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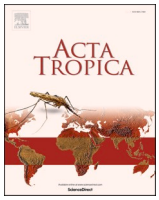

Validation of real-time PCR assays for detecting *Plasmodium* and *Babesia* DNA species in blood samples

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ABSTRACT

Malaria and babesiosis are global health threats affecting humans, wildlife, and domestic animals, particularly in Africa, the Americas, and Europe. Malaria can lead to severe outcomes, while babesiosis usually resembles a mild illness but can be severe and fatal in individuals with weakened immune systems. Swift, accurate detection of these parasites is crucial for treatment and control. We evaluated a real-time PCR assay for diagnosing five *Plasmodium* and three *Babesia* species from blood samples, assessing its sensitivity, specificity, and analytical performance by analyzing 46 malaria-positive and 32 *Babesia* spp-positive samples diagnosed through microscopy. The limit of detection for *Plasmodium* species ranged from 30 to 0.0003 copies/μL. For mixed infections, it was 0.3 copies/μL for *P. falciparum/P. vivax* and 3 copies/μL for *P. malariae/P. knowlesi. Babesia* species had a detection limit of 0.2 copies/μL. No cross-reactivity was observed among 64 DNA samples from various microorganisms. The assay showed good sensitivity, detecting *Plasmodium* and *Babesia* species with 100 % accuracy overall, except for *P. falciparum* (97.7 %) and *B. microti* (12.5 %). The low sensitivity of detecting *B. microti* was attributed to limitations in microscopy for species identification. This technique heavily relies on the proficiency of the examiner, as species within the genus cannot be distinguished under a microscope. Additionally, *Babesia* can be confused with the early trophozoite stage (ring forms) of *Plasmodium* parasites. The findings support multiplex qPCR's diagnostic superiority over the gold standard, despite higher costs. It offers enhanced sensitivity, specificity, and detects mixed infections, crucial for effective monitoring and diagnosis of malaria and babesiosis in endemic regions with significant public health challenges.

1. Introduction

Malaria is a vector-borne parasitic disease endemic in tropical and sub-tropical regions worldwide. This disease is caused by protozoa of the genus *Plasmodium* (Apicomplexa: Plasmodiidae), a diverse group that infects a variety of vertebrate hosts including primates (Muehlenbein et al., 2015). There are six known species of *Plasmodium* that commonly infect humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium knowlesi* and *Plasmodium ovale* which is divided in two genetically distinct sympatric species *P. ovale curtisi* and *P. ovale wallikeri* (Escalante and Pacheco, 2019; Sutherland et al., 2010). Of the six species of *Plasmodium* that infect humans, *P. falciparum* and *P. vivax* have the highest infection and morbidity rates. According to the 2022 World Health Organization (WHO) World Malaria Report, there are an estimated 247 million cases of malaria globally in 2021, marking a slight increase from the 245 million reported in 2020 (Anon., World Health Organization 2022).

On the other hand, babesiosis is a tick-borne disease caused by the protozoan intraerythrocytic parasites of the genus *Babesia.* It poses a significant threat to livestock, pets, and humans worldwide, leading to severe illnesses (Azhar et al., 2023). Out of >100 *Babesia* species/genotypes described so far, only some were diagnosed in infected humans, mostly *B. microti, B. divergens, Babesia divergens*–like species and *B. venatorum* (Vannier et al., 2015; Rozej-Bielicka et al., 2015). Additionally, four larger *Babesia* species, such as *B. canis, B. rossi, B. vogeli*, and the unofficially assigned *Babesia* sp. *(coco)*, are known to

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cause canine babesiosis (Zygner et al., 2023). Babesiosis is currently exhibiting rising incidence trends in the United States and spreading across various regions worldwide, including Europe, Asia, Africa, Australia, and South America (Rollend et al., 2013; Scott and Scott, 2018; Jia et al., 2018). In the U.S. alone, between 2011 and 2019, there were 16,456 reported cases of babesiosis to the Centers for Disease Control and Prevention (CDC). Remarkably, 98.2 % of these cases originated from just 10 states out of the 37 reporting states (SJ Drews et al., 2023).These data underscore the critical significance of promptly and accurately diagnosing malaria and babesiosis, followed by administering effective treatment, in order to effectively control the disease.

One of the main techniques used to detect and identify malaria and *Babesia* parasites is light microscopy, which has been considered the "gold standard" method (Muehlenbein et al., 2015; Krause, 2019; Waked and Krause, 2022; Lee et al., 2020). Microscopy provides a cost-effective solution with rapid results, reasonable sensitivity, and the capacity to detect malaria parasites in densities ranging from 50 to 500 parasites/μl. Previous studies have shown that sensitivity depends on the experience of the microscopist. For example, Moody et al. reported that the thick blood film procedure detects about 50 parasites/μl of blood (assuming a total RBC count of 5×10^6/μl), equivalent to 0.001 % of infected RBCs (Moody, 2002). In contrast, Milne et al. found that most routine diagnostic laboratories achieve lower sensitivity, detecting on average 0.01 % of infected RBCs (500 parasites/μl) (Milne et al., 1994). Similarly, it can identify *Babesia* parasites in blood smears containing 10 to 50 parasites/μl (Krause et al., 1996; Wang et al., 2015). Moreover, this method enables the identification of malaria species and determination of parasite density (Feleke et al., 2017). Yet, it requires interpretation by skilled technicians, is significantly time consuming, and exhibits limitations in sensitivity, particularly in those cases with low parasitemia, such as those found in asymptomatic patients and regions with low endemicity (Galatas et al., 2016; Chen et al., 2016).

Additionally, distinguishing *Babesia* parasites from the early trophozoite stage (ring form) of *Plasmodium* parasites, especially *P. falciparum*, can be challenging. Although there exist characteristics to distinguish *Babesia* parasites from *Plasmodium* as the tetrad forms (known as the Maltese cross), the absence of hemozoin deposit (which appear as brownish pigmentation) in ring forms, and absence of gametocytes (Chan et al., 2021), these features are rare and necessitate the expertise of trained personnel for accurate identification. Furthermore, identifying the infecting *Babesia* species is limited to the genus level based on morphological criteria (Wang et al., 2015). Another frequently employed method is the rapid diagnostic test (RDT), utilizing immunochromatographic strips to detect malarial antigens, such as histidine-rich protein 2 (HRP2) specific to *P. falciparum*, and *Plasmodium* lactate dehydrogenase (pLDH) or aldolase capable of detecting all *Plasmodium* species (Feleke et al., 2021; Srivastava et al., 2023). RDTs offer swift results in contrast to microscopy, are straightforward to administer, have a sensitivity of ~ 100 parasites/μl and operate without the need for electricity. However, it is noteworthy that RDTs provide solely quantitative data and have been reported to yield more false positives than microscopy and its less sensitive at low parasite densities (< 200 parasites/μL) (Nijhuis et al., 2018; Osman et al., 2010). For *Babesia* detection, additional techniques included serological methods such as the indirect fluorescent antibody test (IFAT), competitive enzyme-linked immunosorbent assay (cELISA), immunochromatography (ICT), and complement fixation test (CFT) (Azhar et al., 2023). These methods are more sensitive than microscopy in detecting infection with *Babesia* species (Akoolo et al., 2017; Lempereur et al., 2015). However, they cannot detect acute disease before the adaptive immune response is triggered, nor can they distinguish active disease from past infections. This limitation poses a significant diagnostic challenge, especially in endemic regions (Wormser et al., 2015; Fox et al., 2006).

Conversely, nucleic acid amplification tests (NAATs) offer unparalleled sensitivity and specificity in malaria and babesiosis diagnosis. Importantly among these are the Polymerase Chain Reaction (PCR) and Loop-mediated Isothermal Amplification (LAMP), which both stand out, as proficient approaches at detecting parasite DNA in blood samples (Bell et al., 2016; Lizarazo-Zuluaga et al., 2022; Srionrod et al., 2022). PCR has demonstrated sensitivity and specificity in malaria diagnosis ranging from 75 % to 90.9 % and 91.2 % to 97 %, respectively (Rantala et al., 2010; Wardhani et al., 2020; Fransisca et al., 2015). Additionally, it boasts a remarkable limit of detection as low as 1–5 parasites/μL (Berzosa et al., 2018; Krampa et al., 2017; Mfuh et al., 2019; Berry et al., 2008). For *Babesia* diagnosis, PCR exhibits a sensitivity and specificity of 94.2 % to 100 % and 97.1 % to 100 %, respectively (Rollend et al., 2013; Parodi et al., 2021; Azhahianambi et al., 2018). Notably, PCR has the capability not only to detect submicroscopic and mixed infections but also to distinguish all species currently known to cause human infection, which offers a significant advantage over microscopy (Fradejas et al., 2019; Haanshuus et al., 2019).

To date, real-time PCR (qPCR) technology has shown superior sensitivity compared to traditional PCR methods, with a limit of detection (LOD) below 0.1 parasites/μL for malaria (Kamaliddin et al., 2021) and 1–3 parasites/μl of blood for *Babesia*. (Wang et al., 2015). Additionally, it enables precise quantification of pathogens and helps mitigate the risk of contamination. The majority of malaria and *Babesia* qPCR assays focus on the multicopy 18S ribosomal RNA (rRNA) genes, chosen for their exceptional specificity and conservation across all *Plasmodium* and *Babesia* species (Wang et al., 2015; Mercereau-Puijalon et al., 2002; Kuo et al., 2020; Githaka et al., 2022). These assays typically exhibit to malaria detection limits spanning from 0.002 to 30 parasites/μL, with sensitivity values ranging between 90 % and 97 %, and a consistently high specificity of 100 % (Hanron et al., 2017; Taghdiri et al., 2019; Kumari et al., 2022). For *Babesia*, detection limits typically reach 2.7 parasites/μL of blood, with sensitivity ranging between 95.9 % to 100 % and specificity of 97.7 % to 100 % (Wang et al., 2015; Kuo et al., 2020; Githaka et al., 2022; Troskie et al., 2019; Qurollo et al., 2017; Teal et al., 2012; Kongklieng et al., 2015).

Several studies have detailed the development of a multiplex qPCR assay for detecting targets within the *Plasmodium* and *Babesia* genus, including species-specific markers for different *Plasmodium* and *Babesia* species (Troskie et al., 2019; Rougemont et al., 2004; Mahale et al., 2023; Lazrek et al., 2023; Padmaja et al., 2022; Remesar et al., 2023). Beyond possessing the essential attributes that render qPCR an increasingly appealing diagnostic method, the described multiplex assay was specifically engineered to facilitate high-throughput screening while substantially cutting costs (Lazrek et al., 2023).

Given the persistent threat of malaria and babesiosis as major global health issues, impacting countless individuals annually, especially in high-prevalence regions, and the potential of transmission of those pathogens through blood transfusion accurate diagnosis is paramount. Both parasites not only resemble each other when observed in blood smears but also translate into similar and at times overlapping clinical symptoms, complicating diagnosis (Arsuaga et al., 2018). Therefore, it is crucial to develop accurate diagnostic tools for detecting and identifying these parasites. An inaccurate diagnosis could lead to inappropriate treatment and further complications, emphasizing the urgency for reliable detection methods. (Amexo et al., 2004). Because microscopy, considered as gold standard, exhibits limited sensitivity, struggles to identify mixed infections accurately and may lack precision in detecting low parasitemia scenarios, this study, aimed to evaluate the sensitivity and species-specificity of a qPCR assay targeting the 18S rRNA for detecting *Plasmodium* and *Babesia*. For *Plasmodium* detection, we utilized two real-time multiplex PCR approaches adapted from Rougemont et al., (Rougemont et al., 2004) and Divis et al., (Divis et al., 2010). This method included one assay targeting *P. falciparum* and *P. vivax*, another targeting *P. malariae* and *P. knowlesi*, and a third monoplex-PCR for detecting *P. ovale*. For *Babesia* detection, we implemented three monoplex-PCR assays to detect *B. bovis, B. canis*, and *B. microti*. These tools are essential not only for establishing a timely diagnosis and provide effective treatment to patients but also for carefully monitoring

disease progression and resolution as well as improving diagnostic-based control strategies.

2. Materials and methods

2.1. Ethics approval and consent to participate

Residual clinical specimens were utilized for this study, obtained through the Mount Sinai Pathogen Surveillance Program. This program has undergone review and approval by the Human Research Protection Program at the Icahn School of Medicine at Mount Sinai (ISMMS) (HS#13-00,981).

2.2. Design of in-house multiplex qPCR assay

Different reference sequences of the 18 S rRNA gene of *P. falciparum, P. vivax, P. malariae, P. ovale, P. knowlesi, B. bovis, B. canis and B. microti* were downloaded from the NCBI Nucleotide database (accessible at https://www.ncbi.nlm.nih.gov/nuccore?term). For each sequence, the annealing sites to the forward and reverse primers, as well as the probe, described by Rougemont et al., (Rougemont et al., 2004) and Divis et al., (Divis et al., 2010), Githaka et al., (Githaka et al., 2022) Kuo te al., (Kuo et al., 2020) and Rollend et al., (Rollend et al., 2013) was identified, using the Clustal W algorithm, employing the UGENE version 33.0 software platform (Okonechnikov et al., 2012). This information is visually presented in the Figs. 1 and 2. Subsequently, utilizing these annealing sites, a fragment of 500 bp from each *Plasmodium* and *Babesia* species was retrieved and synthesized by Twist Biosciences, San

Fig. 1. The annealing sites to the forward and reverse primers, as well as the probe in the *Plasmodium* species evaluated. The figure shows the specificity of the primers forward and reverse (a) and probes (b) (highlighted in the dashed black box) for the five *Plasmodium* species tested (*P. falciparum, P. vivax, P. malariae, P. knowles* i and *P. ovale*).

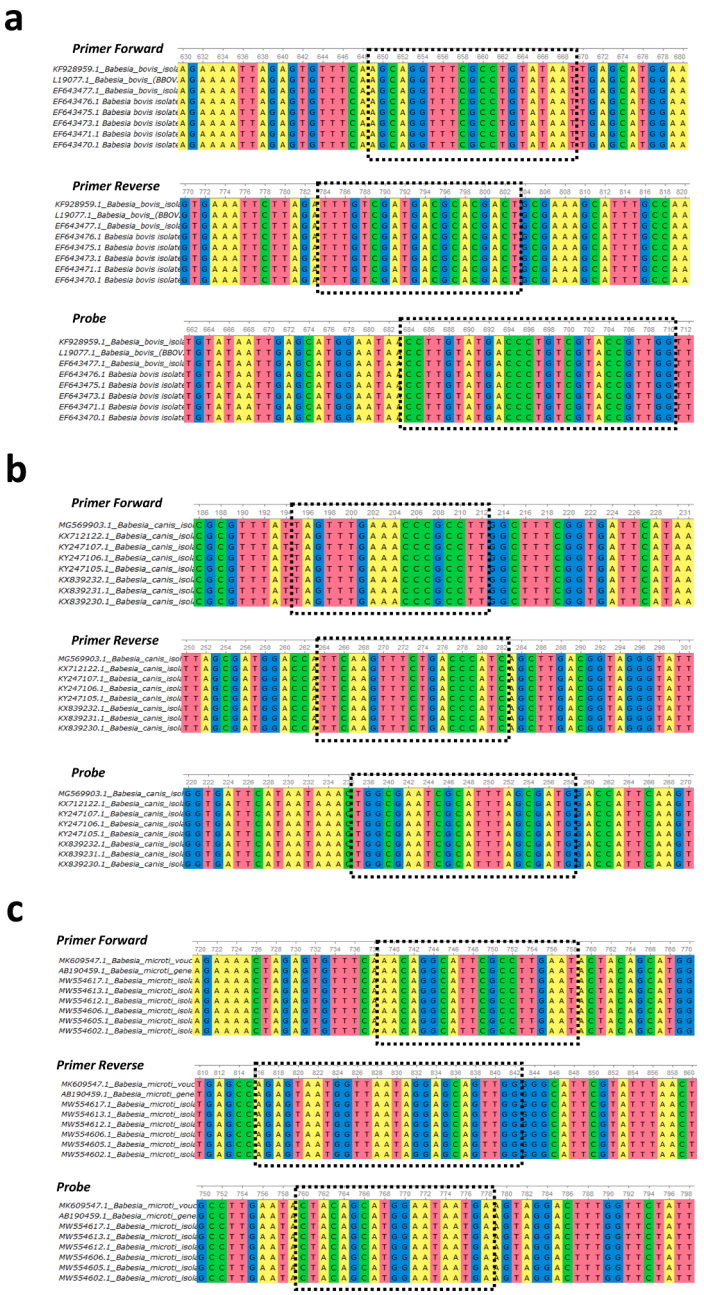

Fig. 2. The annealing sites to the forward and reverse primers, as well as the probe in the *Babesia* species evaluated. The figure shows the specificity of the primers forward and reverse and probes (highlighted in the dashed black box) for *B. bovis* (a), *B. canis* (b) and *B. microti* (c).

Francisco, CA (https://www.twistbioscience.com/products/genes), serving as a positive control for each *Plasmodium* and *Babesia* species. The sequences of each positive control are described in the **Supplemental Fig. S1.**

2.3. Real time PCR

Recognizing the significant advantages of multiplex-PCR, which enables simultaneous detection of multiple pathogens within a single sample, thus proving invaluable in diagnostic research due to its cost-effectiveness and time-saving capabilities, this study devised an approach through two distinct multiplex PCR assays to identify and differentiate various *Plasmodium* species. The first assay targeted the detection and differentiation of *P. falciparum* and/or *P. vivax*, while the second aimed to identify and distinguish *P. malariae* and/or *P. knowlesi*. It is important to note that the multiplex PCR assays did not include the identification and differentiation of *P. ovale*. Instead, a monoplex real-time PCR approach was adopted specifically for detecting *P. ovale*. The real-time PCRs were executed in three simultaneous separate reactions. The selection of these combinations was meticulously carried out, taking into account the specific labels utilized for each *Plasmodium* species. Specifically, *P. falciparum* and *P. vivax* were labeled with 5′FAM and 5′HEX, respectively, similar to what was done with *P. knowlesi* and *P. malariae*. Moreover, these procedures were carefully crafted to prevent any potential interference from intraspecies polymorphic regions, ensuring the accuracy and reliability of the experimental outcomes (Rougemont et al., 2004)

In the first reaction, primers and the two TaqMan probes specific to *P. falciparum* and *P. vivax* were mixed. This PCR assay was performed in a final volume of 25 μL, comprising 12.5 μL of TaqMan Universal Master Mix (Applied Biosystems), 10 μM of each primer, 10 μM each Probe *falciparum* and Probe *vivax* and 4,55 μL of Nuclease Free Water.

Standardization and control of DNA extraction were achieved using a third simplex PCR targeting the endogenous gene RNAseP (10 μM for each primer and 5 μM for the RNAseP Probe). In the second reaction, primers and the two TaqMan probes specific to *P. malariae* and *P. knowlesi* were mixed. The third reaction involved a monoplex real-time PCR designed for detecting *P. ovale.* The sequences of primers and probes used in the study are described in the Table 1 Both the second and third reactions were conducted under the same PCR conditions as previously described.

For *Babesia*, the real-time PCRs were conducted in three separate simultaneous reactions, each targeting a specific species (*B. bovis, B. canis* and *B. microti*). The PCR assay was performed in a final volume of 20 μL, comprising 10 μL of TaqMan Universal Master Mix (Applied Biosystems), 10 μM of each primer, 10 μM of Probe, and 4.2 μL of Nuclease-Free Water. Standardization and control of DNA extraction were achieved using a PCR targeting the endogenous gene RNAseP (with 10 μM for each primer and 5 μM for the RNAseP Probe). The sequences of primers and probes used in the study are described in the Table 1.

The RT-PCR assay was conducted in a Real-Time PCR system Bio-Rad CFX 384-Well Thermal Cycler (Bio-Rad Laboratories). The Thermal cycling conditions for each reaction are described in the **Supplemental Tables S1 and S2.**

2.4. Analytical sensitivity of the real-time PCRs

To assess the analytical sensitivity, we employed a method involving the determination of the limit of detection (LoD). Two distinct assays were conducted to assess the sensitivity of the TaqMan probes targeting *Plasmodium* species examined in this study. Initially, various dilutions of positive controls corresponding to each *Plasmodium* species were performed: ranging from 3×10^{4} to 3×10^{-4} copies/μL, for *P. falciparum* or *P. vivax*, 3×10^{3} to 3×10^{-3} copies/μL for *P. malariae,* 3×10^{3} to 3×10^{-7} copies/μL or *P. knowlesi* and 3×10^{4} to 3×10^{-2} copies/μL for *P. ovale.* Subsequently, to evaluate the assay's capability to co-amplify different DNA targets and to detect mixed infections, different dilutions of a mix containing primers (forward and reverse) and a combination of TaqMan probes were prepared for each pair of species (*P. falciparum/P. vivax* and *P. malariae/P. knowlesi*). For the detection of *P. ovale*, a mix comprising Primers and the TaqMan probe specific to *P. ovale* was prepared (Refer to Table 2 for details). To determine the Limit of Detection (LoD) for *Babesia*, we conducted various dilutions of positive controls corresponding to each *Babesia* species. These dilutions ranged from 2×10^{4} copies/μL to 2×10^{-3} copies/μL (Table 3).

We conducted serial dilutions of the positive controls, ranging from 10 ng/μL to 1×10^{-8} ng/μL, in a human DNA matrix sourced from whole blood. This blood was obtained from individuals living in non-endemic areas, who had tested negative for both *Plasmodium* and *Babesia* by PCR. Initially, 5μL of the positive control [10 ng/μL] was added to 45 μL of human DNA. This process was repeated in ten serial dilutions until reaching the range of 1×10^{-8} ng/μL. Later, each dilution underwent qPCR, as described previously.

All DNA samples from the point-serial dilutions were tested in triplicate using qPCR. The Probit regression analysis was then employed to estimate both the Limit of Detection (LoD) and the limit of quantification (LoQ) (Klymus et al., 2020). The analyses were carried out in RStudio through the script described in "LoD-calculator.R" using the drc package (https://github.com/cmerkes/qPCR_LOD_Calc)

2.5. Analytical specificity of the real-time PCRs

To assess the analytical specificity of the assay, we conducted

Table 2
Range of dilutions conducted, and the Limit of Detection (LOD) achieved through Probit regression analysis for the evaluated *Plasmodium* species.

Mix performed	*Plasmodium* species evaluated	Number of dilutions performed	Range of dilutions (copies/μL)	LOD (copies/μL)
Primers + TaqMan probe	*P. falciparum*	9	3×10^{4} to 3×10^{-4}	0.03
P. falciparum + TaqMan probe	*P. vivax*	9	3×10^{4} to 3×10^{-4}	0.3
P. vivax	*P. falciparum* / *P.vivax*	9	3×10^{4} to 3×10^{-4}	0.3 / 0.3
Primers + TaqMan probe *P. malariae*	*P. malariae*	7	3×10^{3} to 3×10^{-3}	3
+ TaqMan probe *P. knowlesi*	*P. knowlesi*	11	3×10^{3} to 3×10^{-7}	0.0003
	P. malariae / P. knowlesi	7	3×10^{3} to 3×10^{-3}	3 / 3*
Primers + TaqMan probe *P. ovale*	*P. ovale*	7	3×10^{4} to 3×10^{-2}	30

Table 3
Range of dilutions conducted, and the Limit of Detection (LOD) achieved through Probit regression analysis for the evaluated *Babesia* species.

Plasmodium species evaluated	Number of dilutions performed	Range of dilutions (copies/μL)	LOD (copies/μL)
B. bovis	8	2×10^{4} to 2×10^{-3}	0.2
B. canis	8	2×10^{4} to 2×10^{-3}	0.2
B. microti	8	2×10^{4} to 2×10^{-3}	0.2

Table 1
Primers and probes sequences for qPCR detection of *Plasmodium* and *Babesia* species.

Primers	Sequence	Target
18S rDNA Forward	GTTAAGGGAGTGAAGACGATCAGA	18S rDNA-*Plasmodium*
18S rDNA Reverse	AACCCAAAGACTTTGATTTCTCATAA	
18S rDNA Probe-*P. falciparum*	FAM-AGCAATCTAAAAGTCACCTCGAAAGATGACT-BHQ1	
18S rDNA Probe-*P. vivax*	HEX-AGCAATCTAAGAATAAACTCCGAAGAGAAAATTCT-BHQ1	
18S rDNA Probe-*P. malariae*	FAM-CTATCTAAAAGAAACACTCAT-BHQ1	
18S rDNA Probe-*P. knowlesi*	HEX-CTCTCCGGAGATTAGAACTCTTAGATTGCT-BHQ1	
18S rDNA Probe-*P. ovale*	HEX-CGAAAGGAATTTTCTTATT-BHQ1	
18S rDNA Forward	AGCAGGTTTCGCCTGTATAAT	18S rDNA-*B.bovis*
18S rDNA Reverse	AGTCGTGCGTCATCGACAAA	
18S rDNA Probe-*B. bovis*	FAM- CCTTGTATGACCCTGTCGTACCGTTGG-BHQ1	
18S rDNA Forward	TAGTTTGAAACCCGCCTT	18S rDNA-*B.canis*
18S rDNA Reverse	GATGGGTCAGAAACTTGAA	
18S rDNA Probe-*B. canis*	FAM-CATCGCTAAATGCGATTCGCCA-BHQ1	
18S rDNA Forward	AACAGGCATTCGCCTTGAAT	18S rDNA-*B.microti*
18S rDNA Reverse	CCAACTGCTCCTATTAACCATTACTCT	
18S rDNA Probe-*B. microtis*	FAM-CTACAGCATGGAATAATGA-BHQ1	
RnaseP-Forward	AGATTTGGACCTGCGAGCG	RNASeP
RnaseP-Reverse	GAGCGGCTGTCTCCACAAGT	
RnaseP	Cy5-TTCTGACCTGAAGGCTCTGCGCG- BHQ3	

experimental testing with a comprehensive set of 64 DNA samples. These samples were selected to encompass a range of pathogens some of which could potentially mimic symptoms caused by *Plasmodium* or *Babesia* and might exhibit cross-reactivity within the different species of these parasites. The set comprised 47 bacterial DNAs, 11 fungal DNAs, 3 parasites DNAs and 3 virus DNAs. The comprehensive list of organisms included in this panel can be found in Table 4. The DNA samples of these various pathogens were extracted and provided by the Molecular Microbiology Laboratory (MML) located at Mount Sinai Hospital. For each DNA, a species-specific monoplex real-time PCR assay was performed in triplicate.

Table 4
Panel of organisms used in the specificity assay. Including bacteria, fungi, parasites and viruses.

Organism	DNA Concentration (ng/μL)
Bacillus cereus group	298.33
Cutibacterium acnes	14.13
Enterococcus durian atcc 11,576	9.76
Enterococcus faecalis atcc 51,299 (VRE)	12.2
Enterococcus faecalis atcc 29,212 (VSE)	13.31
Listeria monocytogenes	12.36
Micrococcus luteus	34.58
Staphylococcus epidermidis atcc 12,228	23.13
Staphylococcus saprophyticus atcc 15,305	20.95
Staphylococcus aureus atcc 29,213 (OSSA)	16.31
Staphylococcus aureus atcc BAA-977	15.28
Staphylococcus aureus atcc 43,300 (MRSA)	16.38
Staphylococcus aureus atcc 25,923	18.76
Streptococcus mitis/oralis	10.2
Streptococcus pneumoniae atcc 49,619	16.81
Streptococcus pneumoniae atcc 6305	19.17
Streptococcus gallolyticus atcc 49,147	16.81
Streptococcus pyogenes atcc 12,384 (GAS)	19.17
Streptococcus agalactiae atcc 27,956 (GBS)	9.78
Aerococcus viridans	7.68
Streptococcus anginosus (GCS)	9.49
Acinetobacter baumannii	26.08
Bacteroides fragilis	64.52
Enterobacter cloacae subsp. *cloacae atcc 13,047*	8.735
Escherichia coli atcc 35,218	47.73
E. coli atcc 700,728	31.66
E. coli atcc 25,922	38.66
Haemophilus influenzae atcc 10,211	11.29
Klebsiella pneumoniae atcc BAA-1705 (KPC)	7.52
Klebsiella pneumoniae atcc 33,495	11.13
Morganella morganii	13.12
Neisseria meningitidis atcc 13,090	23.9
Oligella urethralis atcc 17,960	11.93
Proteus mirabilis atcc 43,071	28.4
Proteus vulgaris atcc 49,132	43.07
Proteus vulgaris atcc 8427	19.49
Psuedomonas aeruginosa atcc 27,853	30.17
Salmonella enterica subsp. *enterica atcc 14,028*	14.33
Salmonella enterica subsp. *enterica atcc 13,311*	50.41
Serratia marcescens atcc 13,880	170.51
Shigella dysenteriae	32.26
Yersinia enterocolitica atcc 9610	91.39
Streptococcus constellatus (GFS)	10.08
Enterococcus faecium	10.16
Staphylococcus lugdunensis	14.11
Micrococcus lylae	10.93
Klebsiella pneumoniae atcc 700,603 (ESBL)	13.46
Candida albicans atcc 60,193	8.49
Candida auris	33.9
Candida glabrata atcc 15,126	11.65
Candida guilliermondii atcc 6260	13.34
Candida kefyr atcc 2512	16.22
Candida krusei atcc 6258	8.55
Candida lusitaniae atcc 34,449	9.21
Candida parapsilosis atcc 22,019	7.18
Candida tropicalis atcc 9968	7.06
Cryptococcus gattii	23.14
Candida albicans	8.44
Leishmania amazonensis	25.12
Trypanosoma cruzi	18.23
Trypanosoma brucei	31.67
Human Immunodeficiency Virus	771 copies/mL
Hepatitis C virus	1,670,000 IU/mL
Human Papilloma virus	55.10 ng/μL

2.6. Diagnostic performance of the real-time PCRs

A total of 46 malaria-positive samples and 32 *Babesia*-positive samples collected from different patients provided by the Molecular Microbiology Laboratory (MML) at the Mount Sinai Health System were analyzed. Among malaria samples, 42 were positive for *P. falciparum* and 4 for *P. vivax* by microscopy. Regarding the *Babesia* samples, 4 were identified as *B. microti* and 28 as *Babesia spp* by microscopy and PCR.

Given the limited number of positive clinical samples for *P. vivax* and the absence of positive samples for *P. malariae, P. knowlesi, P. ovale, B. bovis* and *B. canis,* whole human blood (confirmed negative for *Plasmodium* and *Babesia)* were spiked with *P. vivax, P. malariae, P. knowlesi, P. ovale, B. canis* and *B. bovis* -positive control (1-fold to 2-fold above the LOD). 30 samples from whole blood negative for malaria and babesiosis, originating from non-endemic areas and 26 samples from patients suspected of having malaria that yield negative results by microscopy were included in the assay. In terms of the diagnostic performance, we tested the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using microscopy results as the gold standard.

The DNA for each clinical specimen was extracted using the Chemagic™ DNA Blood 400 Kit special H96 (CMG-1091; Perkin Elmer) with the automated Chemagic™ 360 instrument (2024-0020; Perkin Elmer), following the manufacturer's protocol and previously established procedures (Paniz-Mondolfi et al., 2023). The DNA underwent qPCR analysis following the instructions described previously.

To quantify parasite load, we constructed a standard curve using serial dilutions (ranging from 10^6 to 10^{11} parasites) of positive-control samples for *P. falciparum, P. vivax,* and *B. microti*. Each amplification plate included both positive and negative controls, with a predefined threshold of 0.1. The parasite load is expressed as the base 10 logarithm (Log10) of the number of parasite genome equivalents (parasite/μL).

2.7. Reproducibility

Inter- and intra-assay reproducibility across clinical specimens was evaluated by utilizing DNA from five positive and negative samples. Each specimen underwent triplicate testing (Intra-run) across three separate runs (days). To assess the reproducibility of *Plasmodium* and *Babesia* species where the number of clinical samples was limited (*P. vivax*) or absent (*P. knowlesi, P. malariae, P.ovale, B. bovis* and *B. canis*), we employed a methodology involving the use of whole human blood (confirmed negative for *Plasmodium* and *Babesia)* spiked with *P. vivax, P. knowlesi, P. malariae, P.ovale, B. bovis* and *B. canis* positive control (1-fold to 2-fold above the LOD). To ensure accuracy, we considered dilutions that were at least two times higher than the limit of detection (LOD). Intra- and inter-assay differences in Ct-values among the tested replicates were statistically analyzed using R software. The normality of continuous values was assessed using the Shapiro-Wilk test. Nonparametric distributions were compared using the Kruskal-Wallis test. All tests were two-tailed, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Analytical sensitivity and specificity of the real-time PCRs

The comparative analysis of the limit of detection showed that the qPCR exhibited high sensitivity to detect parasite at lower levels (higher dilutions). The sensitivity assessment for each *Plasmodium* species revealed values ranging from 30 to 0.0003 copies/μL. Additionally, to evaluate the assay's capacity to simultaneously amplify distinct DNA

targets, the results demonstrated a value of 0.3 copies/μL for each species in the *P. falciparum/P. vivax* mix and 3 copies/μL for each species in the *P. malariae/P. knowlesi* mix. Regarding the sensitivity assessment for each *Babesia* species, the results revealed a sensitivity value of 0.2 copies/μL Detailed information regarding the copies/μL corresponding to each dilution evaluated in this study is provided Tables 2 and 3.

The resulting Ct values of each calibration curve were plotted by means of a linear regression to determine the LOD, linearity and dynamic range of the test. Serial dilution studies demonstrated that the qPCR cycle threshold correlates linearly with copies/μL.

For *P. falciparum, P. vivax* and *P. falciparum/P. vivax* mix the R^2 values ranged from 0.980 to 0.997. Similarly, for *P. malariae, P. knowlesi* and the *P. malariae/P. knowlesi* mix the R^2 values ranged from 0.978 to 0.997. Due to the low detection limit of the positive control for *P. knowlesi*, it was not feasible to graph the linear regression analysis. Lastly, for *P. ovale*, the R^2 value was 0.999 (Fig. 3 and Supplemental Fig. S2). For *B. canis, bovis* and *microti,* the R^2 value were 0.996, 0.996 and 0.976, respectively (Fig. 4). In addition, there were no statistical differences across the triplicates suggesting a high reproducibility of the test and the calibration curves. Finally, we also tested two or three dilutions below the LOD and did not obtain amplification.

Following this, we conducted an evaluation of the specificity of the primers and probes employed in our study. The findings indicated a high level of conservation in the annealing regions of the primers across various *Plasmodium* and *Babesia* species, with the probe exhibiting the utmost specificity (refer to Figs. 1 and 2). Moreover, no amplification was observed for any other DNA samples tested and shown in Table 4.

3.2. Diagnostic performance and reproducibility

To assess the diagnostic performance of the laboratory-developed assay, we compared the qPCR results with microscopy, serving as the reference method. The findings revealed to *plasmodium* a sensitivity of 100 % (95 % confidence interval [CI] 88.4 - 100) in detecting *P. ovale, P. malariae* and *P. knowlesi.* Additionally, the qPCR successfully detected *P. falciparum* in all 42 microscopically positive samples. However, one discrepancy was observed, wherein a sample initially diagnosed as *P. falciparum* by microscopy was instead identified as *P. vivax* by qPCR. As a result, the calculated sensitivity for *P. falciparum* in the qPCR assay was 97.7 % (95 % confidence interval [CI] 87.7 - 99.9) and for *P. vivax* of 100 % (95 % confidence interval [CI] 29.2- 100) (Table 5). Likewise, we observed a positive predictive value (PPV) of 100 % (95 % confidence interval [CI] 88.4 – 100) for *P. ovale, P. malariae*, and *P. knowlesi* with a corresponding negative predictive value (NPV) of 100 % (95 % confidence interval [CI] 47.8 – 100; [CI] 88.8 – 100 and [CI] 88.4 – 100) for these species. For *P. falciparum,* the PPV and NPV were 100 % (95 % confidence interval [CI] 91.6 – 100) and 96.7 % (95 % confidence interval [CI] 82.8 – 99.9), respectively. Similarly, for *P. vivax* the PPV and NPV were 75 % (95 % confidence interval [CI] 19.4 – 99.4), and 100 % (95 % confidence interval [CI] 94.7 – 100), respectively (refer to Table 5).

When comparing the results obtained by microscopy with qPCR for *Babesia*, we found a sensitivity of 100 % (95 % confidence interval [CI] 47.8–100) for *B. bovis* and *B. canis*, with corresponding positive predictive values (PPV) and negative predictive values (NPV) of 100 % (95 % CI: 47.8 – 100) and 100 % (95 % CI: 89.1 – 100), respectively. Regarding *B. microti*, the sensitivity was 12.5 % (95 % CI: 3.5 – 29.0), the PPV was 100 % (95 % CI: 39.8 −100), and the NPV was 15.2 % (95 % CI: 5.1 −31.9) (refer to Table 5). However, when comparing the four samples identifying *Babesia* species by PCR with qPCR, the results were in complete agreement.

Finally, upon analyzing the parasitic load in the 46 malaria-positive samples and 32 *Babesia*-positive samples, the findings revealed a median parasite load of 3×10^4 parasites/uL for *P. falciparum* and *P. vivax*. Additionally, the parasitic load for *B. microti* was found to be 2×10^5 parasites/uL (Fig. 5).

According to the reproducibility, no statistically significant differences in Ct-values were observed across runs and replicates. All specimens that tested negative for *Plasmodium* and *Babesia* by the reference method also yielded negative results on the laboratory-developed assay. Additionally, the intra- and inter-run experiments did not reveal statistically significant differences, underscoring the high level of reproducibility of the assay (Supplemental Tables S3–14).

4. Discussion

For decades, the accurate and sensitive detection of malaria and *Babesia* parasites stands as a pivotal factor in planning, targeting, and evaluating malaria and babesiosis diagnostic-based control efforts. Despite the longstanding status of microscopic examination of blood

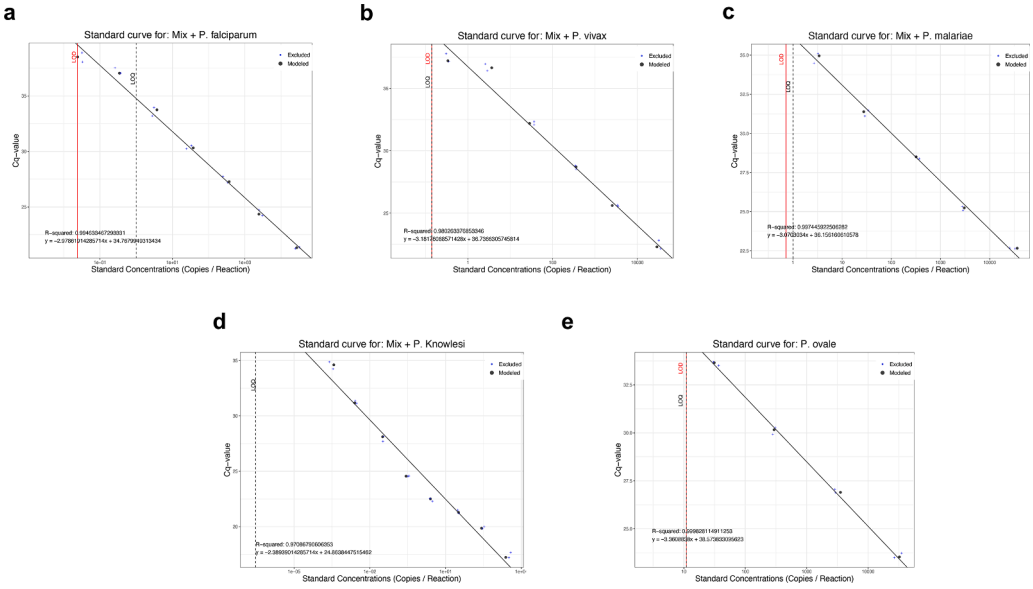

Fig. 3. Linear regression and determination of the limit of detection (LoD) for *Plasmodium* species. **a.** Positive control of *P. falciparum* **b.** Positive control of *P. vivax* **c.** Positive control of *P. malariae*, **d.** Positive control of *P. knowlesi.* and **e.** Positive control of *P. ovale.* All samples were amplified in triplicate for each species with the linear correlation coefficient (R2).

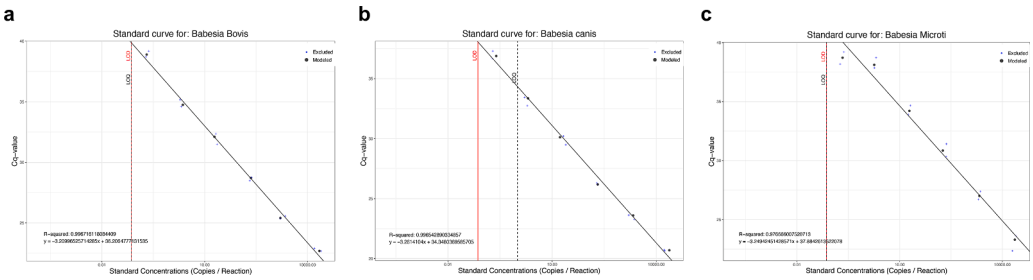

Fig. 4. Linear regression and determination of the limit of detection (LoD) for *Babesia* species. **a.** Positive control of *B. bovis* **b.** Positive control of *B. canis* and **c.** Positive control of *B. microti.* All samples were amplified in triplicate for each species with the linear correlation coefficient (R2).

Table 5
Sensitivity and specificity of the technique for all species analyzed. PPV positive predictive value, NPV negative predictive value.

	Sensitivity% (95 % CI)	Specificity% (95 % CI)	PPV% (95 % CI)	NPV% (95 % CI)
P. falciparum	97.7 (87.7 – 99.9)	100 (88.1–100)	100 (91.6 – 100)	96.7 (82.8 – 99.9)
P. vivax	100 (29.2–100)	98.6 (92.2 – 100)	75.0 (19.4 – 99.4)	100 (94.7 – 100)
P. knowlesi	100 (88.4 – 100)	100 (47.8–100)	100 (88.4 – 100)	100 (47.8 – 100)
P. malariae	100 (88.4 – 100)	100 (88.8–100)	100 (88.4 – 100)	100 (88.8 – 100)
P. ovale	100 (88.4 – 100)	100 (88.4–100)	100 (88.4 – 100)	100 (88.4 – 100)
B. bovis	100 (47.8 – 100)	100 (89.1–100)	100 (47.8 – 100)	100 (89.1–100)
B. canis	100 (47.8 – 100)	100 (89.1–100)	100 (47.8 – 100)	100 (89.1–100)
B. microti	12.5 (3.5–29.0)	100 (47.8 – 100)	100 (39.8–100)	15.2 (5.1–31.9)

smears as the gold standard for diagnosis, it often lacks the desired sensitivity and specificity. Consequently, various molecular diagnostic methods, including nucleic acid amplification tests (NAAT), have been developed. Among these, qPCR which has emerged as a technology demonstrating superior sensitivity compared to traditional PCR methods (Wang et al., 2015; Kamaliddin et al., 2021). Given *(i)* the overlapping symptoms between babesiosis and malaria, *(ii)* the morphological resemblance between *P. falciparum* to *Babesia* species (Gray et al., 2010; Wang et al., 2015), which heighten the risk of misdiagnosing patients, potentially leading to mistaking malaria for piroplasmosis or vice versa (Ayeh-Kumi et al., 2022) *(iii)* the potential of transmission of those pathogens through blood transfusion (Drews et al., 2023; Eskandarian, 2022) and finally *(iv)* the concern about that climate change potentially expanding the geographic parasite range of occurrence (Drews et al., 2023; Gray and Ogden, 2021; Hundessa et al., 2018) a precise diagnostic method is urgently needed, especially in regions where these pathogens are endemic. In this study, we employed a robust, sensitive, and rapid real-time PCR approach to detect the five human clinically relevant *Plasmodium* species and three *Babesia* species from blood samples. Our focus was on assessing the sensitivity, specificity, and overall analytical performance of the assay.

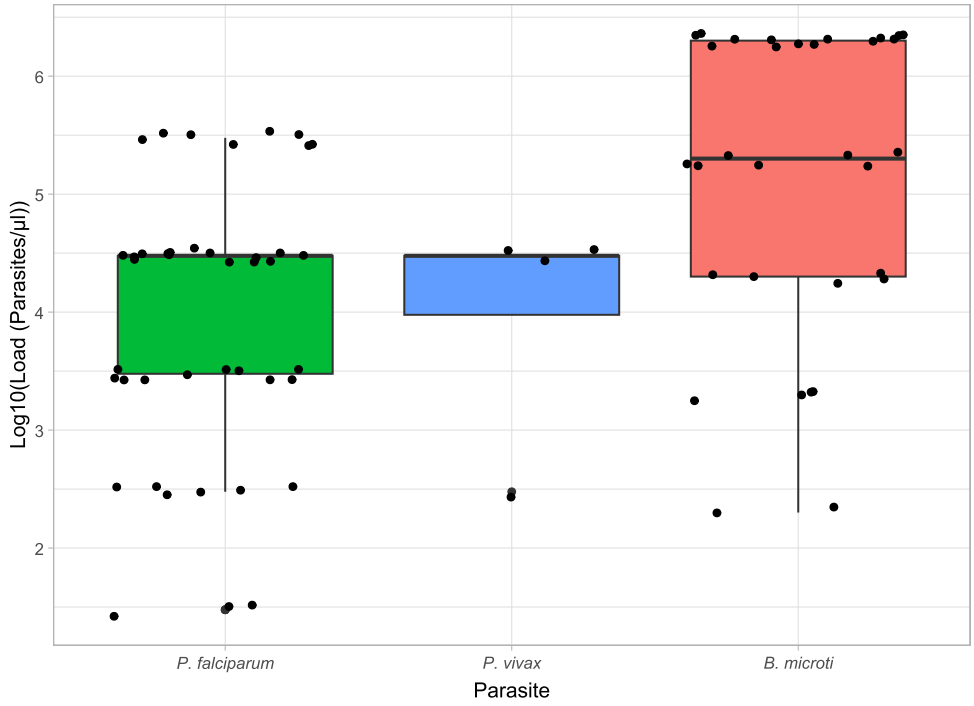

Fig. 5. *Plasmodium* and *Babesia* parasites load expressed as the Log10 of the number of parasite/uL). The horizontal black lines denote the median parasite load, while the vertical dotted lines represent the interquartile range. Each black dot signifies the parasite load of an individual sample analyzed.

5. Analytical sensitivity in *Plasmodium* species

We present evidence showcasing the high analytical sensitivity of this assay, which spans from 0.0003 to 30 copies/μL in monoplex-PCR and 0.3 to 3 copies/μL in the two multiplex-PCR configurations assessed to *Plasmodium* species (refer to Table 2). In addition, a noteworthy correlation between Ct value and the number of copies/μL was consistently observed (Fig. 3). These findings align closely with the parameters of most malaria qPCR assays, which target the multicopy 18S ribosomal RNA (rRNA) genes, exhibiting detection limits ranging from 0.002 to 30 parasites/μL (Rougemont et al., 2004; Divis et al., 2010; Kamau et al., 2011; Das et al., 2023). These indicate that multiplex real-time PCR allows for the detection of single-species infections in monoplex mode or different species when utilized in multiplex mode (via two separate reactions). This capability is particularly crucial in malaria diagnosis, considering two significant aspects. Firstly, the occurrence of co-infections events, especially prevalent in regions where two or more *Plasmodium* species are concurrently circulating (Leonard et al., 2021; Argaw et al., 2016) and secondly, the ongoing challenge of detecting mixed infections using traditional gold standard methods (Leonard et al., 2021; Ayalew et al., 2014).

Likewise, we emphasize the remarkable low limit of detection observed in genomic DNA from *P. knowlesi* positive control (0.0003 copies/ μl) (Fig. 3), which contrast with the threshold reported by Das et al., who documented a LOD of 0.00275 ng/μl [76] or as described by Divis et al., where the analytical sensitivity of this species was 10 copies/μL. These findings underscore the remarkable sensitivity of the qPCR method evaluated in this study for detecting *P. knowlesi*. This sensitivity is especially pertinent given the numerous instances of natural human infection by this species in Southeast Asia (Lempang et al., 2022), as well as infections in travelers returning from these regions (Kantele et al., 2008). Likewise, the underdiagnoses or misdiagnosis using microscopy due to the morphological similarities between *P. knowlesi* and other *Plasmodium* species, especially in endemic regions (Lee et al., 2009). While this assay successfully detects *P. knowlesi* spiked into whole blood, its current limitation lies in assessing its performance on clinical specimens within this study. Therefore, future investigations are warranted to evaluate the test's full potential on samples from patients infected with *P. knowlesi*.

6. Analytical sensitivity in *Babesia* species

The analytical sensitivity for *Babesia* species was found to be 0.2 copies/μL (Table 3), with a consistent correlation observed between Ct value and the number of copies/μL (Fig. 4). These findings contrast with previously reported observations in *Babesia microti, B. canis*, and *B. bovis*. In those instances, the detection limits in whole human blood spiked with plasmid copies varied, with *B. microti* ranging from 20 to 0.36 copies/μL, *B. canis* showing values of twenty copies per microliter (Kuo et al., 2020) and *B. bovis* exhibiting five copies/μL (Zhang et al., 2016). The low analytical sensitivity of this study to detect mainly *B. microti* (0.2 copies/μL = < one parasite/μL, considering the genome's two copies of the 18S rRNA gene (Cornillot et al., 2012)) is notably significant, considering (i) Current assays fail to detect the parasitic load in *Ixodes scapularis* nymphs, the vector, by current assays (Rollend et al., 2013), which is crucial for early diagnosis.(ii) *B. microti* is considered the major cause of human babesiosis, leading to complications such as acute respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC), and liver or renal failure (Fida et al., 2019) (iii) its incidence and global geographical range have increased over the past two decades (Piao et al., 2022) and (iv) it is commonly found in rodents (Guven et al., 2022), mammals that have continuous contact with other mammals, including humans, thereby increasing the transmission range. Hence, further optimization and/or validation of a method enabling the identification on low parasitic loads scenarios remains a pending issue, aiming to enhance the diagnosis of *B. microti*.

7. Specificity in *Plasmodium* and *Babesia* samples

On the other hand, we demonstrate that this assay exhibits high specificity in identifying *Plasmodium* and *Babesia* species. When tested with a panel of bacteria, viruses, fungi, and parasites that could potentially cross-react or present differential diagnoses with any of these species (refer to Table 4), the assay yielded negative results. Furthermore, the primers and probes utilized in this assay demonstrate no annealing with other organisms and show no mismatches thus far, reducing the potential for false negatives.

8. Diagnostic performance in *Plasmodium* samples

To assess the diagnostic performance, the results underscore the superior sensitivity of qPCR for malaria diagnosis in comparison to microscopy. Notably, qPCR demonstrated a high sensitivity of 97.7 %, surpassing microscopy, which typically ranges from 90.4 % to 94 % (Badiane et al., 2015; Manor et al., 2022). Similarly, the PPV for *P. falciparum* was 100 % (95 % confidence interval [CI] 91.6–100) and the NPV was 96.7 % (95 % confidence interval [CI] 82.8 – 99.9). For *P. vivax* the PPV was 75 % (95 % confidence interval [CI] 19.4–99.4) and the NPV was 100 % (95 % confidence interval [CI] 94.7 – 100) (refer to Table 5), which could be attributed to inherent variations in diagnostic tools. It is crucial to acknowledge that the accuracy of microscopic examination heavily depends on the expertise and experience of the microscopist, a factor distinct from the Real-Time PCR method. Some studies indicate that up to 67 % of all *P. vivax* infections are sub-microscopic, potentially eluding detection by light microscopy (Cheng et al., 2015).

9. Diagnostic performance in *Babesia* samples

In terms of the diagnostic performance concerning *Babesia*, we conducted testing for *Babesia spp*. in 32 patient blood samples using the qPCR evaluated in this assay. We then compared these results with those obtained from microscopic examination of Giemsa-stained blood smears, or PCR. In our study, we found a perfect agreement (100 %) between the qPCR assay evaluated and microscopic examination, in detecting both *Babesia spp*.-positive and negative samples, however due to the limitations of microscopic examination in accurately identifying the infecting *Babesia* species beyond the genus level using morphological criteria (Wang et al., 2015), the sensitivity observed in this study was low (12,5 %); which confirm that although the microscopy examination is the easiest and most accessible diagnostic test, it is not very effective for detecting *Babesia* species. When comparing the results from previous PCR assays with our qPCR findings, we observed complete agreement (100 %). Four samples previously identified as *B. microti* were confirmed with the qPCR analysis conducted in this study. These findings align with previous research showing that PCR/qPCR techniques are not only more sensitive than microscopic examination, particularly in detecting *Babesia* infections especially when parasitemia is low (Wang et al., 2015; Akoolo et al., 2017; Teal et al., 2012), but also are more reliable in identifying *Babesia* species (Vannier et al., 2015; Happi et al., 2018). Identifying *Babesia* species is crucial not only for accurately treating infections but also for understanding the epidemiology of the disease and designing effective control and prevention strategies. This includes implementing vector control measures and monitoring disease transmission in both human and animal populations.

Out of the over one hundred known *Babesia* species, five (*B. microti, B. duncani, B. divergens, B. bovis,* and *B. venatorum*) have been linked to human infections (Vannier et al., 2015; Vannier and Krause, 2009). While species such as *B. divergens, B. venatorum, B. crassa-like*, and *B. duncani* have been identified as infecting humans in the United States, Europe, and China, (Drews et al., 2023), *B. microti* remains the primary species of epidemiological and clinical relevance. This is due not only to its widespread global distribution, mainly in United States (Primus et al.,

2018) but also because the infections can result in severe disease, leading to multi-organ failures and fatality rates of up to 20 % in vulnerable populations such as the elderly, immunocompromised individuals, and those without a spleen (Karshima et al., 2022). Considering the significantly higher fatality rate of transfusion-transmitted *Babesia* compared to cases acquired through tick bites, and given that most blood donor studies originate from the northeastern United States where *B. microti* is endemic (Drews et al., 2023), the use of a fast, sensitive, accurate, and reliable method like qPCR becomes a crucial clinical need.

Overall, our study suggests that our qPCR assay presents several advantages over microscopy in detecting *Plasmodium* and *Babesia* infections. Firstly, in terms of sensitivity, our qPCR assay demonstrates a superior ability to detect even low levels of parasite DNA in samples compared to microscopy. This includes parasites such as *P. falciparum, P. vivax*, and *B. microti*, with geometric mean values of detection (parasites/μL) of 3×10^4 (*P. falciparum* and *P. vivax)* and 2×10^5 (*B. microti*) (as shown in Fig. 5), enhancing its effectiveness in diagnosing infections, especially in cases of low parasitemia (Fink and Jones, 2021; Lo et al., 2015). This heightened sensitivity reduces the risk of false negatives, potentially aiding in preventing the spread of transfusion-transmitted parasites and minimizing hidden reservoirs of transmission. Additionally, it ensures more accurate diagnosis and timely treatment initiation, further reducing the risk of transmission. Secondly, our qPCR assay exhibits greater reliability in identifying *Plasmodium* and *Babesia* species. By targeting specific genetic markers, our assay can differentiate between various species with precision, minimizing the likelihood of misidentification or confusion with other pathogens. This specificity is crucial for guiding appropriate treatment strategies, as different *Plasmodium* and *Babesia* species may respond differently to antiparasitic medications. Finally, the qPCR assay offers enhanced speed compared to microscopy. The streamlined qPCR process enables quick detection and quantification of *Plasmodium* and *Babesia* DNA in samples, greatly cutting down on turnaround time for diagnostic results. This rapid turnaround is crucial for promptly informing clinical management decisions and implementing essential control and monitoring measures to prevent disease transmission of malaria and babesiosis in endemic and non-endemic areas alike.

However, this study exhibits several limitations: (i) the restricted availability of positive clinical samples for *P. vivax* and the absence of positive samples for *P. malariae, P. knowlesi, P. ovale, B. bovis* and *B. canis* (ii) the limited geographic diversity of the evaluated samples, and (iii) the lack of information regarding the parasitemia levels of the analyzed samples. Hence, future research endeavors with broader sampling scopes are warranted to deepen our comprehension of the subject matter.

10. Conclusions

In conclusion, our study highlights the effectiveness of a real-time PCR assay in accurately detecting *Plasmodium* and *Babesia* species, offering superior sensitivity and specificity compared to traditional microscopy. Furthermore, the assay's approval by the NYS Department of Health for clinical use as a Laboratory Developed Test underscores its significance as a regional diagnostic contributor within the global disease diagnostic landscape.

Supplemental Fig. S1. Sequences of the positive controls of *Plasmodium* and *Babesia* used in the study.

CRediT authorship contribution statement

Luz Helena Patiño: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sergio Castañeda:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Milena Camargo:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Li Yong Cao:** Writing – review & editing, Resources, Methodology, Investigation. **Bernadette Liggayu:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis. **Alberto Paniz-Mondolfi:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Juan David Ramírez:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Not applicable.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2024.107350.

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