Ticks and Tick-borne Diseases 10 (2019) 371–376

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Development of loop-mediated isothermal amplification (LAMP) assay for detection of *Hepatozoon canis* infection in dogs

Manraj Deep Singh[a], Harkirat Singh[a,*], Nirbhay Kumar Singh[a], Niraj Kumar Singh[b], Neeraj Kashyap[c], Naresh Kumar Sood[d], Shitanshu Shekar Rath[a]

[a] Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, 141004, India
[b] Department of Animal Biotechnology, School of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, 141004, India
[c] Department of Animal Genetics & Breeding, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, 141004, India
[d] Department of Teaching Veterinary Clinical Complex, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, 141004, India

ARTICLE INFO

Keywords:
Detection threshold
Hepatozoon canis
LAMP assay
Microscopy
18S PCR assay

ABSTRACT

The laboratory diagnosis of canine hepatozoonosis, caused by *Hepatozoon canis* is tedious, especially in chronic and latent infections. In the present investigation, a loop mediated isothermal amplification (LAMP) assay was developed and standardized targeting the partial 18S rRNA gene (GenBank accession no. KU096058). The LAMP primers specifically amplified *H. canis* DNA, whereas no amplification was detected in DNA samples from dogs infected with *Babesia vogeli*, *B. gibsoni*, *Ehrlichia canis* and *Trypanosoma evansi*, and no amplification was observed in DNA samples from *H. canis*-free dogs. The threshold sensitivity level of the assay was determined to be 15 fg of genomic DNA of *H. canis*. Furthermore, evaluation of blood samples collected from 250 dogs presented at Small Animal Clinics, Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab (India) was carried out for the presence of *H. canis* by microscopy, 18S PCR assay and LAMP assay. Of the total samples subjected to these tests, LAMP detected *H. canis* in 75 samples, while 18S PCR and microscopy detected *H. canis* in 28 and 9 samples, respectively. The present investigation has developed, for the first time, a highly sensitive, specific and rapid LAMP assay for the detection of *H. canis*, which has practical applications for the screening of field samples.

1. Introduction

Canine hepatozoonosis, caused by *Hepatozoon canis*, is considered to be one of the most prevalent canine vector-borne infections in the world including India (Otranto and Dantas-Torres, 2010; Singh et al., 2017a). The life cycle of the parasite is complex and unique, with the infection being transmitted via ingestion of an ixodid tick harbouring the mature oocysts of the parasite (Baneth et al., 2007). The principal vector of *H. canis* infection is the brown dog tick, *Rhipicephalus sanguineus* sensu lato (s.l.) (Nava et al., 2015). The infections, depending on the level of parasitaemia as well as immune response of the infected dog, may be presented as asymptomatic to mild or severe and potentially fatal (Gavazza et al., 2003). The clinical signs include fever, anorexia, lethargy, weight loss, lymphadenomegaly and pale mucous membranes (Baneth and Weigler, 1997).

Canine hepatozoonosis is routinely diagnosed by conventional parasitological techniques such as microscopic detection of ellipsoidal shaped gamonts within neutrophils or monocytes in stained blood smears and/or visualization of meronts or monozoic cysts in tissues during histopathology (Baneth and Shkap, 2003). Serological tests, such as indirect fluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA) developed for detection of anti-*H. canis* antibodies have high sensitivity, especially in dogs suffering from chronic infections (Shkap et al., 1994; Mylonakis et al., 2005). Although these serological methods may be valuable for epidemiological studies and for diagnosing infected dogs, they need an antigen source consisting of dogs with high parasitaemia and it is rare to find dogs that are heavily infected. Molecular diagnostic techniques in the form of polymerase chain reaction (PCR), real time-PCR assay and Sanger sequencing have been utilized for the diagnosis of *H. canis* infections and identification of isolates from various parts of the world, including India (Otranto et al., 2011; Abd Rani et al., 2011; Aktas et al., 2015; Singh et al., 2017a). The molecular tests have the advantages of higher levels of sensitivity and specificity over microscopy methods for *H.*

* Corresponding author.
E-mail addresses: drharkiratsingh@hotmail.com, harkiratsingh@gadvasu.in (H. Singh).

https://doi.org/10.1016/j.ttbdis.2018.11.016
Received 23 July 2018; Received in revised form 21 November 2018; Accepted 26 November 2018
Available online 27 November 2018
1877-959X/

canis detection in both host and vector (Latrofa et al., 2014; Singh et al., 2017b). However, the field applicability of PCR is limited owing to the high costs of thermocyclers and limited accessibility in field conditions (Mandal et al., 2015).

In this regard, a relatively new generation DNA amplification method, loop-mediated isothermal amplification (LAMP) is being used for early and sensitive diagnosis of various infectious agents, and has been found to be a rapid, specific, sensitive and simple test (Notomi et al., 2000; Parida et al., 2008; Karanis and Ongerth, 2009; Mandal et al., 2015). When compared to conventional PCR and real-time PCR, it has advantages of simplicity and higher throughput amplification efficiency without requiring costly instruments. Furthermore, the results of the assay can be evaluated easily by visualization of the turbidity in positive reactions (Mori et al., 2001). In the field of veterinary parasitology, LAMP assays have been developed successfully for the detection of *Babesia gibsoni* (Ikadai et al., 2004; Mandal et al., 2015), *Trypanosoma evansi* (Thekisoe et al., 2005), *Theileria equi* (Alhassan et al., 2007), *B. caballi* (Alhassan et al., 2007), *Cryptosporidium parvum* (Karanis et al., 2007), *B. orientalis* (He et al., 2009), *Toxoplasma gondii* (Krasteva et al., 2009), *T. parva* (Thekisoe et al., 2010), *T. sergenti* (Wang et al., 2010) and *Tritrichomonas foetus* (Oyhenart et al., 2013). Despite the employment of nucleic acid based detection assays for *H. canis* infections in dogs, there appears to be no published report on the application of LAMP for the detection of this parasite, to date. Therefore in the present study, a LAMP assay was developed and standardized targeting the 18S ribosomal RNA (rRNA) gene for the detection of *H. canis* and the LAMP assay's cross reactivity was also evaluated for other common blood parasites of dogs. Furthermore, the sensitivity of the LAMP assay was compared with microscopy and an 18S PCR assay.

2. Materials and methods

2.1. Geographical area

The study was conducted at Small Animal Clinics (SAC), Teaching Veterinary Clinical Complex (TVCC), Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, a district of Punjab state, India. Ludhiana lies between north latitude 30°34′ and 31°01′ and east longitude 75°18′ and 76°20′. The summer season is very hot and humid while the winter is relatively cold with annual temperatures ranging from 1 °C to 46 °C, and the average annual rainfall of 565.9 mm. These environmental conditions provide favourable and conducive conditions for the survival and propagation of the vector tick, *R. sanguineus* s.l. (Gill and Gill, 1977).

2.2. Sample collection

A total of 250 blood samples were collected aseptically in EDTA coated vials from the cephalic vein of selected dogs presented to SAC, TVCC, GADVASU, Ludhiana, Punjab (India) for a period of one year (May 2017 to April 2018). The dogs were selected on the basis of history and clinical signs suspected to be infected with blood parasites with special emphasis on *H. canis* infection e.g. presence of fever, pale mucus membranes, emaciation, presence of ticks and hind limb paralysis/weakness. The collected blood samples were utilized immediately for the preparation of thin blood smears and the remaining blood was then kept at −20 °C until DNA extraction.

2.3. Microscopy

The microscopic examination of blood samples was performed after staining the prepared thin blood smears with Giemsa as per Juyal et al. (2013). Briefly, a thin blood smear was prepared by placing a drop of blood on one end of a clean microscopic glass slide and was spread by using a spreader slide with clean edges. The smear was immediately air dried by vigorous shaking and the slides were fixed by immersing in methanol for 1–2 min. Thereafter a 10% working Giemsa stain solution (1 part Giemsa stock solution in 9 parts phosphate buffer, pH 6.8–7.2) was poured on the pre-fixed smears and kept for 45 min. The slides were washed twice with distilled water, air-dried and examined under oil immersion (100X) objective of the microscope to detect the gamont stage of *H. canis* in neutrophils and monocytes. At least 100 oil immersion microscopic fields were tested before declaring the sample negative.

2.4. Genomic DNA extraction

For conducting the PCR assays, genomic DNA was isolated from whole blood using the QIAamp® DNA blood mini kit (QIAGEN, GmbH, Germany) following the manufacturer's recommendations with minor modifications as per Singh et al. (2012a). In brief, 200 µL of the blood sample was mixed with 20 µL of proteinase K and 200 µL of lysis buffer and incubated at 56 °C for 10 min. Then, 200 µL of ethanol was added to the sample, and the mixture was applied to the QIAamp® Mini spin columns and centrifuged at 8000 rpm for 1 min. Thereafter, 2 washings were given with 500 µL each of wash buffers 1 and 2. Finally, 150 µL of elution buffer was added to the columns, and DNA was eluted in 1.5 mL Eppendorf tubes after centrifugation and stored at −20 °C until use.

2.5. 18S PCR assay

The 18S PCR assay for detection of *H. canis* was optimized targeting a portion of the 18S rRNA gene of *Hepatozoon* spp. as described by Inokuma et al. (2002). Briefly, the primers Hep-F and Hep-R were used to amplify a 666 bp fragment of the 18S rRNA gene. The names and sequences of the primers are provided in Table 1. The 18S PCR amplifications were performed in Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA) and carried out in a final volume of 25 µL with the reaction mixture composition and cycling conditions as per Singh et al. (2017a). The PCR amplicons were analyzed by electrophoresis in 1.5% ethidium bromide-stained agarose gels (Agarose Low EEO™, SRL), and band sizes were compared with those of the molecular marker (Generuler DNA™ ladder 100 bp, MBI Fermentas) using InGenius® Gel Documentation System (Syngene, UK). Genomic DNA samples isolated from *H. canis*-infected and an infection-free puppy during our previous study (Singh et al., 2017a) were used as positive and negative controls, respectively, and nuclease free water was used as a no-template control.

Table 1
Details of the primers used for *H. canis* DNA amplification by LAMP and 18S PCR assays.

Method	Primer name	Sequence (5′to 3′)	Length (bp)
LAMP	F3	GCAAAGTGAAAACAGGCG	18
	B3	AGAATTGGGTAATTTGCGC	19
	FIP	GCCACGGTAAGCCAATACCATAAATCATTCAAGTTTCTGACCT	43
	BIP	GTGACGGTTAACGGGGGATTGTGGTAGCCGTTTCTCAG	38
18S PCR	HEP-F	ATACATGAGCAAAATCTCAAC	21
	HEP-R	CTTATTATTCCATGCTGCAG	20

2.6. LAMP assay

2.6.1. Designing of primers

The primers were designed using Primer explorer version 5 software (http://primerexplorer.jp/lampv5e/index.html). A partial sequence of the 18S rRNA gene of the Ludhiana isolate of *H. canis* (GenBank accession no. KU096058) was chosen for designing the primers. Four primers *i.e.* outer primers (F3 and B3) and inner primers (FIP and BIP), were selected after being checked for their qualities such as T_m value, length of the primers, length between primers, presence of self and cross dimers and for their specificity using the Basic Local Alignment Search Tool (BLAST) for sequence comparison to the National Center for Biotechnology Information (NCBI). The names and sequences of the primers are provided in Table 1.

2.6.2. LAMP optimization and amplification setup

The LAMP assay was carried out in 25 µL reaction volumes in 0.2 mL PCR tubes as described by Notomi et al. (2000), with few modifications. In order to determine the optimal reaction temperature of the primers, the assay was conducted at 50 °C, 55 °C, 60 °C and 65 °C as the average melting temperature of the primers was 58.1 °C. The concentration of different reagents of LAMP such as mixture of dNTPs (0.2 mM, 0.4 mM, 0.8 mM, 1.2 mM and 1.6 mM), $MgSO_4$ (0 mM, 2 mM, 4 mM and 6 mM) and betaine (0 M, 0.2 M, 0.4 M and 0.6 M) were optimized on the basis of brightness and sharpness of the ladder like pattern of the LAMP products in gel electrophoresis (Alhassan et al., 2007; Oyhenart et al., 2013; Mandal et al., 2015). The assay was also carried out for different time durations ranging from 30 min to 90 min to determine the optimum time for amplification.

Following optimization, the reaction mixture consisted of the following components: 5 pmol of each outer primer (F3 and B3), 50 pmol of each inner primer (FIP and BIP), 0.6 M betaine (Sigma, USA), 6 mM $MgSO_4$ (New England Biolabs, UK), 1.6 mM dNTP mix (MBI Fermentas, USA), 1X ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100) and 1 µL of target DNA. The reaction mixture was heated at 95 °C for 5 min, chilled on ice and subsequently 1 µL (8U) *Bst* DNA polymerase large fragment (New England Biolabs, UK) was added. The amplification reaction was carried out at 55 °C for 90 min and terminated by incubating at 80 °C for 2 min. Genomic DNA isolated from a *H. canis* infection free puppy and nuclease free water were used as negative and no-template control, respectively, while DNA isolated from a *H. canis* positive sample (confirmed using microscopy) was used as positive control in all LAMP assays.

The LAMP products (15 µL) were analyzed by electrophoresis in 2% ethidium bromide-stained agarose gel (Agarose Low EEO, SRL), and visualized using InGenius® Gel Documentation System (Syngene, UK). The LAMP products were also examined with naked eye for the presence of turbidity in the reaction tubes. Formation of insoluble white precipitate/turbidity was directly correlated with amplification.

2.6.3. Sensitivity and specificity of LAMP assay

The 18S PCR assay was employed on the isolated DNA sample and checked for amplification by performing agarose gel electrophoresis. The amplicons were purified with a QIAquick®Gel Extraction Kit (QIAGEN, GmbH, Germany) as per the manufacturer's protocol and DNA was eluted with 30 µL of elution buffer. The eluted product was quantified with a Qubit® dsDNA HS assay kit as per the manufacturer's protocol with the final volume kept at 200 µL. The concentration of the eluted sample was recorded after placing the sample tube in the Qubit® 2.0 Fluorometer.

The eluted sample was then diluted in 10-fold serial dilutions with nuclease free water and the optimized LAMP assay was performed using each dilution as template (1 µL) to estimate the threshold sensitivity level of the assay. The PCR amplicons were checked for amplification by electrophoresis on a 2.0% agarose gel containing ethidium bromide. For testing the threshold sensitivity of the 18S PCR assay, the same eluted sample was diluted in 10-fold serial dilutions and used as template (1 µL) in the assay under the same conditions as per Singh et al. (2017a).

In order to determine the specificity of the LAMP assay, total genomic DNA extracted from whole blood of dogs infected with other canine common haemoparasites e.g. *B. vogeli*, *B. gibsoni*, *E. canis* and *T. evansi* along with leucocyte DNA isolated from a healthy dog were used as templates for the LAMP assay.

2.6.4. Statistical analysis

The statistical analysis was performed by SAS version 9.3. Summary statistics were generated by cross-tabulations of categorical data and statistically significant differences in the proportion of positive samples between the LAMP assay, 18S PCR and microscopy along with the values of sensitivity and specificity of individual assays were determined using the Fischer's exact test. The test results were considered significantly different when p values were < 0.05.

2.7. Ethical guidelines

Approval and necessary guidelines of Institute Animal Ethics Committee (IAEC) was obtained GADVASU/2017/IAEC/39/12 vide Memo No. IAEC/2017/734-760 dated 20.03.2017 for the conduction of the study.

3. Results

3.1. Microscopy

The microscopic examination of Giemsa-stained thin blood smears revealed that 3.6% (9/250) were positive for gamonts of *H. canis* in the neutrophils and/or monocytes (details in Table 2). The gamonts were capsulated, ellipsoidal shaped, and had a large and central nucleus.

3.2. 18S PCR assay

The 18S PCR assay revealed amplicons 666 bp in length in 11.2% of the total samples (28/250) revealing the presence of DNA of *H. canis* in these samples (Fig. 1) (details in Table 2).

3.3. Optimization of LAMP assay

Regarding the optimization, best results were obtained when 5 pmol of each outer primer (F3 and B3), 50 pmol of each inner primers (FIP and BIP), 0.6 M betaine, 6 mM $MgSO_4$, 1.6 mM dNTP mix, and 1 µL of DNA template, respectively, were used and the reaction temperature was kept at 55 °C for 90 min. White precipitates of LAMP products in the reaction tubes containing positive samples were visualized by naked eye under natural light (data not shown). The agarose gel electrophoresis of the LAMP products revealed a characteristic ladder like pattern in positive samples while no such bands were observed in known negative and no-template controls (Fig. 2).

Table 2
Percent positivity of blood samples of dogs for the presence of *H. canis*, evaluated using LAMP, 18S PCR and microscopy.

Assay used	Positive (%)	Negative (%)	Total samples
LAMP	75 (30.0)	175 (70.0)	250
18S PCR	28 (11.2)	222 (88.8)	250
Microscopy	9 (3.6)	241 (96.4)	250

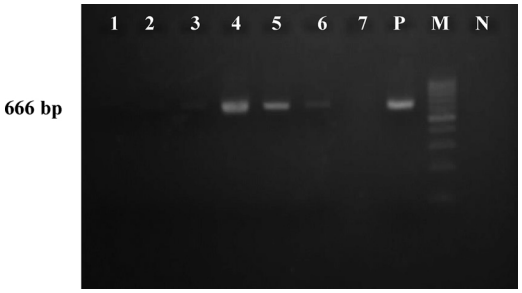

Fig. 1. Field application of 18S PCR assay for detection of *Hepatozoon canis* in dogs.
Lane M: Generuler™ 100 bp Ladder.
Lane 1–7: Field collected samples.
Lane N: Negative control.
Lane P: Positive control.

3.4. Threshold sensitivity and specificity of LAMP assay

The sensitivities of both LAMP (Fig. 3) and 18S PCR (supplementary Fig. 4) assays were determined using 10-fold serial dilutions of quantified eluted product obtained in 18S PCR assay. The characteristic ladder like pattern of LAMP products was detected up to 10^{-7} dilution (15 fg) of the eluted product, while the detection limit of the 18S PCR was10^{-6} dilution (150 fg). Furthermore, the primers used in the present assay did not amplify any product when the genomic DNA of *B. vogeli*, *B. gibsoni*, *E. canis* and *T. evansi* were used as template revealing high specificity of these primers (supplementary Fig. 5).

3.5. Evaluation of LAMP using suspected blood samples

In order to evaluate the usefulness of the optimized LAMP assay as a rapid diagnostic tool, the genomic DNA extracted from the suspected blood samples presented at SAC, TVCC, GADVASU, Ludhiana when tested revealed that 30.0% (75/250) of samples were positive for *H. canis* as revealed by the characteristic ladder like pattern in the positive samples (supplementary Fig. 6) (details in Table 2). There was a statistically significant difference ($p < 0.05$) in the sensitivity of microscopy and these two nucleic acid based amplification methods. All the microscopically positive samples (9/250) were also positive by 18S PCR and LAMP assay. Furthermore, all samples positive by 18S PCR (28/250) were also positive by LAMP assay.

When the sensitivity and specificity values were calculated at 95% confidence intervals (CI) for LAMP assay it was seen that the sensitivity was 96.4% with a range of 89.5%–100.0% which shows the high sensitivity of the LAMP over the 18S PCR assay. The specificity of the LAMP assay was 78.4% with a range of 73.0%–83.8%. The positive and negative predictive values were 36.0% and 99.4%, respectively and all the data was statistically significant at $p < 0.05$.

4. Discussion

Microscopy has been traditionally used to test for and diagnose haemoparasites. Detection of circulating gamonts in stained blood smears is the routine diagnostic approach for detection of *H. canis* infection. However, it has been observed that the absence of parasitaemia does not always indicate absence of infection, as there can be false negative results in dogs with a low parasitaemia or a temporary state of no parasitaemia despite tissue infection with *Hepatozoon* spp. In this regard, the amplification of the parasite's DNA has been stated to be so far more sensitive than the conventional methods because sometimes the lower parasitaemia cannot be detected by microscopy, but as the PCR assays are highly sensitive, they are capable of detecting small amounts of parasitic DNA.

In the current investigation, a LAMP assay targeting the partial 18S rRNA gene was developed and optimized for the sensitive and rapid detection of *H. canis* infection in dogs. The optimal temperature, time as well as concentration of various reagents, which are the critical factors for formation of stem –loop (Notomi et al., 2000), were also standardized. The LAMP primers that were designed and applied were found to be specific to *H. canis* only as DNA of no other blood protozoan parasite evaluated, negative sample and no-template controls were amplified, and the assay specifically produced typical ladder patterns from the

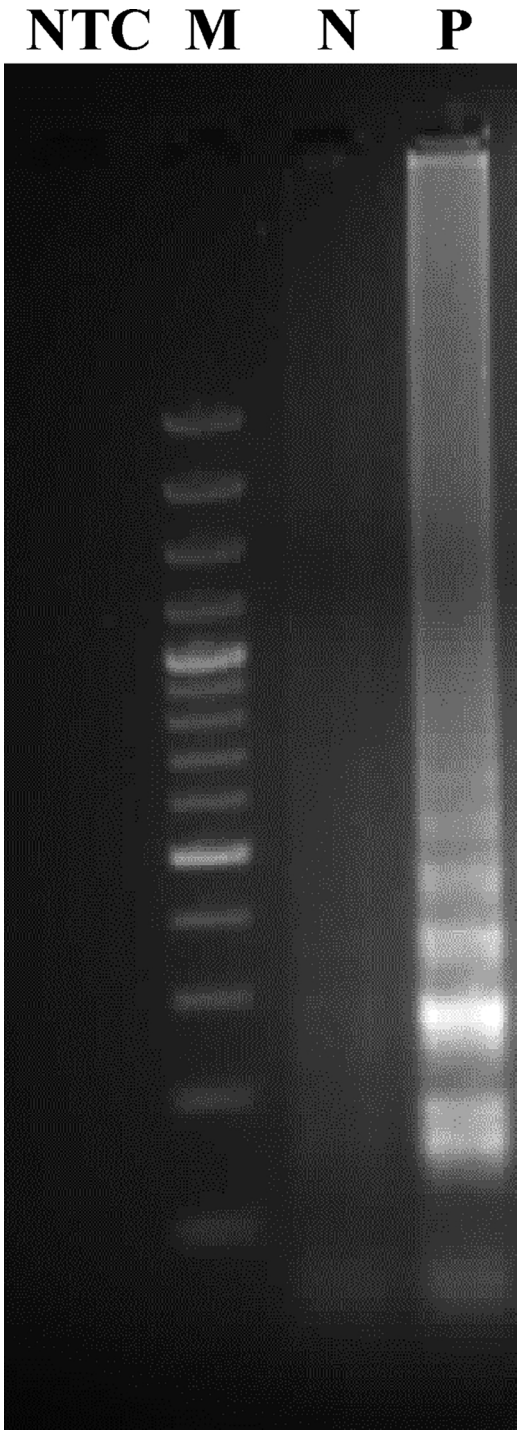

Fig. 2. Standardized LAMP assay for detection of *Hepatozoon canis* in dogs.
Lane M: Generuler™ 100 bp Ladder plus.
Lane NTC: Non-template control.
Lane N: Negative control.
Lane P: Positive control.

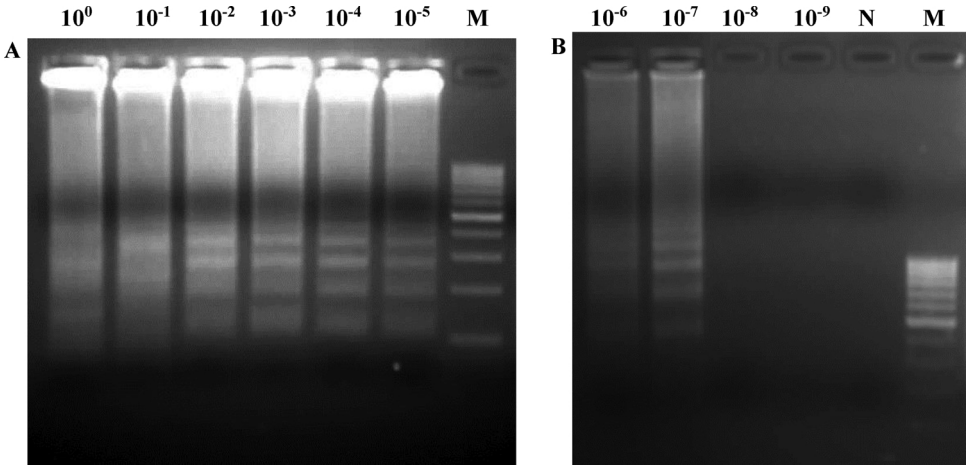

Fig. 3. A and B: Sensitivity of LAMP assay for detection of *Hepatozoon canis* in dogs.
Lane M: Generuler™ 100 bp Ladder.
Lane N: Negative control.
Lane 10°-10^{-9}: 10-fold serially diluted purified product.

target gene of *H. canis* only. This confirmed the absence of self-amplification of the LAMP primers. Results of the present study in respect to the sensitivity and specificity were found to be comparable with previous reports (Alhassan et al., 2007; He et al., 2009; Wang et al., 2010).

The sensitivity of the LAMP assay was 10 times greater than that of the 18S PCR, as it could detect DNA up to 10^{-7} dilution of the eluted product, whereas the 18S PCR detected the DNA up to 10^{-6} dilution only. High sensitivity of LAMP assays over the conventional PCR assays has been reported by various workers targeting different parasites (Wang et al., 2010; Mandal et al., 2015). However, LAMP and PCR assays were found to be equally sensitive for detection of *T. evansi* infection (Alhassan et al., 2007). In the present investigation the threshold detection limit of the LAMP assay was 15 fg which is much higher than that reported by Mandal et al. (2015) for *B. gibsoni*. In two previous studies, it was observed that the LAMP assays could detect cloned plasmid DNA of *T. gondii* and *T. parva* up to 1 fg level (Krasteva et al., 2009; Thekisoe et al., 2010) and this value was considered as the upper limit of the detection threshold of the assay.

In the present study, 3.6% of blood samples were found to be positive for *H. canis* infection by microscopy in and around Ludhiana district, Punjab (India). Recent reports revealed a similar prevalence of hepatozoonosis in dogs in the range of 0.3–5.8% (Singh et al., 2011; Singh et al., 2012b, c; Singla et al., 2016; Singh et al., 2017a) and the results of the current study further established the existence of low prevalence rates from the region. Further, the 18S PCR assay revealed that 11.2% of samples were positive for *H. canis* DNA and this prevalence is similar to our previous report of 13.78% (Singh et al., 2017a), and the 18S PCR assay had a higher sensitivity over microscopy.

In order to determine the applicability of the LAMP assay, 250 blood samples collected from dogs were also subjected to the assay. The 18S PCR and microscopy were used to determine the status *H. canis* infection on these samples. It was observed that the sensitivities of the LAMP and 18S PCR assay were higher than that of the microscopy. Furthermore, 18.8% of samples (47/250), which were negative by 18S PCR, were positive by the LAMP assay, indicating a higher sensitivity over the 18S PCR, and the difference was statistically significant ($p < 0.05$). The observations of the present investigation are in similar to previous reports where the sensitivity of LAMP was found to be higher than conventional PCR for the diagnosis of *T. sergenti* (Wang et al., 2010) and *B. gibsoni* (Mandal et al., 2015).

Therefore, it may be concluded that a simple and rapid LAMP assay was developed and evaluated successfully in field samples for detection of *H. canis* infection in dogs. The assay can be used to detect carrier animals, which act as source of infection for healthy dogs, owing to its high sensitivity and specificity levels, over 18S PCR and microscopy. Furthermore, the LAMP assay can be employed in the field for routine examination, where sophisticated and high-end equipments are not feasible.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors are very grateful to The Dean, Postgraduate Studies, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab for providing the necessary facilities. Thanks are also due to the faculty of Department of Veterinary Medicine and Teaching Veterinary Clinical Complex for the smooth conduct of study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.11.016.

References

Abd Rani, P.A.M., Irwin, P.J., Coleman, G.T., Gatne, M., Traub, R.J., 2011. A survey of canine tick-borne diseases in India. Parasit. Vectors 4, 141–148.
Aktas, M., Özübek, S., Altay, K., Balkaya, I., Utuk, A.E., Kırbas, A., Simsek, S., Dumanli, N., 2015. A molecular and parasitological survey of *Hepatozoon canis* in domestic dogs in Turkey. Vet. Parasitol. 209, 264–267.
Alhassan, A., Thekisoe, O.M., Yokoyama, N., Inoue, N., Motloang, M.Y., Mbati, P.A., Yin, H., Katayama, Y., Anzai, T., Sugimoto, C., Igarashi, I., 2007. Development of loop-mediated isothermal amplification (LAMP) method for diagnosis of equine piroplasmosis. Vet. Parasitol. 143, 155–160.
Baneth, G., Samish, M., Shkap, V., 2007. Life cycle of *Hepatozoon canis*(Apicomplexa: Adeleorina: hepatozoidae) in the tick*Rhipicephalus sanguineus* and domestic dog (*Canis familiaris*). J. Parasitol. 93, 283–299.
Baneth, G., Shkap, V., 2003. Monozoic cysts of Hepatozoon canis. J. Parasitol. 89, 379–381.
Baneth, G., Weigler, B., 1997. Retrospective case-control study of hepatozoonosis in dogs in Israel. J. Vet. Int. Med. 11, 365–370.
Gavazza, A., Bizzeti, M., Papini, R., 2003. Observations on dogs found naturally infected with *Hepatozoon canis* in Italy. Rev. Med. Vet. 154, 565–571.
Gill, H.S., Gill, B.S., 1977. Qualitative district-wise distribution of adult ixodid ticks in the Punjab state. Ixodid ticks of Domestic Animals in the Punjab State. PAU, Ludhiana, pp. 2–14.
He, L., Zhou, Y.Q., Oosthuizen, M.C., Zhao, J.L., 2009. Loop-mediated isothermal amplification (LAMP) detection of *Babesia orientalis* in water buffalo (*Bubalus bubalis*, Linnaeus, 1758) in China. Vet. Parasitol. 165, 36–40.

Ikadai, H., Tanaka, H., Shibahara, N., Matsuu, A., Uechi, M., Itoh, N., Oshiro, S., Kudo, N., Igarashi, I., Oyamada, T., 2004. Molecular evidence of infections with *Babesia gibsoni* parasites in Japan and evaluation of the diagnostic potential of a loop-mediated isothermal amplification method. J. Clin. Microbiol. 42, 2465–2469.

Inokuma, H., Okuda, M., Ohno, K., Shimoda, K., Onishi, T., 2002. Analysis of the 18S rRNA gene sequence of a *Hepatozoon* detected in two Japanese dogs. Vet. Parasitol. 106, 265–271.

Juyal, P.D., Singh, N.K., Singh, H., 2013. Diagnostic Veterinary Parasitology: An Introduction, 1st edition. New India Publishing Agency, New Delhi, India, pp. 30–32.

Karanis, P., Ongerth, J., 2009. LAMP – a powerful and flexible tool for monitoring microbial pathogens. Trends Parasitol. 25, 498–499.

Karanis, P., Thekisoe, O., Kiouptsi, K., Ongerth, J., Igarashi, I., Inoue, N., 2007. Development and preliminary evaluation of a loop-mediated isothermal amplification procedure for sensitive detection of *Cryptosporidium* oocysts in fecal and water samples. Appl. Environ. Microbiol. 73, 5660–5662.

Krasteva, D., Toubiana, M., Hartati, S., Kusumawati, A., Dubremetz, J.F., Widada, J.S., 2009. Development of loop-mediated isothermal amplification (LAMP) as a diagnostic tool of toxoplasmosis. Vet. Parasitol. 162, 327–331.

Latrofa, M.S., Dantas-Torres, F., Giannelli, A., Otranto, D., 2014. Molecular detection of tick-borne pathogens in *Rhipicephalus sanguineus* group ticks. Ticks Tick-Borne Dis. 5, 943–946.

Mandal, M., Banerjee, P.S., Kumar, S., Ram, H., Garg, R., Pawde, A.M., 2015. Development of loop-mediated isothermal amplification (LAMP) for detection of *Babesia gibsoni* infection in dogs. Vet. Parasitol. 209, 50–55.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289, 150–154.

Mylonakis, M.E., Leontides, L., Gonen, L., Billinis, C., Koutinas, A.F., Baneth, G., 2005. Anti- *Hepatozoon canis* serum antibodies and gamonts in naturally-occurring canine monocytic ehrlichiosis. Vet. Parasitol. 129, 229–233.

Nava, S., Estrada-Peña, A., Petney, T., Beati, L., Labruna, M.B., Szabó, M.P., Venzal, J.M., Mastropaolo, M., Mangold, A.J., Guglielmone, A.A., 2015. The taxonomic status of *Rhipicephalus sanguineus* (Latreille, 1806). Vet. Parasitol. 208, 2–8.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Otranto, D., Dantas-Torres, F., 2010. Canine and feline vector-borne diseases in Italy: current situation and perspectives. Parasit. Vectors 3, 2.

Otranto, D., Dantas-Torres, F., Weigl, S., Latrofa, M.S., Stanneck, D., de Caprariis, D., Capelli, G., Baneth, G., 2011. Diagnosis of *Hepatozoon canis* in young dogs by cytology and PCR. Parasit. Vectors 4, 55.

Oyhenart, J., Martinez, F., Ramirez, R., Fort, M., Breccia, J.D., 2013. Loop mediated isothermal amplification of 5.8S rDNA for specific detection of *Tritrichomonas foetus*. Vet. Parasitol. 193, 59–65.

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V., Morita, K., 2008. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev. Med. Virol. 18, 407–421.

Shkap, V., Baneth, G., Pipano, E., 1994. Circulating antibodies to *Hepatozoon canis* demonstrated by immunofluorescence. J. Vet. Diagn. Invest. 6, 121–123.

Singh, H., Jyoti, Haque, M., Singh, N.K., Rath, S.S., 2012a. Molecular detection of *Anaplasma marginale* infection in carrier cattle. Ticks Tick-borne Dis. 3, 55–58.

Singh, H., Haque, M., Jyoti, Singh, N.K., Rath, S.S., 2012b. Occurrence of parasitic infections in dogs in and around Ludhiana, Punjab. Appl. Biol. Res 14, 108–110.

Singh, H., Singh, N.K., Rath, S.S., 2012c. Occurrence of canine hepatozoonosis in and around Ludhiana district (Punjab). Indian J. Canine Pract. 4, 64–66.

Singh, K., Singh, H., Singh, N.K., Kashyap, N., Sood, N.K., Rath, S.S., 2017a. Molecular prevalence, risk factors assessment and haemato-biochemical alterations in hepatozoonosis in dogs from Punjab, India. Comp. Immunol. Microbiol. Infect. Dis. 55, 53–58.

Singh, K., Singh, H., Singh, N.K., Kashyap, N., Rath, S.S., 2017b. Molecular prevalence of *Hepatozoon canis* infection in dog tick, *Rhipicephalus sanguineus*, from Punjab, India. J. Anim. Res. 7, 401–404.

Singh, N.K., Jyoti, Haque, M., Singh, H., Rath, S.S., 2011. Prevalence of canine parasitic infections. Indian Vet. J. 88, 76–77.

Singla, L.D., Sumbria, D., Mandhotra, A., Bal, M.S., Kaur, P., 2016. Critical analysis of vector-borne infections in dogs: *Babesia vogeli, Babesia gibsoni, Ehrlichia canis* and *Hepatozoon canis* in Punjab, India. Acta Parasitol. 61, 697–706.

Thekisoe, O.M.M., Inoue, N., Kuboki, N., Tuntasuvan, D., Bunnoy, W., Borisutsuwan, S., Igarashi, I., Sugimoto, C., 2005. Evaluation of loop-mediated isothermal amplification (LAMP), PCR and parasitological tests for detection of *Trypanosoma evansi* in experimentally infected pigs. Vet. Parasitol. 130, 327–330.

Thekisoe, O.M.M., Rambritch, N.E., Nakao, R., Bazie, R.S., Mbati, P., Naman-gala, B., Malele, I., Skilton, R.A., Jongejan, F., Sugimoto, C., Kawazu, S.I., Inoue, N., 2010. Loop-mediated isothermal amplification (LAMP) assays for detection of *Theileria parva* infections targeting the PIM and p150 genes. Int. J. Parasitol. 40, 55–61.

Wang, L.X., He, L., Fang, R., Song, Q.Q., Tu, P., Jenkins, A., Zhou, Y.Q., Zhao, J.L., 2010. Loop-mediated isothermal amplification (LAMP) assay for detection of *Theileria sergenti* infection targeting the p33 gene. Vet. Parasitol. 171, 159–162.