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Development of loop-mediated isothermal amplification (LAMP) for detection of *Babesia gibsoni* infection in dogs

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ABSTRACT

Diagnosis of canine babesiosis, caused by *Babesia gibsoni* is difficult, especially in chronically infected dogs. A loop mediated isothermal amplification (LAMP) assay was developed and standardized by using four oligonucleotide primers targeting the hypervariable region of 18S rRNA gene (GenBank Acc. no. KC461261). The primers specifically amplified *B. gibsoni* DNA, while no amplification was detected with DNA from non-infected dogs as well as from dogs infected with *Babesia canis vogeli*, *Hepatozoon canis*, *Ehrlichia canis* and *Trypanosoma evansi*. The assay could detect 1.35×10^{-7} parasitaemia and 10^{-4} dilution of recombinant plasmid, equivalent to 12 pg of target DNA. All the samples were tested by nested PCR as well as LAMP assay. LAMP was found to be 10 times more sensitive than nested PCR targeting the same gene. Out of 75 suspected field samples, collected from different parts of the country, LAMP could detect *B. gibsoni* in 43 samples, while nested PCR and microscopy could detect 37 and 23 samples, respectively. High sensitivity, specificity and rapidity of LAMP assay may be exploited for screening large number of samples in a field setting.

1. Introduction

Babesia gibsoni is a tick borne blood protozoan parasite responsible for causing babesiosis in dogs. Since the discovery of *B. gibsoni* from India (Patton, 1910), it has been found to be associated with infection of dogs in Asia, Africa, Australia, Europe and the United States (Irwin, 2010). Acute form of the disease is typically associated with remittent fever, progressive anemia, lethargy, thrombocytopenia, haemoglobinuria, marked splenomegaly and hepatomegaly (Wozniak et al., 1997). However, chronic infection of *B. gibsoni* without apparent clinical manifestation is more common and diagnosis at this stage is very difficult (Irwin, 2010).

Traditionally, diagnosis of *B. gibsoni* is based on light microscopic demonstration of intra-erythrocytic piroplasms in Giemsa-stained blood smears. However, in subclinical or latent infection, this may be difficult due to its poor sensitivity (Goo et al., 2008). Recently, molecular technique, such as polymerase chain reaction (PCR) has been used with good sensitivity and specificity even during early or carrier stage of infection (Ano et al., 2001; Birkenheuer et al., 2003). This method is limited by its requirement of precision equipments, cost involved and accessibility in field condition. Alternatively, serological tests like immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) with whole parasites or native antigen have also been proved to be useful for the diagnosis of chronic infections and in field surveys but they have limited application due to poor quality of antigens and cross reactivity (Aboge et al., 2007). Of late, recombinant protein based ELISA and

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immunochromatographic tests (ICT) have been attempted with satisfactory results (Aboge et al., 2007; Goo et al., 2012). However, these tests fail to detect the antibody titers in early stage of infection.

Recently, a novel new generation DNA amplification method, loop-mediated isothermal amplification (LAMP), a rapid, specific, sensitive and simple technique is being used for early diagnosis of infectious agents (Notomi et al., 2000; Parida et al., 2008; Karanis and Ongerth, 2009). Compared to PCR and real-time PCR, LAMP has advantages of reaction simplicity and higher amplification efficiency and does not require high precision instruments. Beside this, the LAMP products can be evaluated easily by visualization of the turbidity (Mori et al., 2001). For animal parasites, LAMP has been developed successfully for diagnosis of toxoplasmosis, trypanosomosis, theileriosis, babesiosis (*B. orientalis* and *B. caballi*), cryptosporidiosis (Karanis et al., 2007) and tritrichomonosis (Alhassan et al., 2007; He et al., 2009; Krasteva et al., 2009; Oyhenart et al., 2013; Thekisoe et al., 2005, 2010; Wang et al., 2010). There is only one report in which LAMP assay was evaluated for detection of *B. gibsoni* infection in dogs (Ikadai et al., 2004). Despite advances in diagnostic methodologies for infectious diseases, most cases of haemoprotozoan infections are still being diagnosed by microscopic examination only, a technique that is limited by its low sensitivity. Therefore, there is urgent need to develop a highly sensitive and specific diagnostic tool for *B. gibsoni* infection in dogs.

In the present study, an attempt was made to develop LAMP assay targeting the 18S rRNA gene for the diagnosis of *B. gibsoni* infection in dogs and its cross reactivity was evaluated with other common haemoparasites of dogs. The sensitivity of LAMP assay was also compared with the results of microscopy and nested PCR.

2. Materials and methods

2.1. Collection of blood samples

Thirteen whole blood samples from dogs, naturally infected with *B. gibsoni*, as confirmed by microscopy and nested PCR, were collected from different regions of India and were used as known positive control. Out of these, the parasitaemia was determined in one sample as 13.5% by counting the number of infected erythrocytes per microscopic field in a Giemsa-stained blood smears under light microscope. Fifty such fields were examined and the average of the observations was calculated for determining the level of parasitaemia. Thirteen blood samples from healthy dogs reared in the tick free enclosure of Animal Nutrition Division, Indian Veterinary Research Institute, India, and free from *B. gibsoni* infection as confirmed by microscopy as well as by nested PCR, were used as known negative samples. Blood samples from dogs infected with *B. canis vogeli*, *Ehrlichia canis*, *Hepatozoon canis* ($n = 3$ each) and *Trypanosoma evansi* ($n = 2$) were also collected from Referral Polyclinic, Indian Veterinary Research Institute, Izatnagar, Bareilly, UP for studying the cross reactivity of LAMP assay.

All the 75 suspected blood samples of dogs were collected from northern (Punjab, Uttar Pradesh and Uttarakhand) and eastern parts (Assam and West Bengal) of India. All these blood samples were collected from pure-bred dogs with EDTA as anticoagulant and were used for DNA extraction.

2.2. DNA extraction

Genomic DNA was extracted from 300 μl of whole blood of each sample using the Genomic DNA Mini Kit (IBI Scientific, USA) in accordance with the manufacturer's instructions and was stored at −20 °C for further analysis.

2.3. Microscopy and nested PCR (nPCR)

Thin blood smears prepared from all blood samples were stained with Giemsa stain and were examined under oil immersion lens (1000×) for detection of intraerythrocytic small piroplasms. The universal *Babesia* genus specific primer set, B18S-F and B18S-R was used in the primary PCR to amplify the partial sequence of 1665 bp of 18S rRNA gene using 4 μl of extracted DNA following the method described by Ikadai et al. (2004). *B. gibsoni* specific nested PCR was performed to detect 327 bp fragment from primary PCR product using primers PIRO2-F and PIRO2-R, as described by Ano et al. (2001) with certain modifications. In brief, nPCR reaction was carried out in a standard 25 μl reaction mixture containing 2.5 μl of 10× PCR Green buffer, 0.2 μl of Dream *Taq* DNA polymerase (Thermo Scientific, USA), 0.2 mM concentration of dNTPs mixture, 10 pmol of each primer and 1 μl of primary PCR product. The modified PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 30 amplification cycles (95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min) and a final extension step at 72 °C for 10 min. The nPCR products were analyzed by agarose gel electrophoresis using 2% agarose gel stained with ethidium bromide.

2.4. LAMP assay

2.4.1. Primer designing

The best set of four LAMP primers viz., F3, B3, FIP and BIP (Table 1), targeting the hypervariable region of 18S rRNA gene (GenBank accession no. KC461261) of *B. gibsoni*, were designed using Primer explorer version 4 software (http://primerexplorer.jp/e/). All the primers were checked for their qualities such as T_m value, length of the primers, length between primers, presence of self and cross dimmers and for their specificity in BLAST program of NCBI.

2.4.2. Preparation of plasmid DNA

The hypervariable region of 18S rRNA gene of *B. gibsoni* between two outer primers of LAMP, F3 and B3 was cloned in pTZ57R/T vector and sequenced. PCR reaction was optimized in 25 μl reaction mixture of 1X PCR buffer (Thermo Scientific, USA) containing 0.2 μl of Dream *Taq* DNA polymerase (Thermo Scientific, USA), 0.2 mM of each dNTPs mixture, 10 pmol of each primer and 2 μl of template DNA (positive for *B. gibsoni*). The PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 34 amplification cycles (95 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min) and a final extension step at 72 °C for 10 min. The amplification of PCR product was checked by