Molecular Investigation of Canine Babesiosis in and Around Bhubaneswar, India

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Abstract

Canine babesiosis, a hemolytic protozoan disease represents an important veterinary problem caused primarily by large and small forms of piroplasms of Babesia spp. A molecular-based survey on the overall occurrence of natural Babesia infection in stray (n=98) and pet dogs (n=100) from Bhubaneswar and nearby areas using PCR technique targeting 18s RNA gene fragment along with genetic sequence analysis was carried out. A total of 38 (pet:22, stray:16) samples (19.19%) were found positive for babesiosis based on the amplification of 450 bp amplicon region of the gene while 4 samples (0.02%) showed co-infection with Hepatozoon canis. The sequenced PCR products were submitted to NCBI, and on BLAST analysis the isolates with accession no KT246303, KT246306, KT246307 showing similarity with Babesia vogeli, while KT246305 was identical to B.gibsoni isolates and KT246304 was identical to Hepatozoon canis. This is the first report on the molecular diagnosis of canine babesiosis in the state. PCR assay was found to be more precise over microscopic diagnosis, the use of more specific and sensitive tests along with more samples could aid in a better understanding of the epidemiology of canine babesiosis in this region.

Introduction

Canine babesiosis is an important tick-borne life threatening haemo-protozoan disease caused by the intra-erythrocytic protozoan parasites belonging to the genus Babesia. The Babesia species mainly incriminated for causing disease in canids are the small form (Babesia gibsoni, Babesia conrade, Babesia vulpes) and large form (Babesia canis, Babesia vogeli, Babesia rossi) in different parts of the world (Solano-Gallego et al., 2016). The transmission occurs by Dermacentor reticulatus in Europe, Rhipicephalus sanguineus in tropical and subtropical regions, and Haemaphysalis eliptica in South Africa (Uilenberg, 2006). In Indian subcontinent, canids are infested majorly with Rhipicephalus sanguineus and Haemaphysalis spp. of ticks (Raut et al., 2006; Sahu et al., 2013; Ranju Ravindran et al., 2020).

Detection of Babesia spp. is usually achieved using microscopic examination of stained blood smears, but this technique is limited because of low sensitivity, chronic evolution of the disease, and the difficulty of distinguishing morphologically similar strains and species (Irwin, 2005). The serological test such as the immunofluorescent antibody test is useful but has poor specificity as a result of antigen cross-reactivity (Rani et al., 2011) and fails to identify current infection. The employment of recent biotechnological techniques like PCR, nested & semi-nested PCR, PCR-RFLP, and multiplex PCR has lead to advancement in the detection of this protozoan parasite. The true status of canine babesiosis is still not clear in India barring a few reports (Rani et al., 2011; Sarma et al., 2019) and in the region under study (Sahu et al., 2014) which was mostly based on microscopic examination of blood smears. The present study is a molecular-based investigation of canine babesiosis in Bhubaneswar, Odisha.

Materials And Methods
The study was conducted in and around Bhubaneswar (Odisha) located between 20° 14' 0" North, 85° 50' 0" East, and having an average altitude of 45 m (148 ft) above sea level. The average relative humidity and annual rainfall recorded in the region are 70% and 1,542 mm respectively. Blood samples from 198 dogs presented to Teaching Veterinary Clinical Complex and Animal Birth Control Programme, Bhubaneswar (stray:98, pet:100) belonging to either sex and different age groups, showing tick infestation and clinical signs like pyrexia, anorexia or lethargy were selected for this study. Blood smear examination using Giemsa stain was conducted and the blood samples which were found positive or negative for \textit{Babesia} piroplasms were preserved at -20 ° C for further study using molecular techniques. DNA was isolated from 200 µl of blood (with anticoagulant, EDTA) sampled from each dog using QIAamp DNA Mini kit (Qiagen, Germany) following the manufacturer's instructions. A genus-specific forward primer (PIRO A1 5'-AATACCCAATCCTGACACAGGG-3') and antisense PIRO-B (5'-TTAAATACGAATGCCCCACCG-3') were used for PCR of extracted parasitic DNA. Twenty five µL of reaction mixture comprised of 1 µl each of forward and reverse primer (IDT, 10 pico mole concentration), 2 µl of 2.5 mM dNTPS (Fermentas), 2.5 µl of 10X Taq DNA buffer A (tris with 15 mM of MgCl2, Genei, India), 0.5 µl of Taq DNA polymerase (3 U/µl concentration, Genei, India) and 50 ng of template DNA. Then the rest volume was adjusted to 25 µl by addition of nuclease free water (Genei, Bangalore). The amplification was performed in a thermal cycler (Gene Amp PCR System 9700, Applied Biosystem) with cyclic conditions comprising of an initial denaturation at 94 degrees C for 5 min with 35 rounds of denaturation, primer annealing, and polymerization at 94 °C, 62 °C, and 72 °C, respectively for 30 seconds each. A final chain extension at 72 °C for 6 minutes completed the cycle program. Then the amplified PCR products were analyzed on 1.5% agarose gel (Lonza, 0.5 µg/ml) (120 V, 40 min) and visualized under UV transilluminator (Alpha Innotech, New Delhi). PCR products of the expected nucleotide size were purified with QIAquick PCR Purification Kit according to the manufacturer's instruction (Qiagen, Germany). The purified PCR products were sequenced by the automated DNA sequencer (310 Genetic Analyser, Applied Biosystems) using ABI Prism Dye Terminator kit and both forward and reverse primers, and the sequences obtained were subjected to BLAST analysis. Phylogenetic analysis was done by MEGA X using the Neighbour-joining tree method based on the partial gene sequences of 18S rRNA obtained in the study and reference sequences obtained from the NCBI GenBank database. The number at the node was the proportion of 500 bootstraps.

\textbf{Results}

The most consistent clinical signs observed during the examination were elevated rectal temperature above 39.6°C, anorexia, pale mucous membrane, tick infestation, coughing, and vomiting in 85.71%, 68.57%, 65.71%, 57.14%, 37.14% and 8.57% of dogs under study respectively. On microscopical examination of stained blood smears piroplasms of \textit{Babesia} spp. were detected in 8.08% (16/198) samples. The overall detection of babesiosis by conventional PCR technique was 19.19% (38/198) on the basis of presence of 450 bp amplicon (Fig 1). Four samples showed double band after gel purification having 450 and 520 bp amplicon size indicating mixed infection with \textit{Hepatozoon canis} (Fig 2). The infection in pet dogs (22%) was higher than stray dogs (16.23%).
The nucleotide sequence obtained after sequencing of PCR product was submitted to GenBank and accession numbers KT246303, KT246304, KT246305, KT246306, KT246307 were assigned. BLAST analysis of GenBank revealed that the obtained nucleotide sequences of KT246303, KT246306 and KT246307 were 100% homologous to previously deposited 18S rRNA gene sequences of *B. vogeli*. The sequence of KT246305 showed the best matches (100%) with *Babesia gibsoni* sequences from different regions available in NCBI database. The sequence KT246304 showed 99% similarity with the previously deposited 18S rRNA gene sequences of *H. canis*.

On phylogenetic analysis (Fig 3), the sequences clustered into three distinct clades. *B. vogeli* isolates of the study (KT246303 and KT246307) were clustered together to form a well-defined group with other *B. vogeli* strains from different geographical regions (Brazil, China, Japan, Thailand, Zambia). However, the isolate having accession number KT246305 showed close phylogenetic relationship with the *B. gibsoni* isolate from India (eastern and southern India) and other regions (Japan, USA, West Indies) with a high bootstrap value. The *B. vogeli* isolate (KT246306) of the present investigation represented a separate clade. *Hepatozoon canis* isolate (KT246303) showed a close phylogenic relationship with isolates from different parts of the world (China, Taiwan, Israel).

**Discussion**

The overall prevalence rate of babesiosis on the basis of PCR assay ranged from 4.8 - 56.75% as per reports from different parts of India (Laha *et al.*, 2013; Singh *et al.*, 2014; Sarma *et al.*, 2019) and 2.4 - 88.3% from around the globe (Bastos *et al.*, 2004; Foldvari *et al.*, 2005; Porchet *et al.*, 2007; Corali *et al.*, 2018). These differences might have occurred because of the differences in diagnostic techniques, the population sampled in the study, and climatic as well as managemental factors in locations where the research was conducted. A moderate rate of incidence observed during the present research might be due to the hot and humid environmental condition of the state, which favours the survival of tick vectors and a higher availability of stray dogs that act as transporting medium of the parasites due to their uncontrolled movements.

The prevalence of *B. vogeli* has been recorded in stray and pet dogs in north and south India (Jain *et al.*, 2018; Roopesh *et al.*, 2018; Singla *et al.*, 2016), Europe (Cardoso *et al.*, 2008; Ionita *et al.*, 2012), Africa (M’ghirbi and Bouattour, 2008; Salem and Farag, 2014). *B. vogeli* has also been incriminated as the commonest species in Latin America and Caribbean (Panti-May and Rodriguez-Vivas, 2020). A higher presence of *Babesia vogeli* recorded in our study could be due to the dominating prevalence of *Rhipicephalus sanguineus*, among canids of Bhubaneswar (Sahu *et al.*, 2013). *Rhipicephalus sanguineus* is attributed as natural vector for *B. vogeli* and *H. canis* (Dantas-Torres, 2008; Penzhorn, 2020). The present investigation also detected the presence of *Hepatozoon canis* along with *Babesia sp*, which corroborates with earlier reports from North east India (Sarma *et al.*, 2019). This might be due to the primers Piro A1 and Piro B, which is specific to *Babesia spp.* but could also detect *Hepatozoon* species. The use of primers Babesia F and Babesia R by Oyamada *et al.*, 2005 has also revealed cross-reaction between *Babesia* and *Hepatozoon* species.
Based on sequencing results and BLAST analysis, it was also observed that one sequence (KT246305) showed maximum homogeneity with sequences of *B. gibsoni* with a stray match with *B. canis*. In India, *B. canis* is yet to be reported in any molecular diagnosis possibly due to absence of potential vector. *B. gibsoni* infection in dogs has been reported earlier in blood smear examination from Bhubaneswar (Sahu *et al.*, 2014) while molecular detection have been reported in different regions of India (Singh *et al.*, 2014; Jain *et al.*, 2018; Sarma *et al.*, 2019). The molecular identification of *B. gibsoni, B. vogeli* and *H. canis* from canines of Punjab has been previously described (Singla *et al.*, 2016). On phylogenetic analysis the present three isolates showed an affiliation with other *B. vogeli* isolates from different geographical regions. But one of the isolates showed no affiliation with other *B. vogeli* isolates though it was confirmed to be *B. vogeli* from Blast analysis, which might have originated from a different strain. *B. gibsoni* infected dogs exhibit varying clinical manifestation ranging from subclinical to fatal depending on the host body condition. Though *B. vogeli* is less pathogenic exhibiting moderate symptoms in adult dogs, they cause severe condition in puppies and splenectomised dogs (Wang *et al.*, 2018). The study clearly re-established reliability of PCR as a technique over microscopy. Therefore, molecular diagnosis can facilitate pertinent treatment and control regimen.

**Conclusion**

Traditionally, the presumptive diagnosis is based on fever, anemia and thrombocytopenia, while the microscopic examination still remains the most rapid confirmatory method for diagnosis of canine babesiosis under field condition. PCR assay has been found to be more specific method, could detect even in carrier state where there were no clinical signs and symptoms while co-infection with other vector-borne agents were also recorded. Further studies utilising more sensitive tests and species-specific primers along with a larger number of clinical samples needs to be analyzed to get insight into the epidemiological patterns of canine babesiosis as well as devising an effective control program.

**Declarations**

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References

1. Bastos CV, Moreira S M and Passos LMF (2004) Retrospective study (1998-2001) on canine babesiosis in Belo Horizonte, Minas Gerais, Brazil. Annals New York Academy of Sciences, New York. 158-160.

2. Cardoso L, Costa A, Tuna J, Vieira L, Eyal O, Yisaschar-Mekuzas Y, Baneth G (2008) Babesia canis canis and Babesia canis vogeli infections in dogs from northern Portugal. Vet Parasitology 156:199-204.

3. Corali CA, Gabrielli S, Zahirovic A, Nikola M, Milardi GL, Jazic A, Zuko A, Camo D and Otasevic S (2018) First molecular detection of Babesia canis in dogs from Bosnia and Herzegovina. Ticks and Tick-borne Diseases 9:363–368.

4. Dantas-Torres F (2008) Causative agents of canine babesiosis in Brazil. Preventive Veterinary Medicine 83: 210-211.

5. Ionita M, Mitrea IL, Pfister K, Hamel D, Buzatu CM and Silaghi C (2012) Canine babesiosis in Romania due to Babesia canis and Babesia vogeli: a molecular approach. Parasitology Research 110:1659-1664. doi:10.1007/s00436-011-2683-y

6. Irwin PJ (2005) Babesiosis and Cytauxzoonsis; Arthropode-BornInfectious Diseases of Dogs and Cats, Manson Publishing Ltd.Barcelona, Spain, 1st edition.

7. Jain J, Lakshmanan B, Nagaraj HV, Praveena J E, Syamala K and Aravindakshan T (2018) Detection of Babesia canis vogeli, Babesia gibsoni and Ehrlichia canis by multiplex PCR in naturally infected dogs in South India. Vet. Arhiv 88: 215-224.

8. Laha R, Bhattacharjee K, Sarmah PC, Das M, Goswami A, Sarma D and Sen A (2013). Babesia infection in naturally exposed pet dogs from a north-eastern state (Assam) of India, detection by microscopy and polymerase chain reaction. Journal of Parasitic Diseases 38:45-49.

9. M'ghirbi Y and Bouattour A (2015) Detection and molecular characterization of Babesia canis vogeli from naturally infected dogs and Rhipicephalus sanguineus ticks in Tunisia. Vet Parasitology 152:1-7. doi: 10.1016/j.vetpar.2007.12.018.

10. Oyamada M, Bernard D B, Boni M, Dereure J, Bucheton B, Hammad A, Itamoto K, Okuda M, Inokuma H(2005) Detection of Babesia canis rossi, B. canis vogeli and Hepatozoon canis in Dogs in a Village of Eastern Sudan by Using a Screening PCR and Sequencing Methodologies. Clinical and Diagnostic Laboratory Immunology 12:1343-1346.
11. Panti-May J A and Rodriguez-Vivas RI (2020) Canine babesiosis: A literature review of prevalence, distribution, and diagnosis in Latin America and the Caribbean. Veterinary Reports 21:100417. https://doi.org/10.1016/j.vprsr.2020.100417.

12. Penzhorn BL (2005) Don't let sleeping dogs lie: unravelling the identity and taxonomy of Babesia canis, Babesia rossi and Babesia vogeli. Parasite and Vectors 13: 184. https://doi.org/10.1186/s13071-020-04062-w

13. Porchet M J, Sager H, Muggll L, Oppliger A, Muller N, Frey C and Gottstein B A (2007) Descriptive epidemiological study of canine babesiosis in the Lake Geneva region. Schweiz Arch Tierheilkd 149: 457-465.

14. Rani P, Irwin PJ, Coleman GT, Gatne M and Traub RJ (2011) A survey of canine tick-borne diseases in India. Parasites and Vectors 4:1-8.

15. Ranju Ravindran MS, Roberta I, Maria Stefania L, Loredana C, Muthusamy R, Vito C and Domenico O (2020) Canine vector-borne pathogens from dogs and ticks from Tamil Nadu, India. Acta Tropica 203: 105308.

16. Roopesh MP, D'souza EP and Mamatha GS (2018) Molecular Detection and Characterization of Canine Babesia Spp. from South India. International Journal of Pure App. Bioscience 6: 924-930 doi: http://dx.doi.org/10.18782/2320-7051.7160.

17. Sahu A, Mohanty B, Panda MR, Sardar KK and Dehuri M (2013) Prevalence of tick infestation in dogs in and around Bhubaneswar. Veterinary World 6: 982-985.

18. Sahu A, Mohanty B, Panda M and Sardar KK (2014) Incidence of haemoprotozoan parasites in dogs in and around Bhubaneswar. Indian VetJournal 91: 93-95

19. Salem NY and Farag HS (2014) Clinical, Hematologic, and Molecular Findings in Naturally occurring Babesia canis vogeli in Egyptian Dogs. Veterinary Medicine International. http://dx.doi.org/10.1155/2014/270345.

20. Sarma K, Nachum-Biala Yand Kumar M (2019) Molecular investigation of vector-borne parasitic infections in dogs in Northeast India. Parasites and Vectors 12: 122. https://doi.org/10.1186/s13071-019-3389-8

21. Singh A, Singh H, Singh NK, Singh ND and Rath SS (2014). Canine Babesiosis in Northwestern India. Molecular Detection and Assessment of Risk Factors, BioMed Research International, 1(2014):1-5.

22. Singla LD, Sumbria D, Mandhotra A, Bal MS and Kaur P (2016) Critical analysis of vector-borne infections in dogs: Babesia vogeli, Babesia gibsoni, Ehrlichia canis and Hepatozoon canis in Punjab India. Acta Parasitologica, 61697-706. doi: 10.1515/ap-2016-0098.

23. Solano-Gallego L, Sainz A, Roura X, Estrada Pena A and Miro G (2016). A review of canine babesiosis: the European perspective. Parasite and Vectors 9:336.

24. Uilenberg G (2006). Babesia - a historical overview. Veterinary Parasitology 1383-10.

25. Wang J, Liu J, Yang J, Liu, Z., Wang, X., Li , Y., Guan, G. & Yin, H. (2019) Molecular detection and genetic diversity of Babesia canis canis in pet dogs in Henan Province, China. Parasitol Int.71:37-40. doi:10.1016/j.parint.2019.03.011
Figures

Figure 1

Electrophoresis gel showing lanes from left to right, L1 to L13, PCR product showing positive for Babesia spp. (450 bp); 100bp DNA ladder
Figure 2

Electrophoresis gel showing lanes from left to right, L1, double band (450 bp and 520 bp) L2 to L4, PCR product showing positive for B abesia spp. (450 bp); L5, 100bp DNA ladder
Figure 3

Phylogenetic Analysis using MEGA X by Neighbour-joining tree method based on the partial gene sequences of 18S rRNA