher number of carrier animals was also detected with the *B. bigemina cytb*-nPCR assay, though these differences were, with the exception of the samples originating from African countries (panel II), not significant. In a previous study carried out in *R. microplus*-hyperendemic fields of Veracruz, Mexico, very high percentages of *B. bovis* (82.3%) and *B. bigemina* (94.1%) bovine carrier animals were detected using the *cytb*-nPCR assays (Romero-Salas et al.,

2016). This strongly suggests that almost all bovine carriers have been detected by both assays. The difference in sensitivity between *cytb*-nPCRs and the reference nPCR impacts on the classification of *Babesia* spp.-infected field samples as negative or positive. Since babesiosis is characterized as a persistent infectious disease, the ability to correctly identify true-negative samples is a major concern (Goff et al., 2008).

Furthermore, the *cytb*-nPCR assays have been able to detect *B. bovis* and *B. bigemina* strains circulating in five different countries that are situated in three continents, including Mexico (North America), Argentina (South America), and South Africa, Mozambique and Angola (Africa). This is in agreement with the high sequence conservation of the *cytb* gene from *Babesia* spp. isolates from different geographic origin as reported previously (Fahrimal et al., 1992; Buling et al., 2007). This strongly suggests that the primers designed for both *cytb*-nPCR assays are applicable to detect *B. bovis* and *B. bigemina* strains occurring in endemic areas around the world.

Wildlife animals as well as other domestic bovids occasionally raised with bovine cattle, including yak and water buffalo, may play a critical role as reservoirs of tick-borne pathogens in endemic areas by acting as a source of transmission (Ferreri et al., 2008; Saravanan et al., 2013; Weerasooriya et al., 2016; Benitez et al., 2018; Remesar et al., 2019; He et al., 2021). On one hand, wildlife animals, such as white-tailed deer, may disperse *B. bovis* and *B. bigemina*-infected ticks into areas free of bovine babesiosis, as has been observed at the Mexico – USA frontier (da Silveira et al., 2011; Holman et al., 2011; Busch et al., 2014). On the other hand, applying the *B. bovis* and *B. bigemina cytb*-nPCR assays, it has been shown that an increased infection rate of *B. bovis* and *B. bigemina* in bovines was associated with a correspondingly increased infection rate of both pathogens in water buffaloes that were reared on the same pastures (Romero-Salas et al., 2016). This shows that the *cytb*-nPCR assays are a useful tool for direct detection of *B. bovis* and *B. bigemina* parasites in asymptomatic domestic animals and should be also applicable for the detection of wild carrier animals.

Control of bovine babesiosis is primarily achieved by decrease of tick infestation using acaricides and, in some regions of the world, by vaccination with attenuated parasites (Florin-Christensen et al., 2014, 2021). *B. bovis* and *B. bigemina* live vaccines do introduce the parasite into the bovine population and it is desirable to be able to evaluate the circulation of vaccine strains in areas where vaccines are applied (Benitez et al., 2018; Rauf et al., 2020). On the other hand, clinical bovine babesiosis cases are treated with a variety of drugs (Vial and Gorenflot, 2006; Mosqueda et al., 2012). Although effective, these treatments leave undesired residues in the food chain. These shortcomings have prompted an active research effort to identify additional alternative compounds (Li et al., 2020). The presented highly sensitive *cytb*-nPCR assays may be applied to demonstrate parasite elimination following the application of novel therapeutic babesicidial drugs.

A number of factors may influence the performance of PCR-based tests, leading to varied results even when the same assays and identical template DNA is tested (Bustin, 2002). In order to show the reliability of both *cytb*-nPCR assays, a single blinded trial test was performed in three independent laboratories using the same panel of field samples. This interlaboratory comparison showed a highly reproducible detection of *B. bovis* and *B. bigemina* carrier animals by *cytb*-nPCR assays even when carried out by independent operators in different laboratories.

In summary, our results validate the developed *cytb*-nPCRs as useful methods for the direct detection of *B. bovis* and *B. bigemina* parasites in clinical cases and in particular for the identification of an asymptomatic carrier status. Based on our findings and those of previous studies, the *cytb*-nPCR assays exhibit the necessary characteristics for their application to *B. bovis* and *B. bigemina* detection worldwide in regions where bovine babesiosis is endemic. In addition, these assays can be applied for determining a sterile immunity after vaccination trials and/or parasite elimination following the treatment with antiparasitic drugs. The outlined characteristics highlight the *cytb*-nPCR assays as attractive tools for the detection of *B. bovis* and *B. bigemina*.

CRediT authorship contribution statement

Conceptualization: **Leonhard Schnittger**. Formal analysis: **Sabrina Ganzinelli** and **Leonhard Schnittger**. Investigation: **Sabrina Ganzinelli, Charles Byaruhanga, María E. Primo**, and **Zinathi Lukanji**. Resources: **Luis Neves, Daniel Benitez, Batmagnai Enkhbaatar, Arifin Budiman Nugraha**, and **Ikuo Igarashi**. Writing – original draft: **Sabrina Ganzinelli**. Writing – review & editing: **Sabrina Ganzinelli, Leonhard Schnittger, Charles Byaruhanga, Luis Neves, María E. Primo, Kgomotso Sibeko, Tshepo Matjila**, and **Monica Florin-Christensen**. Visualization: **Sabrina Ganzinelli** and **Leonhard Schnittger**. Supervision: **Leonhard Schnittger**. Funding acquisition: **Tshepo Matjila, Monica Florin-Christensen**, and **Leonhard Schnittger**.

Declarations of interest

The authors declare no conflict of interest.

Acknowledgements

We express our gratitude to Anna-Mari Bosman for her invaluable help and assistance. We would also like to thank Ilse Vorster for providing hemoparasite DNA samples. This research was supported by financial assistance from the National Institute of Agricultural Technology (INTA) project Grants 2019-PD-E5-I102-001, 2019-PD-E5-I103-001 and 2019-PE-E5-I109-001 and a Cooperation grant between MINCyT, Argentina and DST, South Africa (SA/17/02).

Declarations of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2022.109686.

References

Alvarez, J.A., Rojas, C., Figueroa, J.V., 2019. Diagnostic tools for the identification of *Babesia* sp. in persistently infected cattle. Pathogens 8, 143. https://doi.org/10.3390/pathogens8030143.

Benitez, D., Mesplet, M., Echaide, I., Torioni de Echaide, S., Schnittger, L., Florin-Christensen, M., 2018. Mitigated clinical disease in water buffaloes experimentally infected with *Babesia bovis*. Ticks Tick Borne Dis. 9, 1358–1363. https://doi.org/10.1016/j.ttbdis.2018.04.012.

Bock, R., Jackson, L., de Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129 Suppl., S247–S269.

Buling, A., Criado-Fornelio, A., Asenzo, G., Benitez, D., Barba-Carretero, J.C., Florin-Christensen, M., 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. Vet. Parasitol. 147, 16–25. https://doi.org/10.1016/j.vetpar.2007.03.031.

Busch, J.D., Stone, N.E., Nottingham, R., Araya-Anchetta, A., Lewis, J., Hochhalter, C., Giles, J.R., Gruendike, J., Freeman, J., Buckmeier, G., Bodine, D., Duhaime, R., Miller, R.J., Davey, R.B., Olafson, P.U., Scoles, G.A., Wagner, D.M., 2014. Widespread movement of invasive cattle fever ticks (*Rhipicephalus microplus*) in southern Texas leads to shared local infestations on cattle and deer. Parasit. Vectors 7, 188. https://doi.org/10.1186/1756-3305-7-188.

Bustin, S.A., 2002. Quantification of mRNA using quantitative reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29, 23–39.

Calder, J.A., Reddy, G.R., Chieves, L., Courtney, C.H., Littell, R., Livengood, J.R., Norval, R.A., Smith, C., Dame, J.B., 1996. Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. J. Clin. Microbiol. 34, 2748–2755. https://doi.org/10.1128/JCM.34.11.2748-2755.1996.

Choopa, C., 2015. Diagnosis of Tick-borne Diseases in Cattle in Bushbuckridge, Mpumalanga, South Africa and Identification of Theileria parva Carriers (Master's dissertation). University of Pretoria.

da Silveira, J.A., Rabelo, E.M., Ribeiro, M.F., 2011. Detection of *Theileria* and *Babesia* in brown brocket deer (*Mazama gouazoubira*) and marsh deer (*Blastocerus dichotomus*) in the State of Minas Gerais, Brazil. Vet. Parasitol. 177, 61–66.

Fahrimal, Y., Goff, W.L., Jasmer, D.P., 1992. Detection of *Babesia bovis* carrier cattle by using polymerase chain reaction amplification of parasite DNA. J. Clin. Microbiol. 30, 1374–1379.

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. https://doi.org/10.1196/annals.1428.036.

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. https://doi.org/10.1016/0304-4017(93)90008-b.

Florin-Christensen, M., Suarez, C.E., Rodriguez, A.E., Flores, D.A., Schnittger, L., 2014. Vaccines against bovine babesiosis: where we are now and possible roads ahead. Parasitology 28, 1–30. https://doi.org/10.1017/S0031182014000961.

Florin-Christensen, M., Schnittger, L., Bastos, R.G., Rathinasamy, V.A., Cooke, B.M., Alzan, H.F., Suarez, C.E., 2021. Pursuing effective vaccines against cattle diseases caused by apicomplexan protozoa. CAB Rev. 16, 24. https://doi.org/10.1079/PAVSNNR202116024.

Ganzinelli, S., Rodriguez, A.E., Schnittger, L., Florin-Christensen, M., 2018. *Babesia* in domestic ruminants. In: Florin-Christensen, M., Schnittger, L. (Eds.), Parasitic Protozoa of Farm Animals and Pets. Springer Nature, Cham, Switzerland, pp. 215–240.

Ganzinelli, S., Benitez, D., Gantuya, S., Guswanto, A., Florin-Christensen, M., Schnittger, L., Igarashi, I., 2020. 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. Transbound. Emerg. Dis. 67 (Suppl. 2), S159–S164. https://doi.org/10.1111/tbed.13435.

Goff, W.L., McElwain, T.F., Suarez, C.E., Johnson, W.C., Brown, W.C., Norimine, J., Knowles, D.P., 2003. Competitive enzyme-linked immunosorbent assay based on a rhoptry-associated protein 1 epitope specifically identifies *Babesia bovis*-infected cattle. Clin. Diagn. Lab. Immunol. 10, 38–43. https://doi.org/10.1128/cdli.10.1.38-43.2003.

Goff, W.L., Molloy, J.B., Johnson, W.C., Suarez, C.E., Pino, I., Rhalem, A., Sahibi, H., Ceci, L., Carelli, G., Adams, D.S., McGuire, T.C., Knowles, D.P., McElwain, T.F., 2006. Validation of a competitive enzyme-linked immunosorbent assay for detection of antibodies against *Babesia bovis*. Clin. Vaccine. Immunol. 13, 1212–1216. https://doi.org/10.1128/CVI.00196-06.

Goff, W.L., Johnson, W.C., Molloy, J.B., Jorgensen, W.K., Waldron, S.J., Figueroa, J.V., Matthee, O., Adams, D.S., McGuire, T.C., Pino, I., Mosqueda, J., Palmer, G.H., Suarez, C.E., Knowles, D.P., McElwain, T.F., 2008. Validation of a competitive enzyme-linked immunosorbent assay for detection of *Babesia bigemina* antibodies in cattle. Clin. Vaccine Immunol. 15, 1316–1321. https://doi.org/10.1128/CVI.00150-08.

Gomes, A.F., Neves, L., 2018. *Rhipicephalus microplus* (Acarina, Ixodidae) in Angola: evidence of its establishment and expansion. Exp. Appl. Acarol. 74, 117–122. https://doi.org/10.1007/s10493-017-0207.

Gubbels, J.M., de Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37, 1782–1789. https://doi.org/10.1128/JCM.37.6.1782-1789.1999.

He, Y., Chen, W., Ma, P., Wei, Y., Li, R., Chen, Z., Tian, S., Qi, T., Yang, J., Sun, Y., Li, J., Kang, M., Li, Y., 2021. Molecular detection of *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. in yaks (*Bos grunniens*) and Tibetan sheep (*Ovis aries*) on the Qinghai-Tibetan Plateau, China. Parasit. Vectors 14, 613. https://doi.org/10.1186/s13071-021-05109-2.

Holman, P.J., Carroll, J.E., Pugh, R., Davis, D.S., 2011. Molecular detection of *Babesia bovis* and *Babesia bigemina* in white-tailed deer (*Odocoileus virginianus*) from Tom Green County in central Texas. Vet. Parasitol. 177, 298–304.

Hutchings, C.L., Li, A., Fernandez, K.M., Fletcher, T., Jackson, L.A., Molloy, J.B., Jorgensen, W.K., Lim, C.T., Cooke, B.M., 2007. New insights into the altered adhesive and mechanical properties of red blood cells parasitized by *Babesia bovis*. Mol. Microbiol. 65, 1092–1105. https://doi.org/10.1111/j.1365-2958.2007.05850.x.

Li, Y., Liu, M., Rizk, M.A., Moumouni, P.F.A., Lee, S.H., Galon, E.M., Guo, H., Gao, Y., Li, J., Beshbishy, A.M., Nugraha, A.B., Ji, S., Tumwebaze, M.A., Benedicto, B., Yokoyama, N., Igarashi, I., Xuan, X., 2020. Drug screening of food and drug administration-approved compounds against *Babesia bovis* in vitro. Exp. Parasitol. 210, 107831.

Mahoney, D.F., 1969. Bovine babesiosis: a study of factors concerned in transmission. Ann. Trop. Med. Parasitol. 63, 1–14. https://doi.org/10.1080/00034983.1969.11686595.

Makgabo, S.M., 2019. Temporal Dynamics of Tick-borne Haemoparasite Infection in Calves in the Mnisi Communal Area, Mpumalanga, South Africa (Master of Science Thesis). University of Pretoria.

Mosqueda, J., Olvera-Ramirez, A., Aguilar-Tipacamu, G., Canto, G.J., 2012. Current advances in detection and treatment of babesiosis. Curr. Med. Chem. 19, 1504–1518.

Nyoni-Phili, S., 2017. Occurrence and Genetic Diversity of Anaplasma marginale in Cattle From Two Dip Tanks in Zambezia Province Mozambique (Master's dissertation). University of Pretoria.

OIE, 2021. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals ⟨https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/⟩, Bovine babesiosis. Chapter 3.4.2.

Rauf, U., Suleman, M., Abid, A., Jamil, H., Menghwar, H., Durrani, A.Z., Rashid, M.I., Akbar, H., 2020. Humoral and cell-mediated immune response validation in calves after a live attenuated vaccine of *Babesia bigemina*. Pathogens 9, 936.

Remesar, S., Díaz, P., Prieto, A., Markina, F., Díaz Cao, J.M., López-Lorenzo, G., Fernández, G., López, C.M., Panadero, R., Díez-Baños, P., Morrondo, P., 2019. Prevalence and distribution of *Babesia* and *Theileria* species in roe deer from Spain. Int. J. Parasitol. Parasites Wildl. 9, 195–201.

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

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. https://doi.org/10.1016/j.vetpar.2015.12.030.

Saravanan, B.C., Das, S., Siju, S.J., Tewari, A.K., Sankar, M., Kataktalware, M.A., Ramesha, K.P., 2013. *Babesia bigemina* infection in yak (*Poephagus grunniens* L.): molecular detection and characterization. Vet. Parasitol. 194, 58–64.

Schnittger, L., Yin, H., Qi, B., Gubbels, M.J., Beyer, D., Niemann, S., Jongejan, F., Ahmed, J.S., 2004. Simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants by reverse line blotting. Parasitol. Res. 92, 189–196. https://doi.org/10.1007/s00436-003-0980-9.

Schnittger, L., Rodriguez, A.E., Florin-Christensen, M., Morrison, D.A., 2012. *Babesia*: a world emerging. Infect. Genet. Evol. 12, 1788–1809. https://doi.org/10.1016/j.meegid.2012.07.004.

Schnittger, L., Ganzinelli, S., Bhoora, R., Omondi, D., Nijhof, A.M., Florin-Christensen, M., 2022. The Piroplasmida *Babesia, Cytauxzoon*, and *Theileria* in farm and companion animals: species compilation, molecular phylogeny, and evolutionary insights. Parasitol. Res.

Sili, G., 2018. Species Composition of Ticks and Tick-borne Pathogens in Domestic Ruminants and Dogs in Tchicala-Tcholoanga, Huambo Province. Angola (Master's dissertation). University of Pretoria.

Sili, G., Byaruhanga, C., Horak, I., Steyn, H., Chaisi, M., Oosthuizen, M.C., Neves, L., 2021. Ticks and tick-borne pathogens infecting livestock and dogs in Tchicala-Tcholoanga, Huambo Province, Angola. Parasitol. Res. 120, 1097–1102. https://doi.org/10.1007/s00436-020-07009-3.

Stoltsz, H., Byaruhanga, C., Troskie, M., Makgabo, M., Oosthuizen, M.C., Collins, N.E., Neves, L., 2020. Improved detection of *Babesia bigemina* from various geographical areas in Africa using quantitative PCR and reverse line blot hybridisation. Ticks Tick Borne Dis. 11, 101415 https://doi.org/10.1016/j.ttbdis.2020.101415.

Suarez, C.E., Noh, S., 2011. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. Vet. Parasitol. 180, 109–125. https://doi.org/10.1016/j.vetpar.2011.05.032.

Vial, H.J., Gorenflot, A., 2006. Chemotherapy against babesiosis. Vet. Parasitol. 138, 147–160.

Wagner, G.G., Holman, P., Waghela, S., 2002. Babesiosis and heartwater: threats without boundaries. Vet. Clin. N. Am. Food Anim. Pract. 18, 417–430 (vi–vii).

Weerasooriya, G., Sivakumar, T., Lan, D.T., Long, P.T., Takemae, H., Igarashi, I., Inoue, N., Yokoyama, N., 2016. Epidemiology of bovine hemoprotozoa parasites in cattle and water buffalo in Vietnam. J. Vet. Med. Sci. 78, 1361–1367.

Byaruhanga, C., Makgaboa, M., Choopaa, C.N., Mulandanea, F.C., Vorstera, I., Troskiea, M., Chaisi M.K., Collins, N.E., 2022. Genetic diversity in *Babesia bovis* from southern Africa and estimation of *Babesia bovis* infection levels in cattle using an optimised quantitative PCR assay. Ticks Tick Borne Dis., in preparation.