Multiplex PCR for molecular screening of *Borrelia burgdorferi* sensu lato, *Anaplasma* spp. and *Babesia* spp.

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▌**Abstract**

Introduction. Ticks transmit a great variety of pathogenic microorganisms to humans and animals. The detection of tick-borne pathogens (TBP) is mainly by molecular techniques based on polymerase chain reactions (PCR).
Objective. To design and evaluate a multiplex PCR for the molecular screening of zoonotic TBP for exploratory studies.
Material and methods. Control DNA from reference strains, DNA from experimentally-infected biological specimens, and from *Rhipicephalus sanguineus* ticks collected from domestic and homeless dogs were used. A multiplex PCR assay to detect the presence of *Borrelia burgdorferi* sensu lato, *Anaplasma* spp. and *Babesia* spp. was designed and optimized using primers previously reported for *B. burgdorferi* sensu lato and *Anaplasma* spp., while for *Babesia* spp. they were designed *in silico*. The multiplex PCR was evaluated on the DNA from biological samples.
Results. A new set of specific primers for *Babesia* spp. was designed. Adjustment of the master mix reactive concentrations and amplification conditions for the multiplex PCR allowed the successful amplification of the specific amplicons for each microbial group from the control DNA and experimentally-infected biological specimens. The efficiency of the multiplex PCR amplifying three DNA targets was confirmed. Individual and co-infection of *Anaplasma* spp. and *Babesia* spp. were detected in the *R. sanguineus* ticks from dogs.
Conclusions. A multiplex PCR assay for the screening of three TBP is available. By using it, *B. burgdorferi* sensu lato, *Anaplasma* spp. and *Babesia* spp. can be detected accurately in one PCR reaction.

▌**Key words**

multiplex PCR, *Borrelia burgdorferi* sensu lato, *Anaplasma* spp., *Babesia* spp., tick-borne pathogen

INTRODUCTION

Ticks transmit a greater variety of pathogenic microorganisms than any other arthropod vector group [1]. Ticks and tick-borne pathogens (TBP) affect animal and human health worldwide, and they are on the increase. The increased risks associated with them are supported by expansion of tick populations into areas where they were previously absent, and by some changes in social habits, increasing the contact between humans and ticks [2]. Ticks cause important economic losses to the livestock industry, mainly affecting tropical and subtropical countries, where ticks constitute one of the main difficulties for the development of the livestock breeding industry [3, 4].

Lyme borreliosis, caused by *Borrelia burgdorferi* sensu lato, is prevalent throughout the northern hemisphere, and the same *Ixodes* tick species transmitting the etiologic agents of this disease also serve as vectors of *Babesia* spp. and *Anaplasma* spp. causing human and animal babesiosis and anaplasmosis, respectively. These pathogens can co-infect one organism simultaneously which makes diagnosis and treatment difficult [5, 6]. The risk of infection with these TBP varies widely across different regions; therefore, knowledge of the local tick infection rates is important for prevention and treatment decisions [7].

New molecular technologies have greatly advanced the knowledge of ticks and TBPs, and developed or refined diagnostic tools have increased the efficacy and accuracy of identifying TBPs [2]. Polymerase Chain Reaction (PCR) combined with Reverse Line Blot (RLB) hybridization is a robust technique, wherein up to 40 different tick-borne pathogens can be detected simultaneously [8, 9]. However, this technique may be expansive and therefore is not always useful in poor-resource countries. As an alternative, multiplex PCR assays or a combination of singleplex PCR have been designed and used for the detection of specific species of TBPs in ticks and clinical samples (i.e. *B. burgdorferi/Anaplasma phagocytophilum*, *A. phagocytophilum/Babesia* spp., *B. burgdorferi/A. phagocytophilum/B. microti*) [10, 11].

In spite of the aforementioned, in some countries the prevalence or incidence of the TBPs is unknown, and require the initial screening of these groups from biological and clinical samples allowing the ulterior identification at species level. Therefore, the presented study aimed to design and evaluate a multiplex PCR for the molecular screening of zoonotic TBPs for exploratory studies. The application of this assay will make a significant difference in achieving the rapid and accurate detection of *B. burgdorferi* sensu lato, *Anaplasma* spp. and *Babesia* spp. in a cost-effective manner.

MATERIALS AND METHOD

Control DNA. For standardization of conditions for the multiplex PCR DNA from *B. burgdorferi* sensu stricto (B31),

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cultured in BSK-H medium (Sigma, USA) at the Laboratory of Eco-Epidemiology of Parasites in Neuchâtel, Switzerland, DNA from *A. phagocytophilum* (Webster strain) kindly provided by Ana Sofia Santos (Instituto Nacional de Saude, Lisboa, Portugal), and *B. microti* (HK) DNA obtained from Jeremy Gray (Dublin, Ireland), were used. Water for molecular biology was used as negative control.

Biological specimens and DNA extraction. DNAs from different artificially infected biological and clinical samples were used. DNA from bovine blood infected with a Swiss *A. phagocytophilum* strain (kindly provided by Jorge Liz) [12] was extracted with NucliSens easyMAG automated extraction platforms (bioMérieux, Switzerland). DNA of four free-living *I. ricinus* nymph ticks collected in Switzerland and artificially infected with *B. burgdorferi* was obtained using DNeasy Blood and Tissue Kit (Qiagen, Germany). Artificially infected ticks were been obtained using capillaries, as previously described [13]. DNA extracts from blood of ten gerbils (*Meriones unguiculatus*) experimentally-infected (intraperitoneal inoculation) with *B. microti* were obtained with QIAamp DNA Blood mini-kit (Qiagen, Germany). All experiments involving gerbils respected the Swiss legislation on animal experimentation and were authorized by the Veterinary Service of the Canton of Neuchâtel (Authorization No. 1/2003).

To evaluate the multiplex PCR, 97 and 55 feeding *Rhipicephalus sanguineus* (nymphs, male and female) were used that had been removed from domestic and stray dogs in different municipalities of Havana, Cuba, respectively. These ticks were conserved in 70% alcohol. Before testing, they were washed and grouped into 55 pools (30 from domestic dogs and 25 from stray dogs) containing 2–4 specimens from the same host.

DNA from tick pools was extracted using ammonium hydroxide (for nymphs, male adults and non-engorged female adults) [14, 15, 16] and potassium acetate procedure (for engorged female adults). For the potassium acetate extraction protocol, briefly, the ticks were frozen with liquid nitrogen and disrupted mechanically using 1.5 mL plastic tubes with a pestle; 500 mL of lysis buffer [0.1 M Tris-HCl (pH 8.25), 0.05 M EDTA, 0.2 M sucrose, 0.5% SDS] was added to each tick lysate. Suspensions were incubated at 65 °C for 10 min. Afterwards, 120 mL of 5M potassium acetate was added to each one and these were incubated in ice for 10 min. They were then centrifuged at 12,000 g for 10 min and supernatants were collected. For the precipitation of nucleic acids, 35 mL of 4M sodium acetate and 0.25% acrylamide mix and 1.0 mL of absolute ethanol were added to each supernatant, which were then incubated for 10 min at -20ºC, followed by centrifugation at 12,000 g for 20 min. The pellets were washed with 500 mL of 70% ethanol and air-dried at room temperature. Finally, the extracts were re-suspended with 25 mL of 1X TE buffer (1 mM Tris-HCl pH 8.0, 1 mM EDTA), and stored at -20ºC until use.

The efficiency of DNA extraction from the ticks was controlled by the amplification of a fragment of the tick mitochondrial 16S rRNA gene using the PCR reported by Halos et al., 2004 [17].

Selection of *B. burgdorferi* sensu lato and *Anaplasma* spp. primers. The primer set described by Postic et al. (1994) (primer 1 [5'-CTGCGAGTTCGCGGGAGA-3'] and primer 2 [5'-TCCTAGGCATTCACCATA-3']) for the detection of *B. burgdorferi* sensu lato [18] was selected given their wide use [19, 20]. It amplifies the variable spacer region between two conserved structures, the 3' end of the 5S rRNA (*rrf*) and the 5' end of the 23S rRNA (*rrl*).

For the detection of *Anaplasma* spp., reported primers targeting fragments of the citrate synthase (*gltA*), heat shock operon (*groESL*), ankyrin repeat-containing proteins (*ankA* and *epank1*), major surface protein 2 (*p44/msp2*) and 16S ribosomal RNA (rRNA) genes [20–24] were evaluated *in silico* using the BLAST (http://blast.ncbi.nlm.gov/Blast.cgi) and OPERON (http://www.operon.com/technical/toolkit.aspx) softwares. A set of primers (B-GA1B-new [5'-CGGGATCCCGAGTTTGCCGGGACTTYTTCT-3'] and 16S8FE [5'-GGAATTCAGAGTTGGATCMTGGYTCAG-3']), unique for members of the *Anaplasma/Ehrlichia* genres that targets the hypervariable V1 region of the 16S rRNA gene, was selected.

Design of *Babesia* spp. primers. Partial sequences of the gene encoding 18S rRNA were obtained from the GenBank (http://www.ncbi.nlm.nih.gov/) in FASTA format, and organized and aligned using Clustal software (http://www.ebi.ac.uk/clustalw/). Conserved sequences around 18–25 bases of length were searched at the beginning and at the end of the alignments in order to generate amplicons with a size superior to that of the other pathogens for a good differentiation of the various amplicons. The specificity of primers and the proposed amplicon were verified by BLAST software. Dimer formation, melting temperatures and GC content were predicted with OPERON. Other parameters, such as 3′ extreme stability and dimer formation among all primers, were also controlled.

Multiplex PCR performance. The singleplex PCR for *B. burgdorferi* sensu lato reported by Postic et al. [18] in a volume of 25 µL was carried out with the following modifications: the extension time was increased by one minute and 5 µL of serial ten-fold dilutions from 1:10–1:1,000 of borrelial reference DNA was used. The same reaction mixture and amplification conditions were used in singleplex PCRs with the specific primer sets for the amplification of *Anaplasma* spp. and *Babesia* spp. at the same serial ten-fold dilutions of respective reference DNA. The PCR amplifications were checked on 1.5% agarose gels stained with gel red and visualized by ultraviolet light.

For the selection of the optimum annealing temperature for the three primer sets, individual PCR with annealing temperature gradients from 51ºC – 61ºC were set in a TProfessional Basic Gradient thermal cycler (Biometra, Germany), keeping the same conditions of reaction mixture and the other parameters of the amplification programme. The criteria selected for the common temperature was that expected more prominent bands without non-specific secondary bands were obtained.

Multiplex PCRs with a combination of the two specific primer sets with reference DNA were run with the aim of detecting non-specific secondary bands.

Finally, a multiplex PCR with the three primer sets was performed. For its optimization, different concentrations of dNTP (Qiagen, Germany) (0.2 mM, 0.3 mM and 0.4 mM), *Taq* DNA polymerase (Qiagen, Germany) (0.03 U/µL and 0.04 U/µL), MgCl$_2$ (Qiagen, Germany) (1.75 mM, 2.0 mM and

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2.5 mM), and primers (Microsynth AG, Balgach, Switzerland) (0.1 µM, 0.2 µM, 0.5 µM and 1.0 µM), were assessed. Clear single bands of expected sizes indicated success, whereas the absence of a band, the presence of multiple bands or the presence of a single band of incorrect size, meant PCR failure. The mixture and amplification conditions providing the best results were kept for further evaluation.

Evaluation of multiplex PCR using different biological specimens. The standardized multiplex PCR was firstly evaluated using each DNA extracted from the specimens artificially infected, and lastly, it was used in a preliminary exploratory study using the DNA extracted from pools of *Rhipicephalus* ticks collected from domestic and stray dogs.

RESULTS

Highly-conserved priming sites from the alignments of *Babesia* spp. sequences were found, which allowed the design of primers Bab1 (5'-TGACACAGGGAGGTAGTGACA-3') and Bab2 (5'-CAGGACATCTAAGGGCATCA-3'). This primer combination targets a 925–995 bp fragment according to the specific species.

The optimum annealing temperature selected for the three primer sets was 53°C. The singleplex and multiplex PCR (with two or three primers sets) reactions were efficient when serial dilutions of the reference DNA were made, and when DNAs from different origins were mixed in the same reaction. No PCR interference/inhibition was caused by the use of multiple primers in the multiplex PCR with the optimal conditions evaluated during the standardization.

The final concentrations of reagents in the amplification reaction mixture are shown in Table 1. They allowed the successful amplification of the specific amplicons for each microbial group (see the controls lanes on Fig. 1).

Table 1. Final concentrations of the reagents in the amplification mixture for the multiplex PCR

Reagent	Final concentration
dNTP	0.4 mM
Taq DNA polymerase	0.03 U/µL
MgCl$_2$	2.0 mM
B. burgdorferi primers	0.1 µM
Anaplasma spp. primers	1.0 µM
Babesia spp. primers	0.1 µM

The results of the evaluation of the standardized multiplex PCR with the biological samples artificially infected are shown on Figure 1. Each specific amplicon with the expected size, according to the pathogen used for the infection, was obtained. Lane 5, interestingly, illustrates the natural co-infection by *Anaplasma* and *Babesia* species in a free-living *I. ricinus* tick that was infected artificially with *B. burgdorferi* in the laboratory. This result confirms the efficiency of the multiplex PCR amplifying three DNA targets into a unique reaction.

No PCR inhibitor was detected in the DNA extracts obtained from the dog ticks. Of the 30 pools from domestic dogs, 7 male and 4 female pools were infected with *Anaplasma* spp. and 1 male and 3 female pools with *Babesia* spp.; while
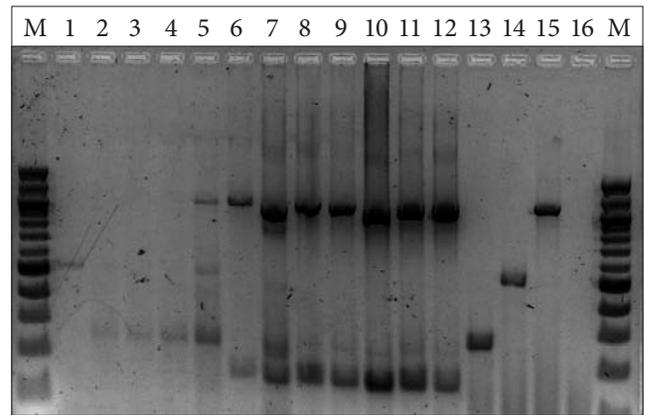

Figure 1. Gel electrophoresis showing PCR results with specimens artificially infected.
Lane M – 100 bp DNA Ladder (BioLabs)
Lane 1 – bovine red blood cells infected with *A. phagocytophilum*
Lanes 2–5 – free-living *I. ricinus* ticks artificially infected with *B. burgdorferi*
Lanes 6–12 – blood of gerbils infected with *B. microti*
Lane 13 – *B. burgdorferi* positive control
Lane 14 – *Anaplasma* positive control
Lane 15 – *Babesia* positive control, lane 16: negative control

of the 25 pools from stray dogs, 1 nymph pool was infected with *Anaplasma* spp. and 1 nymph pool was dually-infected with *Anaplasma* spp. and *Babesia* spp.

DISCUSSION

The present study evaluates a multiplex PCR for the molecular screening of three groups of the most important and frequent TBPs in Europe, Africa, Latin America and Asia [1]. Primers were used which had previously been reported and thoroughly recommended in the literature for the detection of *B. burgdorferi* sensu lato and *Anaplasma* spp., with new designed primers for *Babesia* spp. from a target gene systematically used for PCR reactions, in order to generate amplicons with different sizes for an easier identification of each microbial group. The success of a PCR is particularly contingent upon the primer set used and the target loci, because they will determine the efficiency and accuracy of the pathogen detection. It is important to mention that the *Anaplasma* spp. primers used in this study also allow the detection of *Ehrlichia* spp., given the high degree of similarity in the 16S rRNA genes of these genera [26].

The selection of the annealing temperature is possibly the most critical component for optimizing the specificity of a PCR reaction. High-temperature annealing should result in enhanced specificity, because the hybridization of the primer to the template DNA occurs under more stringent conditions.

To develop and optimize the multiplex assay that can detect the presence of the three TBP groups it was necessary to amplify and detect the amplicons under the same selected PCR parameters. The absence of amplification of the amplicons of each pathogen in the presence of primers of other pathogens, confirmed the specificity of each set of primers for each relevant pathogen template DNA. No PCR interference/inhibition caused by the multiple primers in the multiplex PCR mix was observed.

The use of a well-scheduled amplification programme with a PCR mix with optimized concentration of its components guarantees the efficiency of the PCR reaction, as verified in this research for the detection of each TBP individually as

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well as coinfections. The co-infection of ticks with Lyme borreliosis spirochetes and emerging pathogens like *Babesia* species and *A. phagocytophilum* has been increasing in the endemic regions of tick-borne diseases, and it is very likely that these co-infections will continue to increase steadily in ticks, animals and humans in the near future [27].

R. sanguineus, known as the brown dog tick, is associated with the transmission of canine babesiosis and erhlichiosis [28, 29]. Since there is a common vector for both diseases, co-infections are frequent. *R. sanguineus* has not been associated with borrelial infection, although it is possible because dogs are potential amplifiers of these three pathogen groups [30, 31, 32].

The main *Anaplasma/Ehrlichia* species infecting dog ticks are *E. canis* and *A. platys*. The presented detection of *Anaplasma/Ehrlichia* spp. DNA in *Rhipicephalus* ticks collected from dogs in Cuba is not so surprising, because the infection by *E. canis* was reported previously in Cuban dogs [33]. These infectious diseases are of great importance for small animal clinics and public health, since they are increasingly prevalent in dogs, and because there is evidence that these pathogens can also affect humans [34]. Concerning *Babesia* spp., *B. canis*, *B. gibsoni*, *B. vogeli* and *B. rossi* are the species of haemoprotozoan parasites causing canine babesiosis [35]. *Babesia* spp. DNA was detected in ticks collected from Cuban dogs, although no report of canine *Babesia* spp. has been published. However, some veterinarians consider *B. canis* as responsible for infection in dogs in that country. During this study, it was not possible to use the sequencing of amplicons or other molecular tool for identifying the specific species of *Anaplasma* or *Babesia* detected. Given the zoonotic potential of these pathogens it is advisable to undertake further researches.

Although *R. sanguineus* feeds preferentially on dogs, it can also feed readily on humans and other mammal hosts [36]. Since some of them were collected from dogs in suburban localities where cattle habitually exist, previous parasitic stages of life could develop in a different mammal host, and to carry other *Anaplasma-Babesia* species reported in Cuba, such as *A. marginale*, *B. bigemina* or *B. bovis* [37, 38]. Additional studies should further evaluate the presence of these pathogen species in *R. sanguineus* ticks in suburban localities in Cuba.

Routine diagnoses of these pathogens are based on characteristic clinical and haematological findings. The identification of haemoparasites in blood smears is the most widely used technique in clinical practice, but this method has low diagnostic sensitivity and specificity, and must be supplemented with the use of molecular techniques, such as PCR [34].

The changing global scenario affects the prevalence of existing pathogens and the appearance of new infectious agents among animal populations indicates that exploratory and prevalence studies are needed.

CONCLUSION

A multiplex PCR assay has been developed for the screening of three TBP groups, especially in areas where no information on the occurrence of these taxa is available. By using this assay, *B. burgdorferi* sensu lato, *Anaplasma/Ehrlichia* spp. and *Babesia* spp. can be detected accurately in one PCR reaction; this is both cost-effective for reagents and less labour-intensive, compared to performing three individual PCR reactions.

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