The Pharma Innovation Journal 2023; 12(4): 420-422

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(4): 420-422

www.thepharmajournal.com
Received: 25-01-2023
Accepted: 28-02-2023

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Standardisation of duplex PCR for detection of *Babesia canis vogeli* and *Ehrlichia canis* infection in dogs of Jammu

Sabhyata Sharma, Anish Yadav, Vikas Yadav, Harsh Sharma, Swapnil Magar and Nitin Mishra

Abstract
Babesia canis vogeli and *Ehrlichia canis*, are two canine haemoprotozoans transmitted by ticks, *Rhipicephalus sanguineus* in India. The present report deals with the standardisation and diagnosis of *Babesia canis vogeli* and *Ehrlichia canis* infection in dogs suspected with babesiosis and ehrlichiosis. The canines showing symptoms of high fever, anorexia, debility, anaemia, hind limb swelling were selected and diagnosed using duplex PCR for simultaneous detection of *Babesia canis vogeli* and *Ehrlichia canis*, using species specific primers. The results confirmed the standardisation of duplex PCR for simultaneous detection of *Babesia canis vogeli* and *Ehrlichia canis*.

Keywords: *Babesia canis vogeli*, *Ehrlichia canis*, *Rhipicephalus sanguineus*, duplex PCR, standardisation

Introduction
India having a tropical climate with wide range of flora and fauna, favours propogation of various pathogenic organisms including ectoparasites which harbor and transmit various haemoparasitic organisms. The increasing population of dogs has increased the risks of transmission of various haemoprotozoan diseases which poses serious health problems in dogs (Okubanjo *et al*., 2013 and Vairamuthu *et al*., 2014) [1, 2]. Among the various ectoparasitic infestations, ticks are considered to be the major problem in dogs as they are the leading cause of transmitting pathogenic agents such as bacteria, virus, rickettsia and protozoa. Ticks being haematophagous, attaches to the host and facilitates the transmission of infection by releasing sporozoites in the blood stream of the canines (Kamani *et al*., 2013) [3]. Amongst the various canine haemoprotozoan diseases, Babesiosis is considered as one of the most important, clinically significant and life threatening tick borne haemoprotozoan disease of dogs. The disease is caused by an apicomplexan parasite of genus *Babesia*, which is distributed worldwide, including India. On the basis of geographical distribution and vector specificity, studies revealed that large piroplasms are further subdivided into three species, namely, *B.canis, B.vogeli* and *B.rossi* which are transmitted by *Dermacentor reticulates* in Europe, *Rhipicephalus sanguineus* in Tropical and Sub tropical regions and *Haemaphysalis elliptica* in South Africa, respectively (Bilwal *et al*., 2017 and Jongejan *et al*., 2018) [5, 4] while *Ehrlichia* genus comprises obligate intracellular tick borne rickettsial organism that resides and replicates in circulating monocytes and macrophages. The disease is transmitted by *Rhipicephalus sanguineus* in India, *Amblyomma, Hemaphysalis* and *Dermacentor* in other countries. Three main species that are mostly encountered all over the world are *Ehrlichia canis, Ehrlichia ewingii* and *Ehrlichia chaffeensis*, but *Ehrlichia canis* is the main cause of canine monocytic ehrlichiosis or tropical canine pancytopenia in Indian sub continent (Abd Rani *et al*., 2010) [8].

Material and Method
A total of 50 blood samples were collected from dogs suspected for canine monocytic ehrlichiosis or babesiosis. The samples were selected from dogs having high fever, lethargy, anorexia, debility, anaemia, emaciation, tick infestation, hind limb swelling and coffee colored urine. Blood samples were collected in EDTA vials for molecular studies while a drop of fresh blood was taken on clean grease free slide for conventional microscopy. The slides were stained with Giemsa stain using standard protocol and examined under oil immersion for

microscopic examination and the results were further compared with PCR for confirmatory diagnosis. The genomic DNA was isolated from blood collected in EDTA vials using Favorgen DNA extraction kit following the manufacturer's protocol and kept at -20 ºC till further use. For Duplex PCR assay, the cyclic conditions were optimised using various gradients of annealing temperatures and after standardisation of Duplex PCR, a 20 µl of reaction for simultaneous detection of *Babesia canis* and *Ehrlichia canis*, were prepared using species specific primers for confirmatory diagnosis as described by Kaur *et al*., 2020 [6].

Results

In the present study, Giemsa stained peripheral blood smears revealed that 12% (6/50) samples were positive for piroplasms of *Babesia canis* (Fig.1) while 18% (9/50) were found positive for morula of *Ehrlichia canis* (Fig.2). For standardization of Duplex PCR assay all the samples including the positive cases were subjected to duplex PCR using standardised cyclic conditions targeting *Babesia canis vogeli* and *Ehrlichia canis* which confirmed the diagnosis of both *E.canis* and *B.canis vogeli* in single reaction (Fig.3). The results confirmed that 22% (11/50) and 28% (14/50) of samples were positive for *Babesia canis vogeli* and *Ehrlichia canis* while 8% (4/50) were positive for mixed infection with *Ehrlichia canis* and *Babesia canis vogeli*.

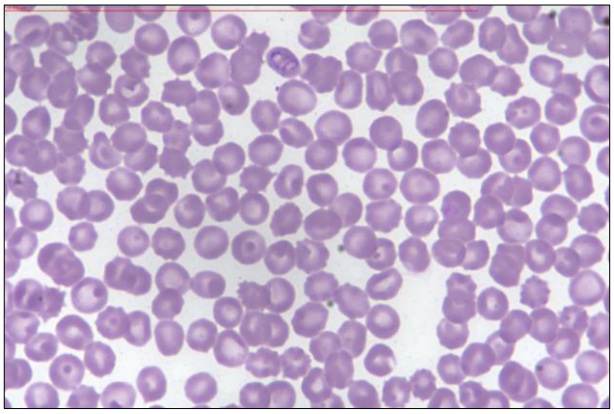

Fig 1: Piroplasms of *Babesia canis* in blood smear (100X)

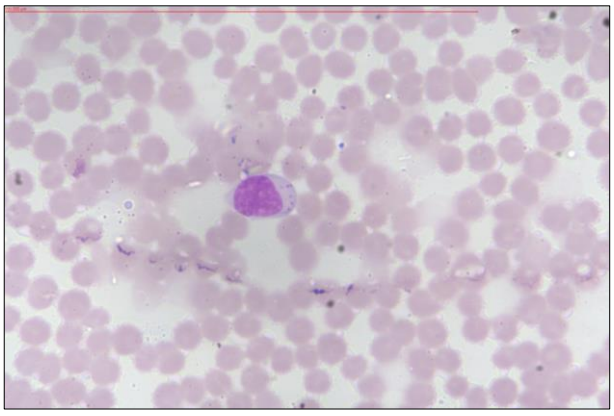

Fig 2: Morula of *Ehrlichia canis* in blood smear (100X)

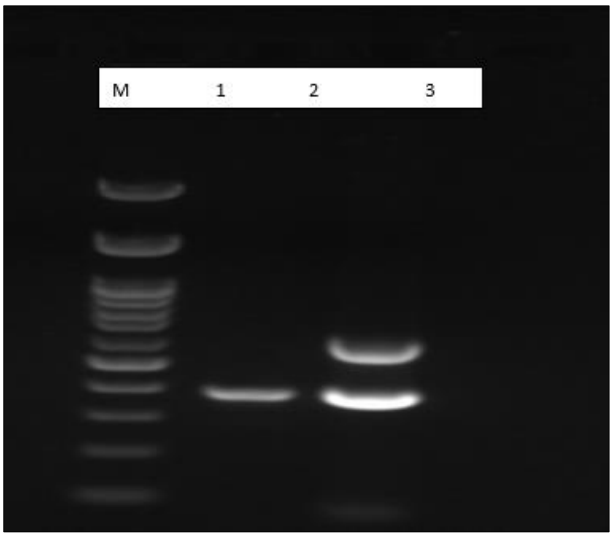

Fig 3: PCR amplification of *E.canis* (16S rRNA) and *B.canis vogeli* (18S rRNA) gene. Lane M: 100 bp DNA ladder, Lane 2: *B.canis* and *E.canis*, Lane 3: Negative control

Discussion

In tropical and subtropical areas of the world, including India, ticks and diseases carried by ticks constitute a significant health barrier for companion dogs. *Babesia canis vogeli* and *Ehrlichia canis* are the most important haemoprotozoan and rickettsial infections of dogs which are maintained and transmitted by brown dog tick, *Rhipicephalus sanguineus* (Aktas *et al*., 2013) [7]. In the present study, (24/50) dogs were found positive for *Ehrlichia canis* and *Babesia canis vogeli* while the *Ehrlichia canis* was recorded with highest prevalence followed by *Babesia canis vogeli.* Similar findings were also reported by Himalini *et al*., (2018) [9] and Kumar *et al*., (2020) [10]. Diagnosis of both *Ehrlichia canis* and *Babesia canis vogeli* by duplex PCR was found helpful in early and quick detection of parasites even in latent or subclinical phases.

Conclusion

The present study reported higher prevalence of *Ehrlichia canis* in canines from Jammu region while standardization of Duplex PCR for simultaneous detection of *Ehrlichia canis* and Babesia *canis vogeli* using species specific primers confirmed that the molecular detection by using duplex PCR is helpful in detecting infection in single cyclic conditions which makes the duplex PCR as quick and fastest method of diagnosis of infection even in latent, subclinical phase of disease. The results of PCR concluded that PCR is highly sensitive, specific and efficient diagnostic assay as compared to conventional blood smear examination and can be used to detect latent infections.

Acknowledgment

The authors acknowledge the support from the Department of Veterinary Parasitology and especially thank Dr. Rajesh Aggarwal and Dr. Indu Bhushan for helping in sample collection presented for the purpose of this study.

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