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Original article

Revisiting the Tams1-encoding gene as a species-specific target for the molecular detection of *Theileria annulata* in bovine blood samples

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ABSTRACT

Tropical or Mediterranean theileriosis is a tick-borne hemoprotozoan disease that poses important health problems in cattle. The etiological agent is the apicomplexan parasite *Theileria annulata* that occurs around the Mediterranean basin, Middle East, and southern Asia. PCR-based assays have been developed for detecting theilerial infections, and the gene encoding an immunodominant major merozoite/piroplasm surface antigen of *T. annulata* (Tams1) is a commonly used genomic target. However, several studies revealed that this gene is highly polymorphic raising questions concerning the suitability of Tams1 gene-targeted primers to detect all *T. annulata* isolates. In this work, we re-evaluated the in silico sensitivity of previously developed primers targeting the Tams1 gene, designed novel and more comprehensive primers for its amplification, and established efficient standard and real-time PCR assays for assessing the presence or absence of *T. annulata* in bovine blood samples.

Introduction

Tropical theileriosis (or Mediterranean theileriosis) is a tick-borne hemoprotozoan disease responsible for causing important health problems in cattle. The agent of the disease is *Theileria annulata*, which occurs around the Mediterranean basin, Middle East and Southern Asia (Gubbels et al., 1999; García-Sanmartín et al., 2006; Branco et al., 2010; Silva et al., 2010). *T. annulata* sporozoites enter the bovine host during tick feeding and rapidly invade macrophages. Ultimately, merozoites are produced and released from the macrophages, invade erythrocytes and develop into piroplasms (Dobbelaere and Heussler, 1999). The animals that survive the acute disease become carriers of *T. annulata* piroplasms and play an important role as reservoirs for the maintenance of the parasite life cycle (D'Oliveira et al., 1995). Identification of these carrier animals is of utmost importance in epidemiological studies, for inferring infection risk and for the implementation and monitoring of control programs. Traditionally, detection of *Theileria* pathogens in infected animals is accomplished by microscopic examination of stained blood smears, which have low sensitivity for the assessment of carrier animals, in which low numbers of erythrocytes remain infected (Altay et al., 2008). Serological methods can be employed to diagnose subclinical infections, but cross-reactions are common, current infections and previous exposures are generally not distinguished, and antibodies tend to disappear in long-term carriers (Altay et al., 2008; García-Sanmartín et al., 2006). A few molecular diagnostic-based assays have also been developed, some of which have proven to be effective in detecting theilerial infections in carrier animals. A reverse line blotting (RLB) assay based on the amplification of the hyper-variable V4 region of the 18S rDNA gene of *Theileria* (and of closely-related *Babesia* parasites) and reverse hybridization of the products with species-specific oligonucleotide probes is currently considered to be the most sensitive test for detecting *T. annulata* (Gubbels et al., 1999; Georges et al., 2001; Bilgic et al., 2010). Nevertheless, since RLB is a relatively cumbersome assay, it is not entirely suitable for use in the routine diagnosis of *Theileria* infections. Some PCR-based assays have also been described for detecting *T. annulata* (e.g. D'Oliveira et al., 1995; Criado-Fornelio et al., 2009). The gene encoding an immunodominant major merozoite/piroplasm surface antigen of this parasite (Tams1) is commonly used as genomic target, and specific primers for the partial amplification of this gene were originally developed by D'Oliveira et al. (1995). These primers, assigned as N516 (forward) and N517 (reverse), have been frequently used by other authors to assess the prevalence and for diagnosis of *T. annulata* infections in bovine populations from different regions (D'Oliveira et al., 1997; Martín-Sánchez et al., 1999; Almeria et al., 2001; Sparagano et al., 2002; Dumanli et al., 2005; Aktas et al., 2006; Durrani and Kamal, 2008; Bilgic et al., 2010; Mahmmod et al., 2010; Shahnawaz et al., 2011). However, it is currently known that Tams1-encoding gene is highly polymorphic (Gubbels et al., 2000), which may constrain the respective PCR-based detection of all *T. annulata* genotypes and potentially contribute to an underestimation of the real prevalence of this

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pathogen. It is noteworthy that many more gene sequences are available in public databases at present than when N516 and N517 primers were originally designed and, therefore, it is possible that the respective complementary targets are located in more variable regions of the gene. In this work, we review the in silico efficiency of N516 and N517 primers, designed novel and more comprehensive primers for amplifying the *T. annulata* species-specific Tams1-encoding gene, and we established an efficient real-time PCR assay for detecting this important pathogenic protozoan in bovine blood samples.

Materials and methods

Samples and DNA extraction

For this study, a total of 63 DNA samples, formerly extracted from bovine (*Bos taurus*) blood samples of various regions of Portugal, were selected on the basis of a previous characterization using an RLB assay (Gomes et al., 2012). DNA samples were classified into 5 groups (Table 1): (I) *T. annulata*-positive samples, for which the hyper-variable region V4 of the 18S rDNA was amplified by PCR, using universal primers for *Theileria* and *Babesia* species, and the presence of the parasite was clearly demonstrated in the same by RLB; (II) *T. annulata*-weakly-positive samples, for which the PCR amplification of the V4 region yielded no detectable fragments in the electrophoresis gel, but the parasite was still detected after reverse hybridization with the species-specific probe; (III) *T. annulata* mixed infections, corresponding to samples in which a mixture of *T. annulata* and other species of *Theileria* and/or *Babesia* was detected by RLB; (IV) samples with other piroplasms, infected with *Theileria* and/or *Babesia* species, but not with *T. annulata*; and (V) negative samples, for which no infection with *Theileria* or *Babesia* was detected. Blood samples were originally collected from apparently healthy animals into sterile tubes with EDTA (Vacutainer, Becton Dickinson). Total genomic DNA was extracted from whole-blood samples by a BioSprint®96 automated workstation (Qiagen), using the BioSprint®96 Blood kit (Qiagen) according to the manufacturer's instructions, and stored at −20 °C until further use. The average total DNA yield in these samples was around 35–40 ng/μL (as assessed using a NanoDrop™ 1000 Spectrophotometer from Thermo Scientific). A DNA sample extracted from a *T. annulata*-infected macrophage culture (sample THA-1), obtained from a calf with clinical diagnosis of theileriosis (confirmed by microscopical examination of Giemsa-stained smears and RLB assay), was used as a positive control. A DNA solution extracted from bovine blood (sample 8182) for which a *T. annulata* parasitemia of 0.03% was observed using optical microscopy was also used as a positive control. DNA samples from *Babesia bovis* and *B. bigemina* kindly provided by Varda Shkap (Kimron Veterinary Institute, Israel) were used as negative controls of amplification. A negative control consisting of sterile distilled water (GIBCO, Invitrogen) was also used.

Sequence analysis and primer design

Comparative analysis of Tams1-encoding gene sequences from *T. annulata*, retrieved from NCBI-GenBank, was performed through sequence alignments using the CLUSTAL X v2.0 software (Larkin et al., 2007). More than 140 gene sequences of diverse geographical origins (e.g. Turkey, Mauritania, Sudan, India, Iraq, Tunisia, Spain, Portugal, and Italy) were analyzed [most of them were obtained and published by Shiels et al. (1995), Katzer et al. (1998), Al-Saeed et al. (2010) and, mainly, by Gubbels et al. (2000)]. Alignments containing tens of sequences were visually inspected for the presence of highly conserved nucleotide segments within *T. annulata* (i.e. present in all available Tams1-encoding gene sequences), providing also enough nucleotide differences when compared to homologous genes from other closely related species (particularly of the genus *Theileria*). A novel set of primers was designed, named Tams1_forw (5′-CAA ATT CGA GAC CTA CTA CGA TG-3′) and Tams1_rev (5′-CCA CTT RTC GTC CTT AAG CTC G-3′), based on selected conserved segments of the gene (Fig. 1), allowing the amplification of a fragment with about 319 bp. Primer properties such as guanine and cytosine content and theoretical melting temperature were assessed using the OligoAnalyzer software, version 1.0.3 (Teemu Kuulasmaa, Kuopio, Finland). The in silico specificities of designed primers were assessed using the BLAST suite of NCBI-GenBank.

Partial amplification of the Tams1-encoding gene using standard and real-time PCR primers Tams1_forw and Tams1_rev were used in standard PCR assays for the detection of *T. annulata* in DNA samples based on the partial amplification of the respective Tams1-encoding gene. Reaction mixture in a 25-μL final volume contained 2.5 mM $MgCl_2$ (Promega), 200 μM of each deoxynucleoside triphosphate (Promega), 1.0 μM of each primer (STAB Vida Lda), 0.5 U of GoTaq®Flexi polymerase (Promega), and 1× of the respective buffer, and 5 μL of the DNA sample solution. The PCR reactions were performed in an automated DNA thermal cycler (MJ Mini, Biorad) with the following program: (i) one initial denaturing step for 10 min at 94 °C; 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C; ending with a final extension step for 10 min at 72 °C. Amplified products were visualized under UV light after electrophoresis in a 1.5% (w/v) agarose gel (in 1× TBE buffer) stained with ethidium bromide. A more sensitive real-time PCR assay was also established using the previous designed primer set. The optimized reaction mixture contained 1× SsoFast™ Evagreen® Supermix (BioRad, CA, USA), 0.3 μM, and 0.15 μM of Tams1_forw and Tams1_rev primer, respectively, for a final volume of 20 μL (including the addition of 5 μL of template DNA sample). The real-time PCR was processed in a CFX96™ Real-Time PCR Detection System (BioRad, CA, USA) with the following optimized program: (i) one initial denaturing step for 2 min at 95 °C; and (ii) 45 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C. An additional step for the determination of the melting curve of the amplified fragments was added at the end of the amplification program, consisting of a 1 °C temperature increase every 5 s (beginning at 55 °C and ending at 95 °C). This additional step allows confirmation that the detection of fluorescence is related to the amplification of specific DNA targets and not with the formation of artifacts such as primer dimers. Data obtained in real-time PCR experiments were analyzed with the CFX Manager™ Software, version 1.5 (BioRad). The nucleotide sequence of selected PCR products was determined using the same above-mentioned Tams1_forw and Tams1_rev primers for the sequencing reactions (STAB Vida Lda, Lisbon, Portugal).

Results

Sequence analysis and primer design

The comparative analysis of more than 140 Tams1-encoding gene sequences of *T. annulata* confirmed the polymorphic feature of this gene observed previously by Gubbels et al. (2000) and others. It also allowed assessing that the N516 primer has a complementary target in a highly polymorphic region of the gene (Fig. 1). This was not possible to assess when this primer was designed in 1995 since only a few sequences of the Tams1 gene were available at that time. Moreover, of the more than 140 *T. annulata* Tams1 gene sequences currently available at NCBI-GenBank, a fully complementary target of N516 was only found in 44 sequences