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3 Short communication

4 **A field evaluation of an isothermal DNA amplification assay for the**
5 **detection of *Theileria annulata* infection in cattle**

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19

20 **Abstract**

21 A loop-mediated isothermal amplification (LAMP) assay was evaluated for the detection
22 of *Theileria annulata* infection in cattle. The results were compared with a real-time PCR
23 used for the quantification of *T. annulata* parasitaemia. One hundred bovine blood
24 samples from 16 cattle farms were tested with LAMP and real-time PCR, with *T. annulata*
25 DNA being detected in 66% and 67% of the samples, respectively. The results showed
26 that the LAMP assay detects a parasitaemia as low as 0.00025%, indicating a high
27 analytical sensitivity of LAMP for clinical diagnosis of bovine theileriosis.

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29 *Keywords:* molecular diagnostics; LAMP; real-time PCR; *Tams1*; *Theileria annulata*;
30 bovine theileriosis

31

32 1. Introduction

33 Tropical or Mediterranean Theileriosis is a tick-borne haemoprotozoan disease caused
34 by the apicomplexan parasite *Theileria annulata*, responsible for important health
35 problems in cattle (*Bos taurus* and *Bos indicus*) and in the Asian buffalo (*Bubalus*
36 *bubalis*). Clinical disease is usually associated with tick vector infestation and the clinical
37 diagnosis is frequently supported by microscopic examination of stained peripheral blood
38 smears for the detection of piroplasm-infected erythrocytes or macroschizont-infected
39 leukocytes in Giemsa-stained lymph node biopsies [1]. These methodologies are useful
40 to detect acute cases, but have low sensitivity for the assessment of carrier animals, in
41 which small numbers of erythrocytes remain infected, and do not allow the morphological
42 discrimination of *T. annulata* from other species of piroplasms in mixed infections [2].
43 Alternative molecular diagnostic-based assays have been developed for the sensitive
44 and specific detection of *T. annulata* infection in cattle, including a Reverse Line Blot
45 (RLB) hybridization assay based on the amplification of the hyper-variable V4 region of
46 the 18S rRNA gene of these parasites [3,4,5,6]. Though considered to be one of the most
47 sensitive tests for detecting *T. annulata* by identifying very low levels of the parasite in
48 carrier animals, the RLB technique is costly and requires a well equipped laboratory and
49 a skilled technician [7].

50 Loop-mediated isothermal amplification (LAMP) is a technique for the amplification of
51 DNA under isothermal conditions with high sensitivity. It is based on a nucleic acid
52 amplification that relies on autocycling strand-displacement DNA synthesis, performed
53 usually with *Bst* (*Bacillus stearothermophilus*) DNA polymerase. This enzyme can
54 synthesize a new strand of DNA, while simultaneously displacing the complementary
55 strand, thereby enabling DNA amplification under isothermal conditions [8]. LAMP has
56 been used successfully for the diagnosis of parasitic infections, such as malaria,
57 trypanosomiasis and toxoplasmosis, being reported as a highly sensitive and specific
58 method [9,10,11]. LAMP assays were also formerly described for the detection of
59 *Theileria* parasites [12,13,14,15,16], including *T. annulata* [17,18].

60 We formerly described the experimental details of a LAMP-based molecular assay
61 allowing the specific detection of *T. annulata* in bovine blood samples by targeting the
62 parasite major merozoite surface antigen (*Tams1*) gene [19]. A field study is described
63 here, where the *Tams1*-targeted assay was validated using a large set of blood samples
64 collected from cattle from a theileriosis endemic area at southern Portugal. A real-time
65 PCR test was used to estimate parasitaemia in naturally infected cattle.

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67 2. Materials and methods

68 Blood samples were collected from individual cattle (of different ages) in EDTA tubes
69 between January and June 2012 from sixteen farms in southern Portugal. One hundred
70 samples were taken from animals older than six months, with no apparent clinical signs
71 of theileriosis. A total of 800 µl of whole blood was used for DNA extraction. Red cells
72 were lysed using distilled water and centrifuged at 4,500 ×g (2 cycles of 5 min). The
73 supernatant was discarded and the remaining pellet was reconstituted with 200 µl of
74 water, and DNA extracted using the High Pure PCR Template Preparation Kit (Roche,
75 Switzerland) according to the manufacturer's instructions. A DNA sample extracted from
76 a *T. annulata*-infected macrophage culture obtained from a calf with clinical diagnosis of
77 theileriosis (confirmed by microscopical examination of Giemsa-stained smears and RLB
78 assay) and a reference blood sample (sample 8182) with an estimated *T. annulata*
79 parasitaemia of 0.03 % were included as positive controls. DNA samples from the blood
80 of an uninfected animal, DNA from each *Babesia bovis*, *B. bigemina* and *Theileria buffeli*
81 and a negative control consisting of sterile distilled water (GIBCO, Invitrogen) were also
82 used. All samples and controls were simultaneously tested with both the LAMP [19] and
83 real-time PCR [6,19] assays as described previously.

84 For the quantification of parasitaemia, a real-time PCR was performed using serial
85 dilutions of an amplified *Tams1* gene fragment. Briefly, a sequence of 851 bp containing
86 the target regions of the *Tams1* gene was amplified as described by Gubbels et al. [20]
87 using *T. annulata* DNA extracted from cell culture as template. The amplification product
88 was purified using illustra GFX™ PCR DNA and the Gel Band Purification Kit (GE
89 Healthcare), the DNA concentration was measured with Nanodrop® 2000 (Thermo
90 Scientific) and the copy number was determined [21]. Logarithmic genomic DNA dilution
91 series from 10⁻² to 10⁻¹⁰ gene copies were tested in triplicate with real-time PCR and the
92 cycle threshold (Ct) values were used for the standard curve elaboration. Since the
93 *Tams1* gene is a single copy gene, the curve equation could be used to quantify the copy
94 number and consecutively to estimate the number of parasites in each sample. Using
95 the average number of bovine erythrocytes of 7.5 X 10⁶ per microliter of blood, the
96 volume used for DNA extraction and the volume for DNA elution buffer, it was possible
97 to estimate *T. annulata* parasitaemia. A reference blood sample with a known *T. annulata*
98 parasitaemia of 0.03 % determined by microscopy was also used as a reference control.
99 The Cohen's kappa coefficient was used to calculate the degree of agreement between
100 LAMP and real-time PCR assays [22].

101 **3. Results**

102 A total of 100 DNA samples extracted from bloods from asymptomatic bovines were
103 screened for the detection of *T. annulata* by LAMP and real-time PCR. A characteristic
104 ladder pattern of a positive LAMP reaction was observed for 66 samples. The real-time
105 PCR assay was able to detect 67 positive samples. Only one positive sample by real-
106 time PCR was not identified by LAMP. Animals infected with *T. annulata* were detected
107 in 14 of the 16 farms sampled. The agreement analysis determined by kappa test gave
108 a value of 0.87, showing an almost perfect agreement between the LAMP and real-time
109 PCR assays.

110 A calibration standard curve was prepared in order to estimate the parasitaemia with
111 real-time PCR. A plot of the threshold cycle versus the log of the initial template copy
112 number (serial dilutions of *Tams1* gene fragments) showed a linear regression. The
113 standard curve presented a high efficiency (98%), an acceptable slope (-3.3708) and a
114 very good correlation (r^2) (0.99) (data not shown). The 66 LAMP-positive samples
115 showed Ct values in the real-time PCR between 21.42 and 31.05, with a mean Ct of
116 25.61 (CI 95%: 26.09 – 27.13) and a standard deviation of 2.11. These Ct values
117 correspond to parasitaemias ranging from 0.00026% to 0.185% and a mean
118 parasitaemia of 0.0001%. The positive sample detected only by real-time PCR showed
119 a Ct of 38.31, corresponding to an estimated parasitaemia of 0.0000018%. The
120 reference sample 8182, with a known parasitaemia of 0.03% determined by light
121 microscopy was shown to be positive for *T. annulata* by both LAMP and real-time PCR,
122 with a Ct value of 25 and an estimated parasitaemia of ~ 0.025%. Ct values obtained for
123 positive and reference samples are shown in Fig. 1.

124

125 **4. Discussion**

126 Bovine blood samples collected in Alentejo, an endemic region of theileriosis located in
127 southern Portugal, were used in a field study to demonstrate the suitability of LAMP as
128 a diagnostic tool and also to detect *T. annulata*-infected carrier animals. The prevalence
129 of *T. annulata*-infected cattle in Alentejo region was recently found to be ~ 29% [23]. Most
130 clinical cases of theileriosis occur in young calves and a high number of older animals
131 are infected without showing clinical signs and are thus considered chronic carriers.

132 LAMP was compared with a targeted *T. annulata* real-time PCR test used in our
133 reference laboratory. The sensitivity of this real-time PCR test was previously assessed
134 using Reverse Line Blot as golden standard [6]. The overall sensitivity was estimated as
135 90%, with negative and positive predictive values of 87% and 100%, respectively [6,24].

136 In the present study, LAMP showed an almost perfect agreement with the real-time PCR
137 results. The LAMP assay was able to detect *T. annulata* DNA in blood samples with a
138 very low parasitaemia, with a value of 0.00026% considered as the detection limit.
139 Infected samples not detected by LAMP might have parasitaemias under the limit of
140 detection or a mixed piroplasm infection [24]. By targeting the *T. annulata*-specific
141 Tams1-encoding gene, we also ensure enhanced specificity of our molecular assays for
142 the detection of this parasite. Former studies regarding the molecular detection of *T.*
143 *annulata* using LAMP, targeting both 18S rRNA and ITS genes and a hypothetical protein
144 gene, estimated the sensitivity of their assays to be between 0.1 pg/ μ l and 10 pg/ μ l
145 [17,18]. In both studies, sensitivity assays were performed using dilutions of template
146 DNA, but none was correlated with a known or estimated parasitaemia, hampering a
147 direct comparison with the results of the present study. *Theileria annulata* is estimated
148 to have only two copies of the 18S ribosomal RNA gene [25]. Consequently, great
149 improvements on the sensitivity of molecular assays are not expected only by targeting
150 this gene, when compared to single copy genes such as the Tams1-encoding gene. In
151 other studies targeting *Theileria* infections, real-time PCR assays were able to detect
152 similar parasitaemia values, such as 0.0009% with *T. parva* and 0.0002% with *T. equi*
153 [26,27]. A LAMP assay for *T. sergenti* was able to detect a positive blood dilution
154 corresponding to 0.000002% parasitaemia [13].

155 Natural infection results in parasitaemias of ~ 0.1 to 0.01% in carrier animals [28]. In the
156 present study, where none of the animals showed clinical signs of disease, the infected
157 animals could be considered healthy carriers. We analysed the Ct distribution and
158 correlated parasitaemia. Excluding the outlier value (Ct = 38.31), it is possible to see a
159 Ct distribution between approximately 21 and 31, with a mean value of 26 (Fig. 1). These
160 values correspond to a parasitaemia of 0.00025% to 0.18%, with a mean value of
161 0.009% and might be evidence of a pattern in the parasitaemia of carrier animals, which
162 are difficult to detect by microscopy of blood smears. The LAMP assay was able to detect
163 all samples within this range of parasitaemia and, thus, we might also expect to detect
164 animals with overt clinical signs in which parasitemias can reach much higher values of
165 10% to 45% [29]. This study indicated the utility of LAMP for the detection of *T. annulata*
166 parasites in carrier animals and also its potential applicability to the clinical diagnosis of
167 the disease.

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283 **Fig. 1.** Representation of cycle threshold values of *Theileria annulata* positive samples
284 from 14 cattle farms and a reference sample (8182). A dashed line indicates the limit of
285 detection of LAMP in this study. All positive LAMP samples are below the dashed line.

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