Development and evaluation of a loop-mediated isothermal amplification assay for detection of *Ehrlichia canis* DNA in naturally infected dogs using the p30 gene

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ABSTRACT. Canine monocytic ehrlichiosis (CME) is a common tick-borne disease caused by the rickettsial bacterium *Ehrlichia canis* (Rickettsiales: Anaplasmataceae). In view of the different stages and variable clinical signs of CME, which can overlap with those of other infections, a conclusive diagnosis can more readily be obtained by combining clinical and hematological evaluations with molecular diagnostic methods. In this study, a loop-mediated isothermal amplification (LAMP) assay targeting the *p30* gene of *E. canis* was developed. The assay was developed using DNA extracted from *E. canis*-infected cultures of the macrophage cell line DH82 and samples from dogs testing positive for *E. canis* DNA by PCR. The LAMP assay was compared to a p30-based PCR assay, using DNA extracted from EDTA-anticoagulated blood samples of 137 dogs from an endemic region in Brazil. The LAMP assay was sensitive enough to detect a single copy of the target gene, and identified 74 (54.0%) *E. canis* DNA-

positive samples, while the p30 PCR assay detected 50 positive samples (36.5%) among the field samples. Agreement between the two assays was observed in 42 positive and 55 negative samples. However, 32 positive samples that were not detected by the PCR assay were identified by the LAMP assay, while eight samples identified as *E. canis*-positive by PCR showed negative results in LAMP. The developed *E. canis* LAMP assay showed the potential to maximize the use of nucleic acid tests in a veterinary clinical laboratory, and to improve the diagnosis of CME.

Key words: *Ehrlichia canis*; Canine monocytic ehrlichiosis; Dog; Loop-mediated DNA amplification

INTRODUCTION

Ehrlichia canis is a tick-borne bacterium and the causative agent of canine monocytic ehrlichiosis (CME). The pathogen is globally distributed, but is particularly frequent in tropical and subtropical regions. The disease is endemic in Brazil, and is highly prevalent in dogs (Vieira et al., 2011). CME manifests as a multi-systemic disease, comprising an acute, subclinical, and, in some cases, a chronic phase. The acute phase manifests after an incubation period of 8 to 20 days post-infection, and is characterized by a wide range of clinical symptoms, including fever, depression, lethargy, anorexia, lymphadenomegaly, splenomegaly, mucosal pallor, conjunctivitis, and ocular discharge. Thrombocytopenia and leukopenia are common laboratory findings of this phase and are often used as a presumptive diagnosis of CME by veterinarians attending to dogs in *E. canis*-endemic areas (Macieira D de et al., 2005; Santos et al., 2009; Vieira et al., 2011). If not treated adequately, dogs can enter the subclinical phase of the disease without showing evident clinical signs; however, laboratory findings include variable persistence of thrombocytopenia, leukopenia, and anemia. This phase can last several months to years, or progress to a mild or severe chronic phase with a potentially fatal outcome. It is characterized by the presence of clinical and hematological signs similar to those seen during the acute phase (Harrus and Waner, 2011).

A conclusive diagnosis of CME can be challenging for veterinarians because clinical symptoms and hematological alterations overlap with those of other tick-borne diseases (Harrus and Waner, 2011). Moreover, co-infection of dogs with *E. canis* and other tick-transmitted pathogens is frequently reported, particularly in subtropical and tropical regions, where vectors generally have a marked presence in urban and rural areas (Mekuzas et al., 2009; Santos et al., 2009; Al Izzi et al., 2013; Eiras et al., 2013). However, the severity of CME pathogenesis may increase, in addition to displaying altered clinical manifestations (Tommasi et al., 2013). In this scenario, molecular techniques are often combined with clinical and hematological evaluation strategies to improve the diagnosis of CME. PCR, nested PCR, and real-time PCR assays, techniques based on the amplification of different genes with variable sensitivity, have been previously used to detect *E. canis* in dogs (McBride et al., 1996; Stich et al., 2002; Doyle et al., 2005; Kledmanee et al., 2009; Nakaghi et al., 2010; Peleg et al., 2010; Cardozo et al., 2011). The 16S rRNA nested PCR assay is the most common assay used in the detection of *E. canis*; however, a nested PCR assay for the detection of the *E. canis p30* gene has been recently shown to be more sensitive (Stich et al., 2002). An advantage of the latter assay is that *E. canis* carries several copies of the *p30* gene. However, despite the high specificity and sensitivity of nested PCR, they are often criticized because of the time-consuming steps and high risk of cross-contamination, which cause false-

positive results (Labruna et al., 2007). Recently, loop-mediated isothermal DNA amplification (LAMP) has been successfully applied to the detection of different pathogens, including *E. canis* and other related rickettsial agents (Nakao et al., 2010; Ma et al., 2011; Faggion et al., 2013). LAMP assays are highly sensitive and specific, and permit rapid generation and visualization of results (Notomi et al., 2000; Francois et al., 2011). In order to offer an alternative to PCR-based methods, the aim of this study was to develop a *p30*-based LAMP assay for the detection of *E. canis* in naturally infected dogs.

MATERIAL AND METHODS

DNA samples for the development of LAMP

DNA from *E. canis* (São Paulo strain)-infected cultures of the macrophage cell line DH82 (Wellman et al., 1988) and samples from dogs that were positive for *E. canis* DNA (diagnosed by PCR assays previously employed in our laboratory) (Santos et al., 2009; Cardozo et al., 2011) were used to develop the LAMP assay. DNA was extracted from blood or a cell suspension (both 200 µL) using the GenElute Blood Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Ultrapure water and total blood DNA extracted from blood collected from healthy dogs were used as negative controls.

LAMP primers and reaction

An internal fragment of the *p30* gene, corresponding to position 1278999-1279216 in the genomic sequence of *E. canis* str. Jake (GenBank ID: CP000107.1) was used to design the *E. canis*-specific LAMP primers, using the PrimerExplorer V4a software (Eiken Chemical Co., Tokyo, Japan) [http://primerexplorer.jp/elamp4.0.0/index.html] (Table 1). PCR fragments of the region of interest previously sequenced in our laboratory from blood samples of dogs were aligned with corresponding sequences deposited in GenBank using the ClustalX program (Larkin et al., 2007) in order to identify sequence conservation. An *EcoR*I restriction site was also created in the Fip-p30 and Bip-p30 primers for subsequent restriction analysis of the LAMP products (Table 1). Each LAMP reaction mixture contained 1 µL extracted DNA, 20 pmol each of the Fip-p30 and Bip-p30 primers, 5 pmol each of the F3 and B3 primer, 0.5 mM dNTP (each), and 1X ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100; pH 8.8). The final volume was adjusted to 19 µL with autoclaved ultrapure water; the mixture was incubated for 2 min at 95°C, and subsequently incubated on ice. *Bst* DNA polymerase large fragment (8 U; New England Biolabs, Ipswich, MA, USA) was added, and the reaction mixture was subsequently incubated at 61°C for 60 min and 80°C for 10 min to terminate the reaction. A 10-µL aliquot of each reaction was electrophoresed on a 2.5% agarose gel in Tris-acetic acid-EDTA (TAE) buffer; the separated bands were visualized under UV light after staining with ethidium bromide. Alternatively, the double-stranded DNA binding dye GelRed™ (Biotium, Hayward, CA, USA) was added to the samples, and the results visualized directly in the reaction tubes under UV light (Nakao et al., 2010).

Detection threshold and specificity of the LAMP assay

The sensitivity of the assay was evaluated as described in a previous study (Faggion et al., 2013). Briefly, the fragment of the *p30* gene encompassing the region amplified by the LAMP reaction was amplified by PCR using the P30-1SPF and P30-1SPR primers, using the

ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich) according to the manufacturer protocols; the following reaction condition were employed: 40 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The amplicons were cloned using the *pGEM-T easy* vector system (Promega, Madison, WI, USA). The identity of the corresponding p*30* gene fragment was confirmed by DNA sequencing. Ten-fold serial dilutions of plasmid DNA containing the *p30* gene fragment were prepared after measuring the concentration and copy number with the Implen Nanophotomer P360 (Implen, Westlake Village, CA, USA). One microliter of each dilution was used to determine the detection threshold of the assay. The LAMP products were digested with *Eco*RI to confirm the lack of cross-reactivity of the amplifications. Cross-reactivity was also evaluated using genomic DNA from *E. chaffeensis* Arkansas strain and *E. muris*, two species closely related to *E. canis*, as well as genomic DNA from *Ehrlichia* sp UFMT-BV strain (a new genotype isolated from cattle and *Rhipicephalus microplus* ticks (Cruz et al., 2012)) and *Babesia vogeli*, a common protozoan found in co-infections with *E. canis* (Santos et al., 2009). DNA samples of *E. canis* obtained from different geographic regions in Brazil were also tested.

Table 1. Nucleotide sequences of the LAMP primers used for the detection of *Ehrlichia canis*.

Primer	Type	Sequence (5'→3')[a]
LAMP		
F3-p30	Forward outer primer	GGCCCAAGAATAGAACTTGA
B3-p30	Reverse outer primer	CCTTCAATTATTATGTCATAGCATG
Fip-p30	Forward inner primer	TGTGTGCGCCGTTCTTATAATT*gaattc*AGTTCTGTACGAGACATTCG
Bip-p30	Reverse inner primer	CATCATAGTTCAGCAACAAACATGT*gaattc*AATGATAAGTCAATTAACCCTTC
PCR		
P30-1SPF	Forward primer	ATGGGTGGCCCAAGAATAGAACTTG
P30-1SPR	Reverse primer	CATCCTGCTATGGTTCCTAGTG

[a]Italic lowercase letters in the *Fip-p30* and *Bip-p30* primer sequences indicate *Eco*RI restriction sites.

Analysis of clinical samples using LAMP and PCR assays

EDTA-anticoagulated blood samples were collected from 137 dogs with a suspicion of CME, referred to a private clinic in the city of Ribeirão Preto, Brazil, between September 2012 and February 2014. DNA was extracted as described in a previous section, and analyzed using the LAMP and PCR assays. PCR was performed using protocols previously developed in our laboratory (Santos et al., 2009; Cardozo et al., 2011), with minor modifications: the forward and reverse primers p30-1SPF and p30-1SPR (Table 1), corresponding to position 1278993-1279419 of the genomic sequence of *E. canis* str. Jake (GenBank ID: CP000107.1), were used instead. The LAMP and PCR products were purified and sequenced to confirm their identity.

RESULTS

The reaction threshold and best results, indicated by the typical ladder-like pattern of LAMP assays after electrophoresis on an agarose gel, were obtained by incubating the samples at 61°C for 60 min. No DNA amplification was detected in the negative control samples up to at least 120 min of incubation (Figure 1). Positive samples were also detected by direct visualization of the LAMP products mixed with GelRed (Figure 2). Agarose gel electrophoresis of the LAMP products obtained with the serial dilution of plasmids containing a fragment of the *E. canis* p30 gene also revealed the typical ladder-like pattern and the detection limit was established at 1 copy of plasmid

DNA, with a threshold time of 60 min. This was also the detection limit for direct visualization of the LAMP products in the test tube with GelRed.

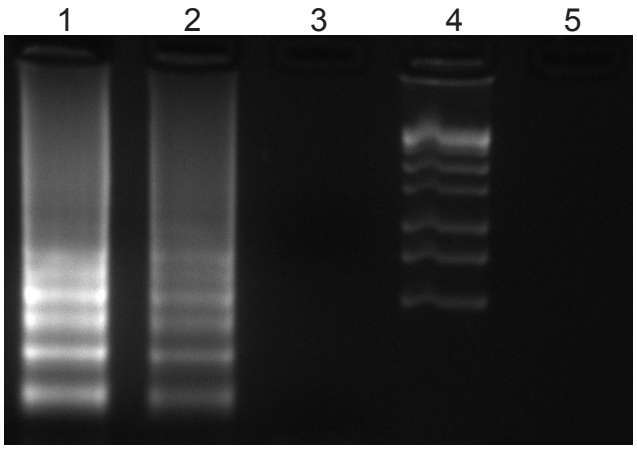

Figure 1. Representative electrophoretic agarose gel showing the results of the LAMP assay for detection of the *Ehrlichia canis* p30 gene. *Lanes 1* and *2*, characteristic LAMP ladder of positive *E. canis* samples; *lane 3*, no LAMP product in a negative sample; *lane 4*, 1-kb DNA ladder (Ludwig); *lane 5*, empty.

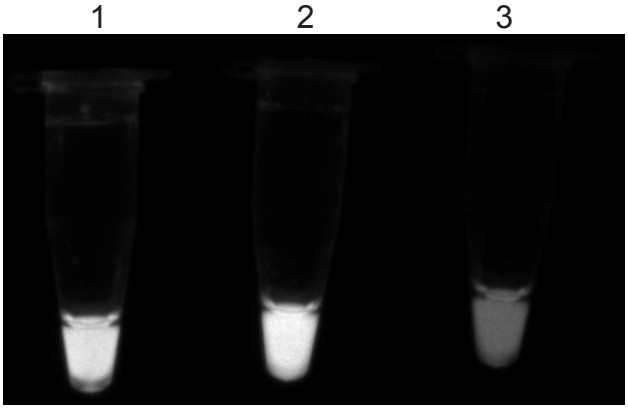

Figure 2. Representative results of the LAMP assay for detection of the *Ehrlichia canis* p30 gene by visual detection with GelRed dye. Tubes 1 and 2, positive *E. canis* samples; tube 3, negative *E. canis* sample.

Digestion of the LAMP products with *Eco*RI and analysis by agarose gel electrophoresis confirmed the correct amplification of the *p30* target sequence. Moreover, the LAMP reactions were positive for all *E. canis* strains obtained from different regions of Brazil, while the three tested *Ehrlichia* species, *Babesia canis*, and healthy dogs showed no such amplification.

The assay was used to detect *E. canis* DNA in clinical samples obtained from 137 dogs with a suspicion of CME referred to a private clinic in the city of Ribeirão Preto, Brazil. Seventy four (54.0%) samples tested positive for *E. canis* DNA in the LAMP assay, while 50 of the samples (36.5%) tested positive in the *p30* PCR assay. The results of the two assays were in agreement for 42 positive samples and 55 negative samples. However, the LAMP assay identified 32 positive

samples that were not detected by the PCR assay, while eight samples identified as positive by PCR were negative by LAMP (Table 1). These results indicate that the sensitivity and specificity of the p30 LAMP assay are 84.0 and 63.2%, respectively. These results also indicate positive and negative predictive values of 56.8 and 87.3%, respectively.

DISCUSSION

The increase in canine population in the world may pose a risk to animal welfare and human health, since dogs are reservoirs of many zoonotic parasites. Doxycycline is the drug of choice for the treatment of dogs infected with *E. canis* and other pathogens. The extensive application of this antibiotic for the treatment of dogs worldwide is cause for concern, as it may lead to the development of resistant strains (Bowman, 2011). It is therefore important to combine clinical findings and serological tests with nucleic acid tests to obtain a conclusive diagnosis of CME, as its clinical signs overlap with those of other diseases. This approach will permit veterinarians to prescribe an appropriate treatment method for, and evaluate pathogen clearance in, infected animals (Harrus and Waner, 2011). Although nested PCR assays are the most sensitive technique for the detection of *Ehrlichia* sp in infected blood samples (Stich et al., 2002; Nakaghi et al., 2010), their application in clinical settings is limited by the need for a high-precision thermal cycler and the excessive number of steps, causing possible cross-contamination (Nakaghi et al., 2010). In contrast, LAMP is performed in a water bath and the results, which can be observed with the naked eye without the need for a specialized gel electrophoresis unit, are obtained within 1 h (Mori and Notomi, 2009). Since its introduction, LAMP has been used in a wide range of applications, including the detection of canine pathogens (Adaszek et al., 2013; Chaouch et al., 2013; Faggion et al., 2013). In this respect, LAMP may be a useful tool for monitoring the treatment of CME in dogs in countries like Brazil, where the disease is endemic and highly prevalent, and veterinary laboratories often have limited access to sophisticated equipment.

In this study, a sensitive LAMP assay specific for a conserved region in the *p30* gene, which specifically amplified *E. canis* DNA and did not cause nonspecific amplification in a negative sample or samples infected with other pathogens, was developed. The assay used dilutions of plasmid DNA containing a fragment of the *p30* gene, and had a detection limit of 1 copy per reaction. This limit is comparable to that of other LAMP assays developed for Anaplasmataceae (Nakao et al., 2010; Ma et al., 2011; Pan et al., 2011; Faggion et al., 2013; Li et al., 2014). The performance of the *p30*-based LAMP assay for detection of *E. canis* DNA in 137 field samples was superior to that of the PCR assay, which was based on the amplification of the *p30* gene. The LAMP assay identified *E. canis*-positive 74 (54.0%), while the PCR assay detected 50 positive samples (36.5%). These results indicated that the performance of the *p30*-based LAMP assay was superior in the detection of *E. canis* DNA than the PCR assay; however, the sensitivity and specificity of LAMP were 84.0 and 63.2%, respectively, when PCR was used as the reference. This finding agrees with those of other studies that displayed the superior performance of LAMP assays over PCR in the detection of Anaplasmataceae DNA (Nakao et al., 2010; Ma et al., 2011; Pan et al., 2011; Muangchuen et al., 2014). The LAMP assay has also given false-negative results wherein PCR is considered the reference method. For example, in a LAMP assay developed for the detection of *Anaplasma ovis*, another bacteria of the Anaplasmataceae family (Ma et al., 2011), samples that were *A. ovis*-positive by PCR were negative in the LAMP assay, with a sensitivity and specificity of 95.45 and 41.61%, respectively. In another study, *Anaplasma phagocytophilum*-positive samples detected by real-time PCR were not positive in the LAMP assay; however, the

LAMP assay showed higher sensitivity (Pan et al., 2011). Recently, Muangchuen et al. (2014) published an article describing a colorimetric LAMP assay for the detection of *E. canis p30*, using gold nano-colloids. The authors also describe a PCR-positive sample that was negative in the LAMP assay, and attributed this result to a possible mutation in the DNA sequence of the target gene. Another possible explanation that was not ruled out by the authors was the inhibitory effect of the DNA components of the field samples used in the LAMP assay.

We have previously developed a LAMP assay for the detection of *E. canis* DNA using the *groESL* operon gene; though this showed a similar sensitivity to the *p30*-based LAMP assay, its performance in the field samples used in this study were not satisfactory (Faggion et al., 2013). The exact reason for these differences between the PCR and LAMP assays must be further clarified; however, despite many articles in literature claiming the superiority of the LAMP assay over PCR, our results and those of others must be considered with caution when applying this technique to field samples.

Another aspect of the study conducted by Muangchuen et al. (2014) is the direct visualization of the LAMP assay results in the test tubes, which is an important aspect of molecular diagnostics, and that makes it more feasible for application in remote areas. Our results regarding the directly visible *p30*-based LAMP assay in test tubes agreed completely with the results obtained by agarose gel electrophoresis. Although the sensitivity of the assay conducted by Muangchen et al. (2014) using field samples (97.5%) was superior to ours (84.0%), we believe that, when developed further, our assay using the DNA binding dye GelRed™ could be cheaper and simpler.

In summary, a new LAMP assay for the detection of *E. canis* was developed, which can be applied to the diagnosis and evaluation of CME treatment in dogs. It is a rapid, straightforward, and low-cost nucleic acid test that can be implemented in veterinary laboratories to improve the diagnosis of CME, and to avoid the unnecessary use of antibiotics due to misdiagnosis as a result of overlapping clinical signs with other diseases.

Conflicts of interest

The authors declare no conflict of interest.

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