Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 11, pp.23265-23267, November, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

USE OF SEMI-NESTED PCR FOR THE DIAGNOSIS AND MOLECULAR IDENTIFICATION OF BABESIAGIBSONI INFECTION IN DOGS

***Vishnurahav, R. B. UshaNarayana Pillai, Alex, P. C., Ajithkumar, S. and Lusy sabu**

Department of Clinical Veterinary Medicine, CV&AS, Mannuthy- 680 651
Kerala Veterinary and Animal Sciences University, Pookode, Wayanad - 673 576

ARTICLE INFO

Article History:
Received 27th August, 2015
Received in revised form
22nd September, 2015
Accepted 07th October, 2015
Published online 30th November, 2015

Key words:

Babesiagibsoni,
18SrRNA, PCR,
Semi-nested PCR.

ABSTRACT

Diagnosis of canine babesiosis, caused by Babesiagibsoni is difficult, especially in dogs with chronic infection or carrier. A Semi-nested Polymerase Chain Reaction assay was developed and standardized by using three oligonucleotide primers targeting the hypervariable region of18SrRNA gene. The specific primers amplified theB. gibsoniDNA, while no nonspecific amplification was detected with DNA from non-infected dogs as well as from dogs infected with other species. All the samples were tested by blood smear examination as well as semi-nested PCR assay. Semi-nested PCR assay was found to be more sensitive than blood smear examination in diagnosis and molecular confirmation of Babesiagibsoni. Out of 273 suspected blood samples, collected from dogs presented to the Teaching Veterinary Clinical complex, Mannuthy, Kerala, India and 34 were confirmed for B.gibsoniby semi-nested PCR.Sequencing of 183bp PCR product revealed that the amplified product was from a region of 18SrRNA gene. The sequence obtained when analysed using BLAST revealed 100 per cent homology with query coverage of 100 per cent with the published B. gibsoni (Asia genotype) gene sequence.

***Citation*:** Vishnurahav, R. B. UshaNarayana Pillai, Alex, P. C., Ajithkumar, S. and Lusy sabu, 2015. "Use of semi-nested PCR for the diagnosis and molecular identification of *Babesiagibsoni* infection in dogs" *International Journal of Current Research*, 7, (11), 23265-23267.

INTRODUCTION

The *Babesia* species belongs to the super kingdom eukaryotes, kingdom Protista, phylum Protozoa, subphylum Apicomplexa, class *sporozoea*, order piroplasmida, family *Babesiidae* and Genus *Babesia*. The family consists of several species and in that four species have been described as important in dogs and cats were *Babesiacanis*, *Babesiagibsoni*, *Babesiavogeli* and *Babesiafelis* (Soulsby, 1982). The relatively larger forms referred to as *B. canis* and a smaller parasite, *B. gibsoni*. The larger forms of *Babesia* spp. include *B. caniscanis*, *B. canisrossi* and *B. canisvogeli*. With regard to the smaller piroplasms, three genetically and clinically distinct species are currently recognized causing the disease in dogs. They are *B. gibsoni*, *B. conradae* and *B. microti* like piroplasm (named *Theileriaannae*). *Babesiagibsoni* has a worldwide distribution and is transmitted by *Haemaphysalis* spp. with a variable degree of virulence (Augustine, 2013). *Babesiagibsoni* was first reported in hounds and jackals in India (Patton, 1910) and since then it was recognized in Asia, America, northern and eastern. Africa and rarely in Europe (Zahler *et al*., 2000). *Babesiagibsoni*, a smaller form of parasite causing canine babesiosis was reported principally in the Middle East, Southern Asia, Japan, North Africa and South America, and considered as an emerging infection in the United States of America as well as having been detected later in Australia, Hungary and Italy (Muhlnickel *et al*., 2002). Canine babesiosis due to *B. canis* and *B. gibsoni* were first reported from Thrissur in Kerala (Sabu *et al*., 2002; Sabu, 2005).*B. canisvogeli* was highly pathogenic agent while *B. gibsoni* was more frequently reported pathogen in the United States (Birkenheur *et al*., 2005). *Babesiagibsoni* gaining importance by its non-vectoral transmission. Macintire *et al*. (2002) observed four differentmodes of *B. gibsoni*transmission *viz.,*transplacental transmission, the direct transmission of blood during dog bites and dog fighting, direct transmission of blood by iatrogenic means and shipping of dogs from endemic areas. *Babesiagibsoni* piroplasms were pleomorphic and exhibited linear, reticulate (network forming), pyriform, amoeboid, and signet ring forms and the latter was reported as the most common form (Fowler *et al*., 1970; Fukumoto *et al*., 2000).

****Corresponding author: Vishnurahav, R. B.***
Department of Clinical Veterinary Medicine, CV&AS, Mannuthy- 680 651.

MATERIALS AND METHODS

Collection of blood samples

Thirty seven whole blood samples from dogs, naturally infected with *B. gibsoni*, as confirmed by microscopy, were collected aseptically in EDTA containing vacutainers and stored at -4ºC initially and then transferred to -20ºC until used for DNA extraction

DNA extraction

Genomic DNA was extracted from 100µl of whole blood of each sample using the DN easy Blood and Tissue Kit in accordance with the manufacturer's instructions and was stored at −20◦C for further analysis

Microscopy

Thin peripheral smears were prepared from ear tip, air-dried and fixed in methanol. The smears were fixed with methanol for one minute and stained with Giemsa stain (1:10) and were examined under the oil immersion (1000x) objective of a light microscope to detect the presence of intraerythrocytic small babesialpiroplasms.

Polymerase chain reaction

Specific primers

1. Primers (Sigma Aldrich Chemicals, Bangalore)
Genus and species specific primers were selected according to Birkenheuer *et al*. (2003).

Genus specific primers:

OFP: GTCTTGTAATTGGAATGATGGTGAC

ORP: ATGCCCCCAACCGTTCCTATTA

Species specific primers:

Bgib Asia-F: ACTCGGCTACTTGCCTTGTC

PCR Amplification Sequence Analysis

The PCR for the detection of *Babesia*spp. in the blood samples was carried out based on the method of Birkenheuer *et al*. (2003). Extracted DNA (5µl) was used as a template to amplify a fragment of the 18S rRNA gene using the following reaction mix. Twenty five microliter of PCR reaction mixture was pipetted out by using 25pM of each primer (forward and reverse) in the presence of 10x PCR buffer, 2mM dNTP, 1.5mM $Mgcl_2$ and one unit of *Taq* polymerase. One cycle of PCR was consisted of denaturation 95˚C for five minutes, followed by 34 amplification cycles (95˚C for 45 seconds, 59.5˚C for 45 seconds, 72˚C for 45 seconds), and a final extension step at 72˚C for 5 minutes. Five microliter of the PCR product was mixed with 1 µl of 6X loading dye and loaded into the well along with 2 µl molecular marker. The agarose 2 per cent gel was electrophoresed at 60V for one hour and on completion, the gel was analysed in Gel Documentation System (BIO-RAD, USA). PCR products of approximately 343 bp (between 300bp and 400bp) in size was analysed, stained by ethidium bromide and visualized under ultraviolet transilluminator.

Semi nested Polymerase Chain Reaction

The amplicons obtained using genus specific primers as per the PCR assay described above were subjected to semi nested PCR for confirmation of the species. For the semi nested reaction, the reverse primer ORP was used with BgibAsia-F for *B. gibsoni*- specific detection under the reaction conditions described above, except for the following: 2.5 µl from the initial reaction was used as DNA template, annealing was at 60ºC for 45 sec. and the reactions were amplified for 30 cycles. Analysis of the semi nested PCR product 183 bp (between 180bp and 200bp) was done as described above. The PCR product obtained was sequenced at Sci genome, Kochi and the sequences were analysed using Basic Local Alignment Search Tool (BLAST).

RESULTS

Microscopy

Giemsa staining

Giemsa stained smears revealed pleomorphic *B. gibsoni* organisms that appeared mostly as signet-ring shaped inside the erythrocytes and measured 1.2 to 1.9 x 0.7 to 1.1 µm (1.4 x 0.8 µm). Both single and multiple forms of annular or oval trophozoites were observed in some of the smears (Fig. 1).

Polymerase Chain Reaction and sequencing of amplicon

Thirty seven samples were subjected to Polymerase Chain Reaction (PCR) analysis by using genus specific outer primer pair's 455-479F, 793-772R which revealeda fragment between 300 and 400 bp (expected product size was 340 bp) amplified product (Fig. 2).The amplicons of the genus specific PCR were used as template in a semi nested PCR reaction using *B. gibsoni* species specific primer, BgibAsia- F revealed a fragment between 100 and 200 bp (expected size was 183 bp) which was considered confirmatory for *B. gibsoni* (Fig. 3).Sequencing of 183 bp PCR product revealed that the amplified product was from a region of 18S rRNA gene. The sequence obtained when analysed using BLAST revealed 100 per cent homology with a query coverage of 100 per cent with the published *B. gibsoni* (Asia genotype) gene sequence.

DISCUSSION

Giemsa Staining

Giemsa stained smears revealed pleomorphic *B. gibsoni* organisms which appeared mostly as signet-ring shaped inside the erythrocytes and measured 1.2 to 1.9 x 0.7 to 1.1 µm (1.4 x 0.8 µm). Both single and multiple forms, annular or oval trophozoites were observed in some of the smears. These observations were in accordance with findings of (Fowler *et al*.1970; Fukumato *et al*. 2000 and Augustine 2013).

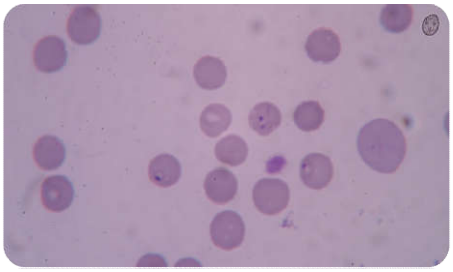

Fig. 1. Signet ring shaped Babesia gibsoni in the erythrocyte, Giemsa's staining (X1000)

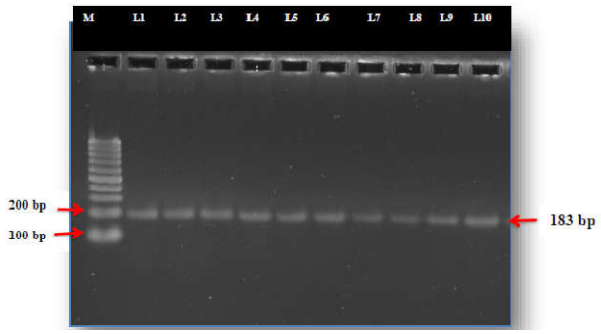

Fig. 2. Agarose gel (2 per cent) showing semi nested PCR amplified product generated with genus and species specific primers. M-100 bp ladder. Lanes, 1, 2, 3, 4, 5,6,7,8,9,10 and 11- Amplified fragment having a size between 100 to 200 bp and 300 and 400bp

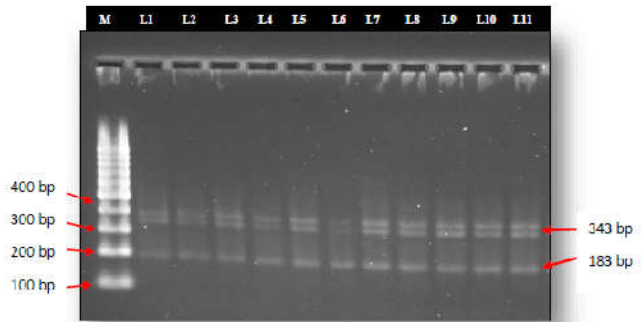

Fig. 3. Agarose gel (2 per cent) showing PCR amplified product generated with B. gibsoni species specific primers. M-100 bp ladder. Lanes, 1, 2, 3, 5,6,7,8,9 and 10- Amplified fragment having a size between 100 and 200 bp

Polymerase Chain Reaction and Sequencing of Amplicon

In the present study, semi nested PCR assay was used for the detection of babesia organism by amplifying a fragment of 18SrRNA gene.

Both genus and species specific amplifications were carried out using primers reported by Birkenheuer *et al*. (2003). Genus specific PCR revealed 340bp fragment for genus *Babesia*. Semi nested PCR using species specific primers produced a 185bp fragment for *B. gibsoni*. Criado-Fornelio *et al*. (2003) had stated that semi nested PCR demonstrated a high level of piroplasms infection in symptomatic animals. Semi nested PCR approach is useful to detect infection of canine babesiosis with low parasitemia including carriers because of its high sensitivity as stated by Birkenheuer *et al*. (2003)

In conclusion, semi-nested PCR assay with high sensitivity and specificity, targeting a fragment of 18SrRNA for detection of *B. gibsoni* infection in dogs was developed and evaluatedin field samples. The assay was able to detect low level of parasitaemia and thus can be used to detect carrier animals, which act as source of infection for healthy dogs in endemic areas. The assay may also be used to screen dogs destined for import in non-endemic countries.

REFERENCES

Augustine, S. 2013. The Morphological and Molecular detection of *Babesia spp*. in dogs in Thrissur.M.V.Sc thesis. Kerala Veterinary and Animal Sciences University, Thrissur. 19, 21, 22p.

Aysul, N., Ural, K., Ulutas, B., Eren, H. and Karangenc, T. 2013. First detection and molecular identification of *Babesiagibsoni* in two dogs from the Aydin Province of Turkey. *Turk.J. Vet. Anim. Sci.,* 37: 226 -229.

Birkenheuer, A.J., Correa, M.T., Levy, M.G. and Breitschwerdt, E.B. 2005. Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2002-2003). *J. Am. Vet. Med. Ass*. 227: 942-947.

Birkenheuer, A.J., Levy, M.G. and Breitschwerdt, E.B. 2003. Development and evaluation of seminested PCR for detection and differentiation of *Babesiagibsoni* (Asian genotype) and *B.canis* DNA in canine blood samples. *J. Clin. Microbiol.,* 41: 4172-4177.

Muhlnickel, C.J., Jefferies, R., Ryan U.M.M. and Irwin, P. J. 2002. *Babesiagibsoni* infection in three dogs in Victoria. *Aus. Vet. J.,* 80: 10.

Patton, W.S. 1910. Preliminary report on a new piroplasam (piroplasmagibsoni sp. nov.) found in the blood of the hounds of the Madras hunt and subsequently discovered in the blood of the jackal *Canisaureus*. *Bull. Soc. Pathol. Exot.,* 3: 274 – 280.

Sabu, L., Baby, P.G., Ajith Kumar, S. and Devada, K. 2002. *Babesiacanis* infections in young pups. *IntasPolivet*., 3: 129-130.

Sabu, L. 2005. Canine babesiosis. *JIVA*. 7:12-14.

Soulsby, E.J.L. 1982. *Helminths, Arthropods and Protozoa of Domesticated Animals.* (7th Ed.). The English language book society and BailliereTindall, London. 809p.

Zahler, M., Rinder, H., Sachein, E. and Gothe, R. 2000. Detection of a new pathogenic *Babesiamicroti*-like species in dogs. *Vet. Parasitol.*89: 241-248.

Fukumoto, S., Xuan, X., Igarashi, I., Zhang, S., Mugisha, J., Ogata, T., Nagasaw, H., Fuijsaki, K., Suzuki, N. and Mikami, T. 2000. Morphological changes of *Babesiagibsoni* grown in canine red blood cells-substituted severe combined immune deficiency mice. *J. Parasito.,* 86: 956-959.

Fowler, J. l., Ruff, M. D. and Hornof, W. F. 1970. Modification of Field's stain for examination of growth forms of *Babesiagibsoni*. *Am. J. Vet. Res.,* 31: 1079 – 1083.

****