

Original Research Article

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First Molecular Detection of *Anaplasma platys* and Coinfection with *Babesia gibsoni* in Dogs from Bengaluru, India

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ABSTRACT

Keywords

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Anaplasmosis, a tick-borne disease in dogs, caused by obligate intracellular pathogens, *Anaplasma platys* and *Anaplasma phagocytophilum*. Anaplasmosis is characterized by a variety of clinical symptoms including pyrexia as well as non-symptomatic thrombocytopenia. Advances in molecular techniques have enabled physicians and diagnosticians to use polymerase chain reaction for identification of these pathogens in dogs. A total of 80 samples collected from the veterinary clinics in Bengaluru were considered for the study for screening *Anaplasma platys* and *Anaplasma phagocytophilum* by polymerase chain reaction. Seventeen out of eighty samples were positive for *A. platys*. Co-infection of *A. platys* with *Babesia gibsoni* and *Ehrlichia canis* was also reported in this study. *Anaplasma platys* positive samples were confirmed by sequencing and submitted to GenBank. No samples were found positive for *Anaplasma phagocytophilum* in the current study.

Introduction

Anaplasmosis is an emerging infectious tick-borne disease caused by intracellular rickettsial organisms of the genus *Anaplasma* (Harvey *et al.*, 1978). To date, *Anaplasma platys* and *Anaplasma phagocytophilum* have been reported in dogs. *Anaplasma platys*, known to cause canine infectious cyclic thrombocytopenia, where in *Anaplasma phagocytophilum* causes granulocytic anaplasmosis (Abd Rani *et al.*, 2011). Most commonly observed clinical signs include weight loss, anorexia, pale mucous membranes, high fever, lethargy, lymphadenopathy and splenomegaly (Harrus *et al.*, 2005).

In India, most canine tick-borne diseases are diagnosed by traditional methods, as in microscopic examination of blood smears and serological tests (Abd Rani *et al.*, 2010). These approaches have limitations and do not allow reliable identification of the parasites as both false positive and false negative results may interfere with the interpretations. Thus, methods based on analysis of DNA sequences are very efficient tools for the detection and characterization of pathogenic agents in Dogs. Literatures or reports on prevalence of Anaplasmosis in dogs of Bengaluru is scant or not reported so far.

This study aims to determine the prevalence of *Anaplasma platys* and *Anaplasma phagocytophilum* in domestic dogs in Bengaluru, which were submitted for routine haemoprotozoan screening in dogs by molecular analysis of 16srRNA sequence comparison.

This test panel (canine tick fever panel) included screening of *Babesia canis. canis*, *Babesia gibsoni*, *Babesia canis. vogeli*, *Ehrlichia canis*, *Anaplasma platys* and *Anaplasma phagocytophilum*.

Materials and Methods

Blood samples

A total of 80 whole blood samples were submitted to Vetlesions Veterinary Diagnostic Laboratory, Bengaluru, India for screening canine haemoprotozoan diseases from various veterinary clinics in Bengaluru. From the whole blood (1mL) thin smears were made and stained with Giemsa for evaluation of intracellular parasites by standard microscopic methods. Following which the whole blood samples were processed for DNA extraction.

DNA extraction

For each sample, DNA was extracted from 250µL of whole blood according to the manufacturer's instructions provided by M/s. Omega, genomic blood DNA purification kit. Extracted DNA samples were stored at 4°C until use.

PCR assay

The primers targeting 16s rRNA of *Anaplasma platys* and *Anaplasma phagocytophilum* developed by Hancock *et al.*, 2001; Beall *et al.*, 2008 were used in this study. Extracted DNA of 1.5µL was added to 48mL reaction mixture comprising M/s. Takara Bio, EmeraldAmp GT PCR master mix and primers. Amplification was performed using a GeneAmp 9700 thermal cycler (Applied biosystems). An initial denaturation step at 95°C for 5min was followed by 40 cycles of denaturation at 95°C for 1min, annealing at 58°C for 1min and extension at 72°C for 1min. Final extension was done at 72°C for 5min followed by a hold step at 4°C. Amplified DNA was subjected to electrophoresis in a 1.2% agarose gel (100V, 1h), pre-stained with ethidium-bromide and viewed under ultra-violet light.

Sequencing

Two PCR products (378bp) positive of *A.platys* was randomly selected and were sequenced. After purification with QIAquick Gel Extraction Kit (Qiagen), Big Dye Terminator BDT™ v3.1 kit (Applied biosystems) was used for DNA sequencing reactions. Samples were then analysed using an Applied Biosystems 3730xL genetic analyser. Obtained sequences were checked with ABI analyser and compared to the sequence data available from GenBank, using NCBI blast. New sequence was submitted to GenBank database.

Results and Discussion

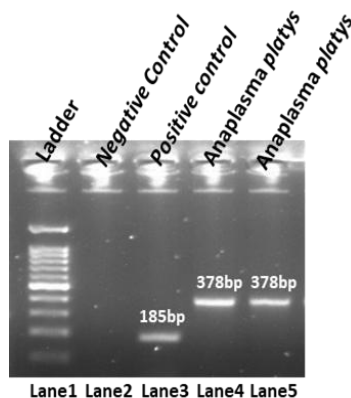
In total, 80 whole blood samples of dogs were screened for haemoprotozoan diseases using both conventional bright field microscopy and PCR. Blood smear examination of all the samples were negative for *Anaplasma* sp. Whereas, 17 out of 80 dogs (n=17/80) were

positive for *Anaplasma platys* by PCR with a band evident at 378bp (Fig.1). No amplicons were detected for *Anaplasma phagocytophilum* from the above sample size. Two *Anaplasma platys* positive samples were sequenced. The two sequences showed 100% homology to each other. Obtained sequence was compared to the sequence data available from GenBank, using NCBI blast and revealed the highest similarity (100%) with the available *Anaplasma platys* 16s rRNA partial sequence bearing accession number MH620179.1. The New sequence obtained from the current study was submitted to GenBank (accession number: MN994319).

Co-infection

Out of seventeen(n=17/80) *Anaplasma platys* positive samples, seven(n=7/17) showed *Babesia gibsoni* infection and five(n=5/17) showed *Ehrlichia canis* infection, which were diagnosed by PCR.

Fig.1 The PCR amplified products were run on 1.2% agarose gel depicting the amplified products of *Anaplasma platys* in Lane 4 and Lane 5. *Babesia gibsoni* from canine was used as a positive control in this experiment



Diagnosis of Haemoprotozoan infections is difficult to be achieved in a clinical setting. Inconsistent microscopic examination and unstandardized serologic assays does not

support species specific diagnosis of the piroplasm's, further complicating the condition in dogs (Birkenheuer *et al.*, 1998). In the present study, we describe a PCR

protocol for the diagnosis of *Anaplasma platys* and *Anaplasma phagocytophilum* in dogs. Out of 80 samples collected from dogs, suspected for Haemoprotozoan infections, seventeen (n=17/80) were positive for *Anaplasma platys* by PCR. None of the samples turned out to be positive for *Anaplasma phagocytophilum*. These findings support the conclusion that *Anaplasma platys* is an etiologic agent of canine anaplasmosis in Bengaluru, India. *A. platys* was first reported in the United States in 1978 (Harvey *et al.*, 1978). Since then *A. platys* has been described in several countries as the etiologic agent of cyclic thrombocytopenia in dogs (Abarca *et al.*, 2007). Clinically, manifestations of canine anaplasmosis ranges from mild to severe symptoms and often asymptomatic (Harvey *et al.*, 1978; Aguirre *et al.*, 2006; Fuente *et al.*, 2006; Huang *et al.*, 2005), hence it becomes important to consider *Anaplasma platys* as one of the etiological agent in tick fever panel for diagnosis. A study reported that *Anaplasma platys* are often coinfecting with *Ehrlichia canis* in dogs causing pronounced anaemia and thrombocytopenia, when compared to the sole infection with either pathogen (Simpson *et al.*, 1991). In our study, among seventeen *Anaplasma platys* positive samples, seven showed *Babesia gibsoni* infection and five showed *Ehrlichia canis* infection potentiating disease pathogenesis, thereby altering clinical manifestations typically associated with singular infections. These factors complicate diagnosis, treatment and can adversely influence prognosis. But in this study we reported co-infection of *Anaplasma platys* with *Babesia gibsoni*, which has not been reported so far. Reports on co-infection of *Anaplasma platys* and *Babesia gibsoni* is scant or unavailable. Hence this study becomes the first such report on detection of coinfection of *Anaplasma platys* and *Babesia gibsoni*. Blood smear examination for all samples were negative, but still turned out to be positive for PCR, thus serving as an

efficient tool in the diagnosis of *Anaplasma* sp. in Dogs. This False negative blood smear results could be due to initial stages of infection and treatment given prior to sample collection. The results indicated that the PCR was specific for *Anaplasma platys* and displayed enhanced sensitivity to reduce the incidence of reporting false negative results. The sequence analyses confirmed a higher integrity of specificity. Thus, PCR would improve the diagnostic capabilities for the detection and differentiation of canine *Anaplasma* spp. in clinical samples and facilitate future research studies that assess canine infection with these organisms.

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