INVESTIGATION OF THE 18S RRNA GENE SEQUENCE OF *Hepatozoon canis* DETECTED IN INDIAN DOGS

SINGLA L.D., DEEPAK SUMBRIA*, AJAY MANDHOTRA, BAL M.S.[A] AND PARAMJIT KAUR

Department of Veterinary Parasitology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004

* sumbria.deepak31@gmail.com, deepak_sumbria@yahoo.com

ABSTRACT. Canine hepatozoonosis is a growing tick-borne disease in Punjab. Two canine hepatozoonosis cases, one clinical and one subclinical, in Punjab were analyzed by PCR targeting 18S rRNA gene (666 bp). After sequence analysis of the PCR products, both of them were found almost identical to each other and were closely related to the *Hepatozoon canis* strain found in Saint kitts and Nevis and Brazil with 100% (442/442) and 99% (440/442) nucleotide identity respectively. Isolates from Malta and Philippines of *H. canis* were distantly related to Indian *H. canis* with 437/442 and 436/442 match identities. These results suggest that *H. canis* detected in north Indian dogs might have closer ancestral relationship with Saint Kitts and Nevis followed by Brazil strain. This is the first molecular characterization of *Hepatozoon* from Punjab, India.

Keywords: Hepatozoon canis, canines, phylogenetic analysis, PCR, Punjab

INTRODUCTION

Canine hepatozoonosis is caused by *Hepatozoon canis* and *Hepatozoon americanum* the two intracellular hemoprotozoan parasites of phylum Apicomplexa, order eucoccidiorida, suborder adeleorina and family Hemogregarinidae (Hepatozoidae). Its prevalence synchronizes with the existence of the ixodid tick-vector (Spolidorio *et al.,* 2009). In contrast to other tick-borne protozoa, *H. canis* infects leukocytes and parenchymal tissues and is transmitted to dogs by the ingestion of ticks containing mature oocysts. Out of the two major causative agents, *H. americanum* is more virulent than *H. canis* (Vincent-Johnson, 2003). In India *H. americanum* has never been reported but *H. canis* was reported first time in 1905 (James, 1905), and was incriminated as the cause of mild disease, mainly characterized by anemia and lethargy (Baneth and Weigler, 1997). Dogs infected with *H. canis* generally appear healthy, since the disease is usually unapparent and subclinical, and the diagnosis is occasionally confirmed

during laboratory examinations (Paludo *et al.,* 2005). *H. canis* has been reported in Africa, Southeast Asia, Middle East, Europe, and South America. The brown dog tick, *Rhipicephalus sanguineus* (Ixodida: Ixodidae), is the main vector of *H. canis* (Baneth *et al.,* 2001, 2003), although *Haemaphysalis longicornis* and *Haemaphysalis flava* in Japan (Murata *et al.,* 1991) and *Amblyomma ovale* in Brazil (Rubini *et al.,* 2009).

Generally, the disease is diagnosed on the basis of clinical evidences augmented with some parasitological, serological tests or molecular tests. The clinical signs are variable and often non-specific. In thin blood smears intracytoplasmic ellipsoidal-shaped gamonts are visible within neutrophils and monocytes (Ibrahim *et al.,* 1989). Moreover, in intermittent parasitaemia or when the number of circulating gametocytes may be very low, gametocytes are not always detectable (Baneth and Shkap, 2003). Serological methods fail to differentiate between past and current infection. For the diagnosis of subclinical and latent infection, molecular techniques (PCR) give the promising result (Baneth *et al.,* 2000). Although PCR is considered the most sensitive detection method as compared to microscopic slide examination, but only a few studies have compared these methods (Karagenc *et al.,* 2006) and a diagnostic gold standard has not been clearly established. No genetic analysis has been done for the pathogen of canine hepatozoonosis in Punjab state. Thus, we analyzed the partial 18S rRNA gene sequences of *Hepatozoon* detected in two canine cases in Punjab, one clinical and the other latent, to elucidate the phylogeny of the Indian *Hepatozoon* in dogs.

MATERIAL AND METHODS

Blood sampling and blood film

Blood samples received from two canine cases in the clinical diagnostic laboratory of TVCC (Teaching veterinary clinical complex) GADVASU Ludhiana was analyzed for haemoprotozoan infections. Blood of case number Case I was received in the month of October 2014 and case was without any relevant clinical signs. Whereas blood from case number II was received in the month of November 2014. This case was reported to have relevant clinical signs of some haemoportoza/hepatozoonosis. Giemsa stained thin blood smears (GSTBS) were prepared immediately after the blood sample was received (Coles, 1986). The smears were observed for the presence of gametocytes of *H. canis* in white blood cells. Morphological characteristics of *H. canis* were identified according to the key described by Soulsby, (2005).

DNA Extraction and PCR assay

Genomic DNA was extracted from the blood samples by using the protocol of Himedia® HiPura™ Blood Genomic DNA Miniprep Purification Spin Kit. Primers were procured from Bangalore Genei, India Pvt. Ltd. PCR reaction

mixture (25 µl) was constituted by 12.5 µl of KAPA2G® Fast HotStart ReadyMix (2X containing KAPA2G® Fast HotStart DNA polymerase, KAPA2G® Fast HotStart PCR buffer, 0.2 mM dNTP each, 1.5 mM $MgCl_2$), 1.5 µl of 10 pmol HepF: 5'-ATACATGAGCAAAATCTCAAC-3'/ HepR: 5'-CTTATTATTCCATG CTGCAG-3' primers (Inokuma *et al.,* 2002) and 5 µl of DNA template suspended in 4.5 µl of nuclease-free water. The reaction was set in thermocycler with the following programme: initial denaturation at 95 °C (5 min), 35 cycles of denaturation at 95 °C (30 sec), annealing at 57 °C (30 sec), and extension at 70 °C (1.30 min) and final extension at 72 °C (5 min). The PCR products were electrophoresed on 1.0% agarose and visualized under UV for 666 bp band (Figure 1). A non template control i.e. control having no DNA amplicon was also used to rule out any contamination of the products.

Figure 1. Agarose gel electrophoresis (1.5%) showing amplified DNA of 666 bp for *H. canis*. Lane M: 100 bp plus DNA ladder; Lane P: positive control; Lane N: negative control; Lane 1-2, 3: clinical sign showing samples in triplicate, Lane 4-5: subclinical sample in duplicate.

Nucleotide sequence analysis

Amplicons of the PCR products were custom sequenced from Xcelris Genomics, Ahmedabad, India. The nucleotide sequences were subjected to BLASTn analysis (Altschul *et al.,* 1990) for determining the similarity with the sequence present in the nucleotide database i.e. NCBI (National Centre for Biotechnology Information). Phylogenetic analysis was conducted on MEGA 6 (Tamura *et al.,* 2013). The evolutionary history (Figure 2) of haemoparasites was inferred using the best evolutionary model. The evolutionary distances were computed using the Maximum Likelihood method (Tamura *et al.,* 2013) and the bootstrap consensus tree inferred from 1000 replicates. MSA was done using muscle in MEGA 6. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

RESULTS AND DISCUSSION

As per the clinical report the case II was showing fever, lethargy, anorexia, anemia, pale mucous membrane. Hematological parameters showed anaemia with haemoglobin 2 g/dl, total leukocyte count 12,800/µl; differential leukocyte count

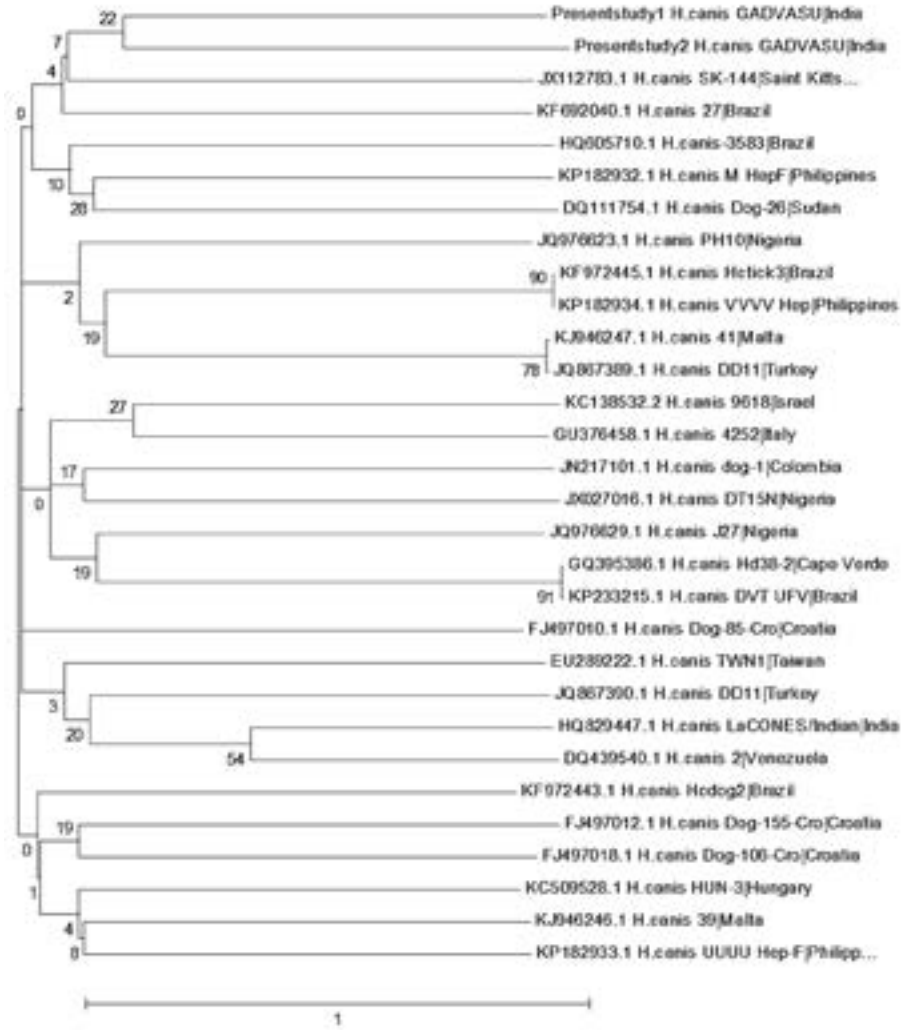

Figure 2. Phylogenetic tree of *H. canis.* This tree was constructed by Neighbor-Joining method with MEGA 6 program. This phylogenetic analysis showed high genetic diversity among different strains of *H. canis.* Consensus sequences obtained in this study is indicated as ‘‘Presentstudy1 *H. canis* GADVASU |India and Presentstudy2 *H. canis* GADVASU |India”.

(DLC) of the blood revealed neutrophilia with neutrophil 90% and lymphocyte 10% and thrombocytopenia was also observed, these findings were in concordant with Inokuma *et al.* (2002). Gametocytes were found in white blood cells in peripheral blood stained with Giemsa (Figure 3). Molecular analysis was performed to clarify the phylogeny of the Indian isolate of *Hepatozoon.* After standardization of PCR expected band size of 666 bp was obtained in clinical and subclinical case with no false amplification in negative control depicting the presence of *H. canis* in dogs. Analyzing the nucleotide sequences of the PCR product excluding the primer regions revealed that both sequences were 100% identical to each other. Phylogenetic analysis depicted that the sequence of the Indian *H. canis* was analogous to that of *H. canis* from Saint kitts and Nevis (JX112783.1) and Brazil (KF692040.1) with 100% (442/442) and 99% (440/442) nucleotide identity respectively (Figure 2). Both isolates i.e. KJ946246.1 from Malta and KP182933.1 from Philippines of *H. canis* were distantly related to Indian *H. canis* with 437/442 and 436/442 match identities, respectively. These results suggest that *H. canis* detected in north Indian dogs might have closer ancestral relationship with Saint kitts and Nevis strain. The suspected vectors of Indian *Hepatozoon* are *R. sanguineus* ticks (Chhabra *et al.,* 2013); analyses of other gene sequences and antigens were needed to explain the differences in the pathogenesis and vectors of *Hepatozoon* in other countries. As partial sequences (666 bp) of the 18S rRNA gene of the agent from only two dogs were analyzed in the present study, pathogens from more dogs should be analyzed over longer sequences of the gene to confirm the results in this study. More future epidemiological studies using molecular tools are also required to clarify the status of canine hepatozoonosis in Punjab, India.

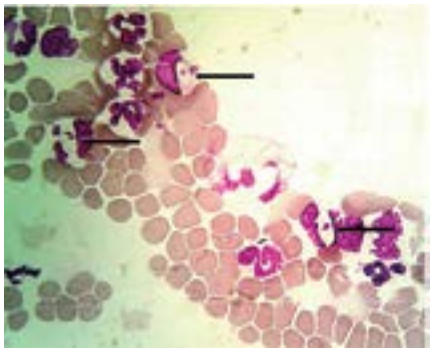

Figure 3. Microscopic image of *H. canis*

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CONFLICT OF INTEREST. All authors are having no conflict of interest.

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