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Full Length Article

First Report on Molecular Diagnosis and Phylogenetic Analysis of *Hepatozoon canis* in Naturally Infected Domesticated and Stray Dogs from Jhang, Pakistan

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Abstract

Canine diseases, particularly caused by tick-borne hepatozoons are responsible for high morbidity and mortality and are the reason for attracting significant focus. The current study was aimed to survey the occurrence of *H. canis* in domesticated and stray dogs of Jhang city (Punjab) with its molecular identification. For this, blood samples from 300 dogs (n=200 domesticated; n=100 stray) were collected and assayed through PCR for the detection of *H. canis* supported by sequencing analysis. The results demonstrated, 15.66% (47/300) of samples positive for *H. canis*. A significantly (P<0.05) higher occurrence of *H. canis* was observed in stray dogs (27%) than domesticated dogs (10%). Evaluation of the various risk factors showed that the age, sex, breed, dog category (domestic or stray), body coat, environmental settings (rural or urban) and ectoparasitic infestation were significantly ($P < 0.05$) associated with the occurrence of infection. The phylogenetic analysis of the PCR confirmed specimens revealed a very close homology of the detected strains with the ones diagnosed earlier in China and Malta. It was concluded that present moderate prevalence of *H. canis* among the dog populations in the area of Jhang may rise with stray dogs being most vulnerable hosts and potential source of vectors spread.

Keywords: *H. canis*; PCR and phylogenetic analysis; Prevalence; Stray dogs

Introduction

Canine hepatozoonosis is a tick-borne disease of carnivores affecting both the wild and domestic animals. More than 300 hepatozoon species have been known so far, out of which 46 infect mammals. *Hepatozoon* spp. belonging to phylum hepatozoid apicomplexa are the blood parasites of vertebrate intermediate hosts (Baneth *et al*. 2003). These intracellular protozoans affect the leukocytes chiefly neutrophils and monocytes of animals (Baneth 2011). *Hepatozoon canis (H. canis)* and *Hepatozoon americanum (H. americanum)* are the two reported species acting as sole source of infection among dogs (Baneth *et al*. 2000). *H. canis* is the cause of Old World canine hepatozoonosis and has been transmitted by *Amblyomma ovale* (Forlano *et al*. 2005; Rubini *et al*. 2009), *Haemaphysalis (H.) longicornis, H. flava* (Murata *et al*. 1995) and *Rhipicephalus sanguineus sensu lato* (Baneth *et al*. 1998). Epidemiological studies have reported the prevalence of *H. canis* in Asia, Europe, Southeast Asia, Africa, Middle East and South America, while *H. americanum* has been limited to the United States (Ewing and Panciera 2003). *H. americanum* can only be transmitted by *Amblyomma maculatum* and has been found in the Central and South American countries (Vincent-Johnson 2003).

Pathogenesis of *H. canis* is considered relatively weaker as subclinical infections are predominant, manifesting milder disease affecting the spleen, lymph nodes, and bone marrow, culminating in anemia and lethargy (Baneth and Weigler 1997). Transplacental transmission of *H. canis* is possible (Murata *et al*. 1993). Infection can be diagnosed by PCR or sequencing (Baneth *et al*. 2003; Criado-Fornelio *et al*. 2007). Only a limited studies on *H. canis* have been reported in Pakistan till to date (Qamar *et al*. 2017; Ahmad *et al*. 2018). It was hypothesized that *H. canis* is prevalent in the dogs of this region and the current study was planned to investigate the prevalence and risk factors associated with the protozoon infection in domestic and stray dogs from Jhang, Punjab, Pakistan. It is one of the very few studies exploring the

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phylogenetic sequence of 18S rRNA gene of *H. canis* in domestic and stray dogs from Pakistan, providing baseline information for effective control of this malady in dogs.

Materials and Methods

Study area and blood sampling

Blood samples (n= 300) were collected randomly from 03 different dog breeds *i.e.*; German Shepherd (n=100), Pointer (n=100) and non-descript stray dogs (n=100) from Jhang, Pakistan. About 5 mL of blood was collected aseptically from cephalic or saphenous venipuncture using 5 mL disposable plastic syringe. The collected blood samples were immediately transferred to purple capped vacutainer (BD Vacutainer® spray-coated K_2 EDTA) tubes and appropriately labeled. Subsequently, the samples were transported in cold chain to Postgraduate Medicine Laboratory at College of Veterinary and Animal Sciences (CVAS), Jhang for initial analysis, and were preserved at -20°C till the DNA was extracted. The study design was permitted by the Committee on Animal Ethics at CVAS (Sub-campus University of Veterinary & Animal Sciences, Lahore), initially with the final approval of the content by Directorate of Advanced Studies (DAS/7550, on 23 July 2019) of the University. Verbal and written consents were acquired by each dog owner prior to blood sampling of their animals. Data pertaining to possible contributing risk factors such as age (evaluated the age via canine teeth and incisor changes), sex, breed, dog category either domestic or stray, environmental settings (rural or urban) and ectoparasite control practices were collected using a predesigned questionnaire proforma.

Molecular detection

The extraction of genomic DNA of protozoan (*H. canis*) was carried out from 200 μL of EDTA anticoagulated blood specimens using a commercially available QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) as per manufacturer's instructions. The quality of DNA was measured by electrophoresis on an agarose gel. The primers PIRO-A1(50-AGGGAGCCTGAGAGACGGCTACC-30) and PIROB (50-TTAAATACGAATGCCCCCAAC-30) (manufactured by Gene Link™) were used to amplify an approximately 450 bp region of the 18S rRNA gene (Földvári *et al.* 2005). PCR was performed in a total of 25 μL volume of reaction mixture having 12 μL Master mix (VizPure™ PCR 2X Master), 2 μL forward and reverse primers each, 4 μL of DNA and 5 μL of nuclease free water.

The PCR amplification was accomplished in a thermal cycler (Applied Biosystems® Veriti®, Foster city, California). The initial denaturing temperature was set at 95°C for 10 min with subsequent 40 cycles at 94°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 30 sec and the final extension was obtained at 72°C after 7 min. The amplified DNA (Fig. 1) was examined through 1.3% agarose gel electrophoresis. A 100 bp marker was also run to ascertain the size of amplified DNA (Thermo Scientific®, Waltham, Massachusetts).

Sequencing and phylogenetic analysis

For the confirmation of PCR results, a total of 10 randomly selected samples were subjected to sequencing, out of which 4 were recognized as *H. canis*. The PCR selected products were cleaned up using a commercial kit method. For DNA sequencing reaction, Big Dye® Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems® Division) was used. For examination of sequencing reactions, ABI Prism® 3730xl Genetic Analyzer (Applied Biosystems®, Foster city, California) was used. The sequences obtained were checked with Chromas v.1.45 and compared to sequence data available in the GenBank1, using the BLAST at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The newly identified sequences of the partial 18S rRNA gene of *H. canis* identified in the current study were submitted to GenBank (Accession numbers: MN900602, MN900603, MN900610 and MN900692).

The phylogenetic analysis was completed using the software MEGA X 10.0.5 to compare the DNA sequences of the current study with the ones previously deposited in the GenBank from the studies conducted in the other countries. The neighbor-joining algorithms using the Tamura 3 Parameter trees were formulated. Nodes with bootstrap values of greater than 30% after 1,000 replicates are indicated (Fig. 2).

Statistical analysis

The data pertaining to prevalence of *H. canis* among dogs were analyzed using Pearson Chi Square statistic at 95% confidence interval using the OpenEpi program (https://www.openepi.com/TwobyTwo/TwobyTwo.htm).

Results

Overall, 15.66% (47/300, ± 4.1 at 95% CI) of the samples amplified exhibited 450-bp band specific for 18S rRNA gene of *H. canis.* Analysis of the possible risk factors associated with *H. canis* occurrence was carried out using Chi-square statistics (Table 1) It showed that the prevalence of *H. canis* was significantly ($P < 0.05$) associated with various risk factors, namely, age, sex, breed, dog category (domestic or stray), body coat, environmental (rural or urban) settings and ectoparasitic infestation. The prevalence was high in the males and young dogs of age below one year with long body coat and dogs kept in the rural areas. The male dogs depicted relatively higher prevalence ($P < 0.05$) than females. The stray or abandoned dogs infested with ticks represented a significantly ($P < 0.05$) higher

Table 1: Descriptive statistics and results of a Chi-square testing for the association between selected potential risk factors and *H. canis* prevalence (at 95% CI)

Risk Factors	Category	Samples Tested (n)	Positive (n)	% prevalence	*P*-value
Age	<1 Year	166	34	20.48	0.010
	>1 Year	134	13	9.70	
Sex	Male	214	40	18.69	0.022
	Female	86	7	8.13	
Breed	German Shepherd	100	14	14	0.003
	Pointer	100	8	8	
	Non-descript	100	25	25	
Dog Category	Pet Dogs	200	20	10	0.000
	Stray Dogs	100	27	27	
Body Coat	Long	207	40	19.32	0.009
	Short	93	7	7.52	
Environmental setting	Urban	193	23	11.91	0.016
	Rural	107	24	22.42	
Ectoparasitic infestation	Yes	185	35	18.91	0.049
	No	115	12	10.43	

P values ≤ 0.05 are statistically significantly different

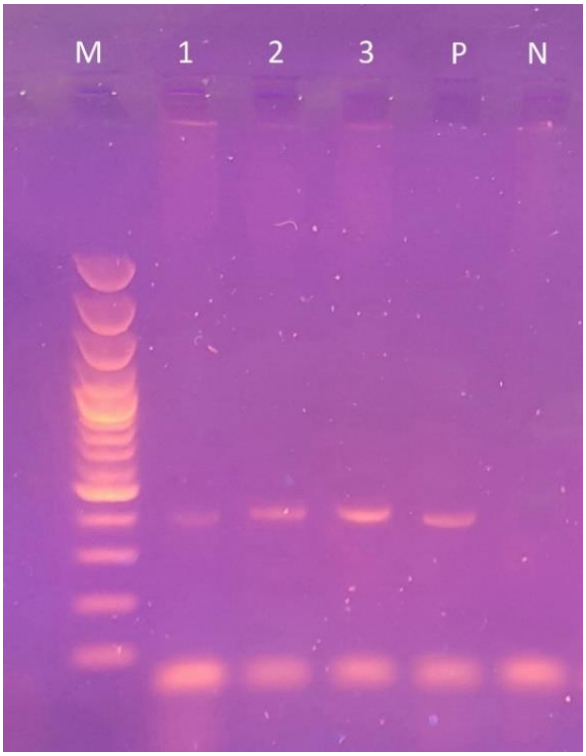

Fig. 1: PCR amplification of 450 bp of 18S rRNA represent *H. canis*, Lane 1-3 represents *H. canis* samples M=100 bp DNA ladder/marker, P= Control positive and N= Control negative

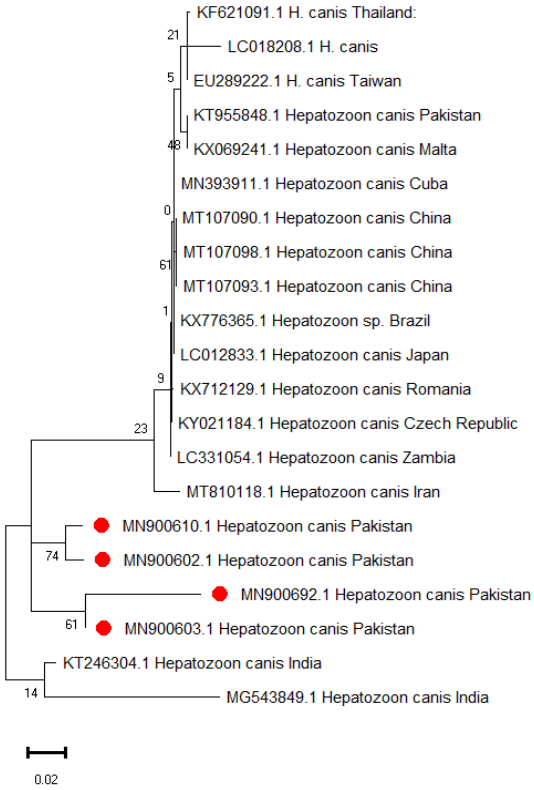

Fig. 2: Phylogenetic relationship of *H. canis* detected during present study to *H. canis* (red dots) reported from other countries based on the partial sequence of the 18S rRNA gene. The evolutionary history was inferred by the Neighbor-Joining method based on the Tamura-Nei mode. Boot strap analysis, used to estimate the node reliability of the trees, was conducted with 1000 replicates as implemented in MEGA X 10.0.5. Hepatozoon spp., host species, country of origin from where these sequences were derived and the GenBank accession numbers are included for each sequence

prevalence than the domestic dogs kept as pets by the owners irrespective of their area of living *i.e*., either in rural or urban ambiance.

A total of 10 PCR amplicons were randomly sorted out for performing the DNA sequencing. The outcome of sequencing of the amplicon displayed 4 distinct 18S rRNA sequences (466–496bp). The nucleotide BLAST analysis confirmed the similarity among the obtained sequences at large (i.e., sequence homology >92%) to already existing *H. canis* spp. in the data bases. The alignment of 4 confirmed sequences was submitted to GenBank (GenBank accession numbers MN900602, MN900603, MN900610 and MN900692).

Discussion

The current study enabled the first insight of the genetic characterization of *H. canis* from domesticated and stray dogs in the oldest district of Jhang, Punjab (Pakistan). Canine hepatozoonosis is a tick-borne disease of increasing importance in dogs worldwide. Besides the microscopic and serological methods of diagnosis, molecular techniques are quite specific and sensitive. In the current study, dogs reared in the Jhang district were investigated showing a prevalence of 15.66%. The prevalence of *H. canis* recorded in the current study was significantly ($P < 0.05$) higher than an earlier study in Pakistan (Qamar *et al*. 2017) reporting a prevalence of 11.9% but lower than 45.5% Ahmad *et al*. (2018). This variation in the prevalence of *H. canis* may be attributed to many factors, including the distribution (Spolidorio *et al*. 2009), population status of the vector (Otranto *et al*. 2011), methodology of sampling and the traits of the dog population being studied (de Azevedo Gomes *et al*. 2016). The differences of environmental settings either rural or urban, the status of look after extended by the owner to their pets and a stray category in the present study of dogs were observed as the significantly contributing risk factors in the incidence of this infection.

Surveys of *H. canis* in dogs in different countries have shown varying prevalence rates such as Brazil 3.8%, Croatia 11.8% (Vojta *et al*. 2009), Costa Rica 7.5% (Rojas *et al*. 2014), India 30% (Singla *et al*. 2016), Iran 23% (Dalimi *et al*. 2017), Qatar 1.6% (Alho *et al*. 2017), Thailand 11.4% (Jittapalapong *et al*. 2006) and Turkey 3.6% (Aydin *et al*. 2015). The prevalence recorded in the present study also lies in between the highest and lowest prevalence of 3.6% and 30%. However, further broad studies are required to make the scenario clearer.

Contrarily, some of the investigations from Brazil have reported alarmingly high prevalence (58.7 and 66.4%) of *H. canis* in dogs (Spolidorio *et al*. 2009; de Castro Demoner *et al*. 2016). The variations in the reported prevalence may plausibly be owing to various risk factors including the traits of the target dog population under investigation, season of specimens collection, social and husbandry facilities (same species animals and tick preventive measures), geoclimatic characteristics influencing the abundance and spread of tick vector species (Stich *et al*. 2014).

As far as the role of various risk factors in the prevalence of canine hepatozoonsis is concerned, many of authors have reported higher prevalence among young dogs under the age of one year than older ones (Abdullahi *et al*. 1990; Vezzani *et al*. 2017) resembling with the findings of the current study. This may be due to deficient immune competency and vulnerable exposure of infection at young age. In pertinence to dogs' categories as pets or stray, a significantly higher ($P < 0.05$) prevalence among stray dogs was encountered than their pet counterparts. The most plausible reason in this scenario seems to be the keen observation and vigilance of domestic dogs by the owners contrary to the sheer abandonment of ownership on the part of stray dogs. The stray dogs wander here and there having maximum chances of getting tick infestation and subsequent infection (Bashir *et al*. 2009). It has been also seen that contact with other animals (domestic or wild) can pose a high risk of tick infestation. Stray dogs have been mostly infected with vector borne diseases than other pet breeds (Hornok *et al*. 2006; Amuta *et al*. 2010; Singh *et al*. 2014).

In terms of sex of the host, male dogs were found to be affected significantly ($P < 0.05$) high than females being in consensus with a previous report (Vezzani *et al*. 2017). Male dogs, owing to their wandering, aggressive and fighting temperament seem to be affected higher while the females have been observed to remain isolated after mating during the term and post-whelping in fostering the pups. Here, it was seen that contact with other animals may be the source of transmission as the other livestock animals at the farm may already have tick load. Anyhow, this risk factor was not evidenced as a significant contributor possibly because the animals coming in contact with these dogs were either free of ticks or were least exposed owing to good management. Dog category either pet or stray was significantly ($P < 0.05$) different in current study, and also is not in line with the findings of previous studies which may possibly be due to difference in the breeds as the susceptibility to the disease may be associated with the breed genetics.

As the coat of body is concerned, it was noted that dogs with long coat are more susceptible than short coated dogs. In the current study it was seen that environmental setting influences the prevalence of *H. canis* as the dogs in rural areas are more infected than the urban areas. The livestock population is significantly high in the rural areas and in contact dogs may get the ticks transferred from other animals and become the source of infection to nearby dogs as described by (Pacifico *et al*. 2020). The ticks attached hide themselves in long coat and remain unnoticed from the owner's observation. This is the very first report of molecular detection and characterization of *H. canis* in naturally infected dogs (pet and stray dogs) from Jhang, Pakistan.

Conclusion

In conclusion, the prevalence of *H. canis* among the dog populations is considerable with a potential to rise with soaring trends of dog keeping in the region. PCR coupled with sequencing analysis provides a reliable confirmatory test for such emerging infections in the non-reported areas for undertaking effective therapeutic and control measures.

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Author Contributions

MA AN and AS planned the experiments; SEH and MAZ interpreted the results; MK statistically analyzed the data and made illustrations; MA contributed in the write up.

Conflict of interest

The authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethics Approval

Not applicable in this paper

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