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A novel time-saving multiplex PCR assay for detecting and discriminating the most common canine *Babesia* species in Europe

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ABSTRACT

In Europe, most cases of canine babesiosis are caused by *Babesia canis, Babesia vogeli* (large piroplasms) and *Babesia vulpes* (small piroplasm). Molecular diagnosis is recommended due to its high sensitivity. Species identification after sequencing allows applying a rapid and efficient treatment, leading to a better prognosis; however, it is expensive and time-consuming. Thus, the objective of the present study was to develop a time-saving multiplex polymerase chain reaction (PCR) for simultaneously detecting and discriminating between large and small forms without sequence analysis. A new multiplex PCR was designed and tested using blood samples from 79 dogs showing clinical signs compatible with babesiosis which were previously analysed using blood smears and molecular methods. Multiplex PCR successfully discriminated between both *Babesia* groups showing bands of 700 and 890 bp for *B. canis/B. vogeli* and *B. vulpes*, respectively. No significant differences in the results of both PCR were detected and a substantial agreement between protocols ($\kappa = 0.64$) was found. Our multiplex PCR represents a reliable tool for detecting infections by the major *Babesia* spp. in dogs from Europe. Since no sequence analysis is required for identifying the species involved, this PCR allows the rapid administration of an appropriate treatment, thus improving the survival rate of the infected animals. In addition, it will represent a helpful tool for unravelling the real prevalence and distribution of *B. vulpes* and its implication in clinical cases.

1. Introduction

Canine babesiosis is a tick-borne disease with worldwide distribution caused by several protozoa of the genus *Babesia.* Three major *Babesia* spp. have been detected in dogs from Western Europe: *Babesia canis*, *Babesia vogeli* and *Babesia vulpes* (syn. *Babesia microti*-like and *Theileria annae*); other species such as *Babesia gibsoni* have been sporadically detected in this area (Solano-Gallego et al., 2016). These species show a different geographic range determined by the distribution of their main tick vector species. Thus, *B. canis*, the most distributed *Babesia* spp. in Europe, is mainly transmitted by *Dermacentor reticulatus* which is present along the continent, being more abundant in central Europe (Estrada-Peña et al., 2017). In contrast, *Rhipicephalus sanguineus* s.l., abundant in tropical and subtropical regions (Estrada-Peña et al., 2017), is considered the main vector of *B. vogeli* and probably of *B. gibsoni* in this continent (Solano-Gallego et al., 2016). Although *B. vulpes* has been associated to endophilic species of *Ixodes* such as *Ixodes hexagonus* and *Ixodes canisuga*, their vector competence has not been completely proven (Solano-Gallego et al., 2016). Its geographical range has not being completely unravelled, although this species has been detected infecting dogs from a number of European regions (Dezdek et al., 2010; Cardoso et al., 2013; Duscher et al., 2014; Farkas et al., 2015; Gabrielli et al., 2015; Hodžić et al., 2015; Rene-Martellet et al., 2015). This could be associated to the patched distribution of their associated vectors which have a burrow-dwelling cycle being common in areas with a high population of red foxes (*Vulpes vulpes*) and hedgehogs (*Erinaceus europaeus*) (Estrada-Peña et al., 2017).

In Spain, *B. canis* and *B. vogeli* are the most widely distributed *Babesia* spp. affecting dogs, being the former more common in northern areas and the latter in southern areas (Solano-Gallego et al., 2016). In addition, *B. vulpes* is endemic in northwestern Spain (Miró et al., 2015), being detected in more than half of the dogs with clinical babesiosis from that area (Miró et al., 2015). In contrast, reports of *B. gibsoni* are very sporadic in this country (Criado-Fornelio et al., 2003).

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The four canine *Babesia* species present in Europe were classified into two groups according to the diameter of the merozoites in the red blood cells (Laha et al., 2015); thus, *B. canis* and *B. vogeli* are considered large piroplasms (2.5–5 μm) and *B. vulpes* and *B. gibsoni* small piroplasms (< 2.5 μm). Canine babesiosis is a life-threatening disease and its clinical manifestation is mostly acute; however, it has been reported that adult dogs typically survive with an appropriate therapy (Taboada and Lobetti, 2006). The identification of the species involved in canine babesiosis has a special clinical interest since the treatment and prognosis depends on the infecting species. In this regard, imidocarb dipropionate is the treatment of choice for *B. canis* and *B. vogeli* infections (Vial and Gorenflot, 2006) whereas, the combination of atovaquone and azithromycin is the only effective treatment against *B. vulpes* and *B. gibsoni* infections (Solano-Gallego et al., 2016). In addition, large *Babesia* species are usually considered less virulent than small ones, leading to a better prognosis (Solano-Gallego et al., 2016).

The diagnosis of babesiosis in veterinary clinics is mainly based on both the clinical signs and the observation of intra-erythrocytic parasites in a stained thin blood smear. However, the sensitivity of this technique is poor, especially in animals with low parasitemia (Solano-Gallego et al., 2016) and an experienced eye is needed for detecting small *Babesia* spp. (Onchan et al., 2022). In this respect, molecular methods show a higher sensitivity and thus are strongly recommended for diagnosis of canine babesiosis (Solano-Gallego et al., 2016). Although generic *Babesia* polymerase chain reaction (PCR) protocols can be used, species identification is only achieved after sequence analysis (Solano-Gallego et al., 2016), which is expensive and time-consuming. In addition, *Babesia* spp. can be identified without sequencing using specific PCR protocols, but several PCR assays should be performed in this case, entailing higher economic costs. In this way, molecular methods allowing a reliable and rapid identification of the species involved are needed for establishing an appropriate treatment as soon as possible (Solano-Gallego et al., 2016).

For all these reasons, the objective of the present study was to develop a time-saving multiplex PCR for simultaneously detect and distinguish *B. canis* and *B. vogeli* from *B. vulpes* without subsequent sequencing.

2. Materials and methods

2.1. Study area and animal inclusion

A total of 79 dogs from the province of Lugo (North-western Spain) were included in this study. All animals had clinical signs or laboratory abnormalities compatible with canine babesiosis such as pale mucous membranes, apathy, anorexia, anaemia and/or hematuria. In order to achieve a diagnosis, veterinarians collected individual blood samples via cephalic venipuncture using a sterile syringe. An aliquot of blood was later transferred to tubes with EDTA (Vacuette K2E K2EDTA tube, Greiner Bio-One GmbH, Kremsmünster, Austria) and sent to the Laboratory of the INVESAGA Group within 12 h from collection.

2.2. Babesia spp. detection and molecular identification

Each blood sample was firstly analysed using microscopic (thin blood smears) and molecular (single PCR) techniques for detecting the presence of *Babesia* spp. within 24 h after reception.

Individual thin blood smears were performed, air-dried, fixed and stained with a commercial kit (Quick Panoptic, QCA S.A., Amposta, Spain). Intra-erythrocytic protozoa were microscopically detected at 1000 × magnification and measured using an EP50 digital camera and the software EPview (Olympus, Allentown, Pennsylvania, USA).

For molecular analysis, DNA was firstly extracted from 200 μl of blood using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH®, Mannheim, Germany) following the manufacturer' instructions. DNA samples were stored at −20 °C until analysed. All samples were tested using a single PCR for amplifying a ≈ 500-bp fragment of the 18S rRNA gene of genera *Babesia* and *Theileria,* using previously reported primers and protocols (Soares et al., 2011). DNA of *B. canis* obtained from an infected dog, and nuclease free water were included as positive and negative controls, respectively.

Since the size of the obtained amplicon was similar regardless the piroplasm species, sequence analysis was needed for identifying the species present. Positive samples were purified and sequenced in both senses on an ABI 3730xl (Applied Biosystems, Foster City, California, USA) using a Big dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA) at the Sequencing and Fragment Analysis Unit of the Santiago de Compostela University (Lugo, Spain). Sequences were aligned and edited using ChromasPro (Technelysium, Brisbane, Australia), and consensus sequences were scanned against GenBank database using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Multiplex PCR primer design

Partial nucleotide sequences encoding for the 18S rRNA of *B. canis, B. vogeli* and *B. vulpes* (Supplementary table 1) were used for designing the primers; these sequences were firstly aligned using the online tool GenomeNet Multiple Sequence Alignment by CLUSTALW (https://www.genome.jp/tools-bin/clustalw). Specific primer sets were designed using the online software Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?GROUP_TARGET=on). Thus, three novel primers (BvcF, BvR and BcR) were designed (Table 1). The quality of the obtained primers was checked using the online tool Multiple Primer Analyzer (ThermoFisher Scientific) (https://www.thermofisher.com/es/es/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html), results were summarized in Table 1.

The amplification mixture for multiplex PCR contained 3.5 mM of $MgCl_2$, 200 mM of dNTPs, 1.2 μM of the forward primer and 0.6 μM of each reverse primer and 0.5 units of NZYTaq II DNA polymerase (NZYTech, Lisboa, Portugal) in a final volume of 25 μl. Amplification was carried out in a T100 Thermal Cycler Bio-Rad (Hercules, California, USA). The cycling conditions were set as follows: denaturing at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 1 min, and a final elongation for 7 min at 72 °C. Since *B. vogeli* has never been detected in the studied area, a DNA sample from an infected dog from Central Spain -kindly provided by Exopol (Zaragoza, Spain)- was included in one of the assays to test the designed primers. An amplicon size of 700 bp and 890 bp for large and small *Babesia* spp., respectively, was expected. For confirming species identification, a subset of PCR positive samples was further purified and sequenced as indicated above.

2.4. Statistical analysis

The degree of agreement between both PCR assays and between each PCR and the results obtained through observation of the blood smears was determined through McNemar's test. In addition, a Cohen's kappa coefficient (κ) was carried out to measure the inter-rater reliability between both PCR assays (Altman, 1991). Statistical analyses were performed using the statistical software R 4.2.1 (R Core Team, 2022). The level of significance was set at P-values <0.05.

3. Results and discussion

A total of 50 (63.29%) dogs were positive to *Babesia* spp. in at least one of the two PCR protocols. The single PCR assay described by Soares et al. (2011) allowed the detection of 45 positive animals (57.0%), while 41 animals (51.9%) yielded positive results with the multiplex PCR (Table 2). Merozoites were observed in the blood smear of 33 (41.8%) dogs of which 32 and 29 were detected by single and multiplex PCR

Table 1
Primers used for detection and identification of *Babesia canis / Babesia vogeli (BcR)* and *Babesia vulpes (BvR)*.

Gene target	Name	Primer sequence 5′- 3′	Fragment size	nt*	Tm °C	CG%	Self-dimers	Cross primer dimers
18S RNA	BvcF	GTCTTGTAATTGGAATGATGG	–	21	58.9 °C	38.1	None	None
	BcR	AATCCTACCGTTTGTCTGGA	700 bp	21	60.1 °C	38.1	None	None
	BvR	ACTTCCTTGCGTTTTATGAAC	890 bp	20	61.4 °C	45.0	None	None

* Number of nucleotides.

Table 2
Number of samples in which *Babesia* spp. (including *Babesia canis* and *Babesia vulpes*) DNA was detected using blood smears, a single PCR assay (Soares et al., 2011) and the multiplex PCR assay described in this paper.

	Blood smear			PCR single			PCR multiplex		
	Total positive samples	*B. canis* (n)	*B. vulpes* (n)	Total positive samples	*B. canis* (n)	*B. vulpes* (n)	Total positive samples	*B. canis* (n)	*B. vulpes* (n)
Blood smear	–	–	–	32/33 (97.0%) 82.28%* κ = 0.65	19/19 72.41%*	13/14 71.43%*	29/33 (87.9%) 79.75%* κ = 0.60	18/19 65.52%*	11/14 71.43%*
PCR single	32/45 (71.1%) 82.28%* κ = 0.65	18/27 72.41%*	14/18 71.43%*	–	–	–	36/45 (80%) 82.28%* κ = 0.64	25/27 86.21%*	11/18 52.38%*
PCR multiplex	29/41 (70.73%) 79.75%* κ = 0.60	17/27 65.52%*	12/14 71.43%*	36/41 (87.81%) 82.28%* κ = 0.64	25/27 86.21%*	11/14 52.38%*	–	–	–
Total positive samples	33/50 (66%) 78.48%* κ = 0.60	18/29	15/21	45/50 (90%) 93.67%* κ = 0.87	27/29	18/21	41/50 (82%) 88.61%* κ = 0.77	27/29	14/21

Cohen's kappa coefficient (κ).
* Observed agreement.

protocols, respectively. Cohen's kappa coefficient showed a substantial agreement (79.75–82.3%) between the smear and the single (κ = 0.65) and multiplex PCR protocols (κ = 0.60). Eighty percent of the positive samples detected by the conventional PCR assay were detected by the new multiplex PCR; similarly, 87.8% of the samples detected by the multiplex PCR were detected using the conventional PCR (Table 2). The differences in the results of both assays were not significant (McNemar's chi-squared = 0.64, df = 1, p = 0.42). In addition, Cohen's kappa coefficient showed a substantial agreement between both PCR protocols (κ = 0.64). These data indicate that both PCR assays give similar results and the multiplex PCR protocol designed in the present study is a reliable tool for the detection of *Babesia* spp.

Sequence analysis of PCR products obtained with the single PCR allowed identifying two *Babesia* spp. showing a similar occurrence. In this way, *B. canis* and *B. vulpes* were detected in 58% and 42% of the positive samples, respectively. *Babesia canis* samples had a percentage of similarity between 99.7 and 100% with the reference sequences KC593878.1, MG569903.1, MK571834.1 and MK591947.1, whereas all *B. vulpes* sequences were identical to the reference sequence MK591948.1.

Multiplex PCR successfully discriminated between both species showing bands of 700 bp and 890 bp for *B. canis* and *B. vulpes*, respectively (Fig. 1). In those samples positive to both PCR assays, no species identification discrepancies were detected. Species identification was confirmed after sequence analysis of six samples (three showing bands of 700 bp and three showing bands of 890 bp). Thus, those samples identified as *B. canis* were identical to the references sequences MN173223.1 and MG569903.1 and those identified as *B. vulpes* were identical to the reference sequence MT509981.1. These results have importance from an epidemiological point of view since the use of a multiplex PCR assay could be very helpful for unravelling the real prevalence and distribution of *B. vulpes,* allowing identifying areas involving a high risk of infection and increasing the clinical suspicion.

Although *B. canis* and *B. vogeli* are considered the most abundant and

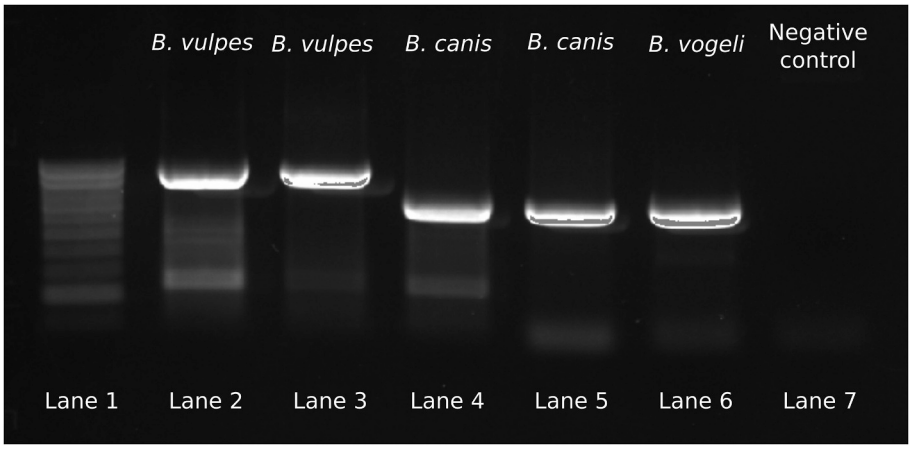

Fig. 1. Gel electroforesis showing multiplex PCR results (primer set BvcF, BcR and BvR). Lane 1, ladder (100 bp); lanes 2–3, *Babesia vulpes* (890 bp); lanes 4–5 and 6, *Babesia canis* and *Babesia vogeli* (700 bp), respectively and lane 7, negative control.

distributed species in dogs from Europe (Solano-Gallego et al., 2016), the involvement of *B. vulpes* in canine babesiosis cases cannot be ruled out since it is difficult to detect through blood smear and thus its prevalence may be underestimated (Solano-Gallego et al., 2016). This pathogen has been detected in dogs from Spain (Miró et al., 2015), Portugal (Simoes et al., 2011), France (Rene-Martellet et al., 2015), Croatia (Beck et al., 2009), Serbia (Gabrielli et al., 2015) and Hungary (Tuska-Szalay et al., 2021) with prevalences ranging from 0.7% to 62.5%. In addition, it was detected in foxes from almost all European countries (Tampieri et al., 2008; Dezdek et al., 2010; Cardoso et al., 2013; Duscher et al., 2014; Farkas et al., 2015; Hodžić et al., 2015). *Babesia vulpes* has also been detected in dogs from Russia (Radyuk and Karan, 2020), Canada (Arsenault et al., 2022) and USA (Barash et al., 2019) and in foxes from these two American countries (Birkenheuer et al., 2010; Clancey et al., 2010) suggesting that it shows a wide distribution.

The discrimination between large (*B. canis* and *B. vogeli*) and small (*B. vulpes*) piroplasms through the same PCR assay and without performing the subsequent sequencing has a special interest from a clinical point of view. As it has been previously indicated, the treatment and prognosis of large and small *Babesia* spp. are different (Vial and Gorenflot, 2006; Solano-Gallego et al., 2016) and the acute manifestation of the disease requires fast and accurate results; consequently, techniques involving DNA sequencing are not the most suitable in these cases. Multiplex PCR is a time-saving and relatively inexpensive solution to these disadvantages.

4. Conclusions

The multiplex PCR assay developed in this study represents a reliable and time-effective tool for detecting the presence of both large and small *Babesia* spp. affecting dogs from Europe. Our protocol has an important application from a clinical point of view, since it allows differentiating infections caused by large (*B. canis/B. vogeli*) and small (*B. vulpes*) *Babesia* spp. using a single PCR assay and without the subsequent sequencing of the PCR products. This data has also importance from an epidemiological point of view since the use of a multiplex PCR assay is useful for determining the distribution and distribution of *B. vulpes,* which is of particular interest for identifying areas involving a high risk of infection and increasing the clinical suspicion.

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Ethics approval and consent to participate

The Ethics Committee of the University of Santiago de Compostela (USC) considers that this investigation does not fall under the legislation for the protection of animals used for scientific purposes, national decree-law RD53/2013 (2010/63/EU Directive) since non-experimental clinical veterinary practices (blood samples were collected for clinical diagnosis) falls into the exceptions referred in Article 2 (5.b) of the mentioned legislation. In consequence, this project was exempted from ethics review and did not require the approval of the USC Ethics Committee.

Declaration of Competing Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.04.001.

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