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ORIGINAL PAPER

Molecular characterization of *Hepatozoon canis* from farm dogs in Pakistan

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Abstract

Hepatozoon canis is a tick-borne pathogen of canids, which is distributed worldwide. However, very little is known about this protozoan parasite in Pakistan. This study provides the first molecular evidence of *H. canis* from farm dogs from three agro-ecological zones of Punjab, Pakistan. A conventional PCR targeting the 18S rRNA gene was used to characterize *H. canis* from farm dogs from three districts, namely Kasur, Rawalpindi and Muzaffargarh, in Punjab. Of 341 blood samples tested, 155 (45.5%) were positive for *H. canis*, 73 (61.3%) from Kasur, 46 (42.5%) from Rawalpindi and 36 (31.5%) from Muzaffargarh. Phylogenetic analyses revealed that 18S rRNA sequences of *H. canis* from this study clustered in three clades with those of *H. canis* from previously published studies to the exclusion of all other *Hepatozoon* spp. included in the analysis. This study provides the first insight into *H. canis* from farm dogs in Pakistan. Furthermore, it lays a foundation for future studies of the parasite to assess the impact of canine hepatozoonosis in dogs from various agro-ecological zones in Pakistan where pet ownership of dogs is increasing.

Keywords *Hepatozoon canis*, 18S rRNA gene molecular characterization, prevalence, dogs, Pakistan

Introduction

Hepatozoon represents an important genus of apicomplexan protozoa, and consists of more than 300 species with at least 46 of them infecting mammals (Smith, 1996). *Hepatozoon canis* and *Hepatozoon americanum* are important tick-borne pathogens which cause canine hepatozoonosis in domestic and wild canids (Gomes et al., 2016). Ixodid ticks are known to transmit *H. canis* in canids, including *Ambylomma ovale* in South America (Rubini et al., 2009), and *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* in different parts of the world (Baneth et al., 2007; Giannelli et al., 2017), whereas *Ambylomma maculatum* is the only vector known for *H. americanum* in the USA (Mathew et al., 1998). The transfer of infection has also been reported by vertical transmission for *H. canis* (Murata et al., 1993) and by predation for *H. americanum* (Johnson et al., 2009).

H. canis and *H. americanum* infections may result in marked elevation of leukocytes, leading to clinical signs such as pyrexia, inappetence, hyperesthesia, muscle wasting and weight loss (Baneth, 2011). Most *H. canis* infected dogs have subclinical infection or mild clinical signs, although a certain subset experience severe and potentially fatal disease (Baneth, 2011).

As the name indicates, *H. americanum* has only been reported to cause clinical disease in dogs in the USA so far (Potter and Macintire, 2010), whereas the occurrence of *H. canis* has been described from various tropical, sub-tropical and temperate regions of the world, including Africa, Europe, Asia, and the USA (Aydin et al., 2015; Vojta et al., 2009). However, very limited information is available on the prevalence and characterization of canine hepatozoonosis from Pakistan (Qamar et al., 2017).

Pakistan is located in South-Asia, between 30º 00’ North and 70º 00’ East with a total land area of 796,095 km^2 (Farooqi et al., 2005; Maps, 2017). Although 10 agro-ecological zones with different climatic conditions exist in the country, sub-tropical and partially temperate

regions constitute the major proportion, extending from the Arabian Sea in the south to the Himalayas in the north (Farooqi et al., 2005). Due to such favorable climatic conditions for the transmission of tick-borne diseases, a high prevalence of *H. canis* would be expected in this region. However, there is paucity of information on the prevalence of *H. canis* from Pakistan, especially from farm dogs. Therefore, this study was designed to survey and genetically characterize *H. canis* from farm dogs in three different agro-ecological zones of Punjab, Pakistan.

Materials and methods

Study area and blood sampling

Blood samples (*n* = 341) were collected from the cephalic vein of apparently healthy farm dogs of both sexes in EDTA tubes and stored at -20°C until DNA extraction. The sample collection was performed between June and October 2016 from three different districts in the province of Punjab, including Kasur (31° 12′ 21″N, 74° 45′ 81″E; number of samples = 119), Muzaffargarh (30º 07′ 36″N, 71º 18′ 05″E; n = 114) and Rawalpindi (33º 59′ 84″N, 73º 04′ 41″E; n = 108) (Fig. 1). The average rainfall and temperature in these districts are summarized in Table 1 (PMD, 2016). This study was approved by the animal ethics committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 200 µl of each blood sample using the GeneAll Exgene Genomic DNA Micro extraction kit (Cambio Ltd., Cambridge, England) following the manufacturer's guidelines. DNA was stored at -20ºC until further use.

For the identification of *H. canis*, a 666 bp fragment of the 18S rRNA gene was amplified using conventional PCR by employing the HepF (5'-ATACATGAGCAAAATCTCAAC-3') and HepR (5'- CTTATTATTCCATGCTGCAG-3') primers (Inokuma et al., 2002). PCRs were performed in 25 µl volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega, Madison, WI, USA), 3.5 mM $MgCl_2$, 250 µm of each deoxynucleotide triphosphate (dNTP), 25 pmol of each primer and 1 U of Go*Taq* polymerase (Promega). PCR cycling conditions were: initial denaturation at 95ºC for 12 min followed by 35 cycles of denaturation at 95ºC for 30 sec, annealing at 57ºC for 30 sec and extension at 72ºC for 30 sec, and the final extension was carried out at 72ºC for 7 min. PCR reactions were run using a T100 Thermal Cycler (BioRad, Hercules, CA, USA). Positive (*H. canis*) and negative (double distilled water) controls were included in each PCR run. Following PCR, aliquots (5 µl) of individual amplicons were examined by agarose gel electrophoresis (1.5% gels in 0.5X TAE buffer [20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA]). Gels were stained using GelRed Nucleic Acid Gel Stain (Biotium, Inc., Fremont, CA, USA), subjected to transillumination and photographed using a gel documentation system (Kodak Gel Logic 1500 Imaging System, Eastman Kodak Company, Rochester, NY, USA).

Ten PCR amplicons from each district were randomly selected for DNA sequencing. Each amplicon was treated with shrimp alkaline phosphatase and exonuclease I (Werle et al., 1994) and then subjected to automated DNA sequencing using the 96-capillary 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA) at Macrogen Incorporation, South Korea. Sequencing of the 18S rRNA gene was conducted using the PCR primers (HepF and HepR) in separate reactions. The quality of nucleotide sequences was appraised using the program Geneious R10 (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012), and polymorphic sites were designated using International Union of Pure and Applied Chemistry (IUPAC) codes.

DNA sequence and phylogenetic analyses

Consensus sequences from Geneious software were aligned with the MUSCLE V 3.8.31 program (Edgar, 2004) and then adjusted manually using the Mesquite V 3.03 software (Maddison, 2008). Sequence differences were calculated based on pairwise comparisons using the program BioEdit (Hall, 2011).

Phylogenetic analyses were performed using Bayesian inference (BI) and Neighbor-Joining (NJ) methods. Previously published 18S rRNA sequences of *Hepatozoon* spp. (Alho et al., 2017; Aydin et al., 2015; Baneth et al., 2013; Criado-Fornelio et al., 2003; Inokuma et al., 2002; Kongklieng et al., 2015; Loftis et al., 2013; Maia et al., 2014; Mathew et al., 2000; Pawar et al., 2012; Qamar et al., 2017; Rubini et al., 2009; Vojta et al., 2009) available on GenBank and those determined herein were aligned (575 bp) and used for phylogenetic analyses. *Toxoplasma gondii* was used as the outgroup (Luton et al., 1995). The BI analysis was conducted, employing the Markov Chain Monte Carlo (MCMC) method in Mr Bayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The likelihood parameters for BI were based on the Akaike Information Criterion (AIC) and Bayesian Inference Criterion (BIC) tests in jModeltest v2.1.5 (Darriba et al., 2012). For all datasets, AIC revealed the Kimura 3-parameter model of evolution, with gamma-distribution (TPM3uF+G) as the 'best' model. Estimates of the base frequencies, the substitution rate model matrix, and the proportion of invariable sites, were fixed. Posterior probabilities (pp) were calculated using 2,000,000 generations, employing four simultaneous tree-building chains, with every 100^{th} tree being saved. A consensus tree (50% majority rule) was constructed based upon the remaining trees generated by BI. The NJ analyses were performed employing the online software, MEGA 7.026 (Tamura et al., 2013) and the nodes were tested for robustness with 10,000 bootstrap replicates. The phylogenetic trees produced from the BI

and NJ analyses were compared for concordance in their topologies using the software FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

A partial fragment of 18S rRNA was successfully amplified by PCR from 45.5% (155/341) of the samples. The prevalence of *H. canis* in three districts of Punjab is summarized in Table 1. Based on location, the highest prevalence of *H. canis* was found in Kasur (61.3%; 73/119) followed by Rawalpindi (42.5%; 46/108) and Muzaffargarh (31.5 %; 36/114).

A total of 30 PCR amplicons (i.e., 10 from each district) were randomly selected for DNA sequencing, and the sequence analysis revealed 15 different 18S rRNA sequences (578-626 bp). The nucleotide BLAST analysis revealed that all these sequences were most similar (i.e., sequence identity of >97%) to those of *H. canis* available in public databases. An alignment of 15 sequences determined herein (GenBank accession nos. MG209580-MG209594) displayed two transitions either C↔T at position 10 or G↔A at positions 17, 41, 274, 429 and 470 (see supplementary Fig. 1). However, at five positions, three IUPAC ambiguity codes, including M (alignment position numbers 380, 409, 478), R (28) and X (315) were present (see supplementary Fig. 1). Pairwise comparisons of nucleotide sequences showed nucleotide differences ranging between 0.2% to 15.1% (Table 2). Furthermore, a comparison of partial 18S rRNA sequences determined herein and those of selected previously published sequences revealed that two sets of six sequences each were 98.8 to 99.6 identical to *H. canis* 18S rRNA sequences from Thailand (GenBank accession no. KF621082 vs MG209581, MG209583, MG209584, MG209588, MG209590, MG209591 found herein) and Japan (GenBank accession AF418558 vs MG209580, MG209582, MG209585, MG209586, MG209587, MG209593), respectively (Table 2). The remaining two sequences determined in

this study (GenBank accession nos. MG209592, MG209594) were 94.2 to 96.4% identical to *H. canis* sequences from Rawalpindi district of Pakistan (KU535870) while our last sequence (GenBank accession no. MG209589) was 94.8% identical to *H. canis* sequence previously reported from the Lahore district of Pakistan (Table 2).

DNA sequences were aligned over 537 positions and subjected to phylogenetic analyses. The analyses revealed that the topologies of both trees produced separately using BI and NJ methods were similar (data not shown); hence, only the NJ tree is presented here (Fig. 2). Phylogenetic analyses of 18S rRNA data revealed that *H. canis* sequences determined herein clustered into three clades (Fig. 2). Clade 1 contained nine sequences (GenBank accession nos. MG209581, MG209583, MG209584, MG209588, MG209590, MG209591-MG209594) which grouped together with those from Pakistan (Rawalpindi), Portugal, Taiwan, Thailand and the West Indies (Fig. 2). Clade 2 contained only one sequence (GenBank accession no. MG209582). Clade 3 comprised the remaining five sequences (MG209580, MG209585-MG209587, MG209589) which clustered with those from Brazil, Croatia, Japan, Pakistan (Lahore) and Spain (Fig. 2). Clade 4 comprised only previously reported a sequence from Turkey. Despite the variation observed in the 18S rRNA sequences of *H. canis* from farm dogs, all of them grouped together to the exclusion of all other *Hepatozoon* spp. included in the analyses (Fig. 2).

Discussion

This study provides the first insights into the genetic characterization of *H. canis* from farm dogs in three agro-ecological zones of Punjab (Pakistan). *H. canis* is a tick-borne protozoan parasite known to cause hepatozoonosis in dogs in tropical and subtropical regions and presents a major clinically underestimated vector-borne disease in dogs across the tropical and subtropical areas globally health issue across the globe (Aydin et al., 2015; Baneth et al.

2011). However, there is paucity of information on the prevalence and infection intensities of *H. canis* in dogs from Asia. *H. canis* is frequently diagnosed using microscopic examination for the detection of gamonts in stained blood smears (Ibrahim et al., 1989). Tools such as enzyme-linked immunosorbent assay and indirect fluorescent antibody test have also been employed for the serological diagnosis of antibodies against *H. canis* (Baneth et al., 1998; Gonen et al., 2004). We used PCR for the detection of *H. canis* in dogs as this is the most reliable diagnostic tool for the detection of *H. canis* infection in dogs (Otranto et al., 2011; Vojta et al., 2009).

In this study, the prevalence of *H. canis* was higher (45.5%) than that of a recent study (11.9%) from Pakistan that also used PCR for the detection of the parasite (Qamar et al., 2017). These differences in the prevalence of *H. canis* could be due to (i) the difference in geographic locations of dogs (samples were collected from Islamabad, Lahore, and Multan districts in the previous study) (ii) differences in the environmental background of dogs sampled (farm dogs in this study versus pet dogs in the previous study), and (iii) differences in care for the dogs, including tick prophylaxis, i.e., the presence of more ticks on farm dogs as compared to pet dogs (Ahmad et al., unpublished data) . Farm dogs are permitted to roam unsupervised and observed to interact more frequently with other farm and stray dogs when they accompany grazing animals. This increases their likelihood of acquiring *H. canis* infection. Contrarily, pet dogs are better cared for, are usually kept within the confines of the home and backyard and walked using a leash under strict supervision. Better tick management by urban dog owners could explain less tick-borne disease infestation and may be the likely cause of the lower prevalence of *H. canis* in a previous study from Pakistan (Qamar et al., 2017).

From a global perspective, the overall molecular prevalence (45.9%) of *H. canis* in dogs in this study falls within those reported in previous studies (0.9 to 67.7%) (Criado-Fornelio et

al., 2009; Rubini et al., 2005). For example, the prevalence of *H. canis* (45.5%) in this study was comparable to a study from Spain (44%; Criado-Fornelio et al., 2007). However, this level of prevalence was relatively higher as compared to some reports from Brazil (3.8%; de Miranda et al., 2014), Croatia (11.8%; Vojta et al., 2009), Costa Rica (7.5%; Rojas et al., 2014), India (0.26% and 30%; Abd Rani et al., 2011; 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). Contrarily, some studies from Brazil have reported relatively higher prevalence (58.7% and 66.4%) of *H. canis* in dogs than that found in this study (Demoner et al., 2016; Spolidorio et al., 2009). The differences in infection rates could be due to various contributory factors such as the characteristics of target dog population, sampling seasons, social factors (e.g. canine husbandry, use of tick preventers), geographical location and climatic conditions which ultimately impact the abundance and distribution of vector tick species (de Miranda et al., 2014; Stich et al., 2014).

The frequency of dogs infected with *H. canis* was variable in different regions of Pakistan. The highest prevalence was found in the Kasur district (61.3%) followed by Rawalpindi (42.5%) and Muzaffargarh (31.5%). These variations in infection rates could be due to differences in climatic conditions and geographical location of the three districts (see Table 1). For instance, Rawalpindi is located in the proximity of foothills of Himalayas and exhibits lower average temperatures (both summer and winter) and approximately five-times higher annual rainfall than the flat alluvial planes of Muzaffargarh (PMD, 2016). However, despite the presence of more conducive environmental conditions for the transmission via ticks of hepatozoonosis in Rawalpindi, the prevalence of *H. canis* in this district was lower than that of Kasur (Dantas-Torres, 2010; Ul-Hasan et al., 2012). This could possibly be due to stall feeding practices for livestock in Rawalpindi, which result in farm dogs being confined within the farm premises. Contrarily, farm dogs in Kasur are likely to interact more

frequently with other farm and stray dogs during grazing of livestock. Under these conditions, dogs can get infested with ticks which could increase the likelihood of acquiring *H. canis* infection (de Miranda et al., 2014). The lowest prevalence in Muzaffargarh could be due to harsh environmental conditions (e.g. high temperature and low rainfall 0.77 mm during summer) in this region (PMD, 2016); hence, a possible lower survival rate for tick vectors involved in the transmission of *H. canis*. As this is the first report of *H. canis* infection from farm dogs in Pakistan, almost all the above listed factors warrant further investigation. Furthermore, farm dogs are likely to acquire infection by eating a tick vector (e.g. *R. sanguineus*) directly or potentially by ingesting a small rodent infected with the vector harboring the infective protozoan (Demoner et al., 2016). However, the role of these tick vectors and rodents in the transmission of *H. canis* in Pakistan remains poorly understood.

The 18S rRNA sequences *H. canis* clustered in three different clades, although with low statistical support for clade separation (see Fig. 2). Nine (out of 15; GenBank accession nos. MG209581, MG209583, MG209584, MG209588, MG209590, MG209591-MG209594) 18S rRNA sequences determined here showed a high similarity with those from previously published sequences from Pakistan (Rawalpindi), Portugal, Taiwan, Thailand and West Indies. Similarly, five other sequences (GenBank accession nos. MG209580, MG209585-MG209587, MG209589) were closely related to those from Brazil, Japan, Pakistan (Lahore) and Spain while only one sequence grouped separately in between clades 1 and 3 (see Fig. 2). The weak statistical support for separation between clades in the phylogenetic tree suggests that *H. canis* genotypes represent a single species. However, the occurrence of 15 distinct genotypes of *H. canis* (found in this study) in Pakistan suggests a high genetic variability within this parasite population. Moreover, the distribution of our *H. canis* genotypes among these clades does not follow any specific pattern i.e. the sequence variation is distributed

among all three districts. Collectively, these data suggest a frequent minor strain variation and vast geographical spread of the protozoan parasite, not only in Pakistan but also throughout Asia. One possible explanation for the genetic diversity of *H. canis* could be the international movement of dogs from other countries (Ul-Hasan et al., 2012; Vojta et al., 2009). However, it is also possible that these genotypes might have diverged within the country and not introduced as such from other countries. The inter-continental movement of humans as well as animals due to natural disasters and warfare (e.g. World wars) and social factors could also be considered as potential means for the spread of vectors and vector-borne diseases (Stich et al., 2014; Sutherst, 2004).

Conclusion

The present study provides the first molecular characterization of *H. canis* using 18S rRNA gene fragment from farm dogs in Pakistan. The prevalence of the parasite was much higher in all three agro-ecological zones studied as compared to a recent study using blood samples collected from Pakistani dogs living in urban settings. A molecular-phylogenetic analysis revealed that *H. canis* sequences grouped into three clades. Future studies should focus on testing *H. canis* in domestic and wild canids covering various climatic regions of Pakistan and evaluating the effect of different factors on its prevalence. Furthermore, studies are required to characterize the tick vector(s) involved in the transmission of *H. canis* in Pakistan.

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Compliance with ethical standards

This study was approved by the animal ethics committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1 Map of Pakistan showing different states and the study sites (indicated by black stars) within Punjab province.

Fig. 2 Genetic relationships of *Hepatozoon canis* from Pakistan (bold) with reference sequences selected from previous studies. The relationships were inferred based on phylogenetic analyses of 18S rRNA sequence data using the Neighbour Joining (NJ) and Bayesian inference (BI) methods, and *Toxoplasma gondii* was used as an outgroup. The country of origin of each unique sequence of *H. canis* is given in parentheses. Nodal support values are indicated: bootstrap for NJ (first), and posterior probability for BI (second). The scale bar indicates distance.

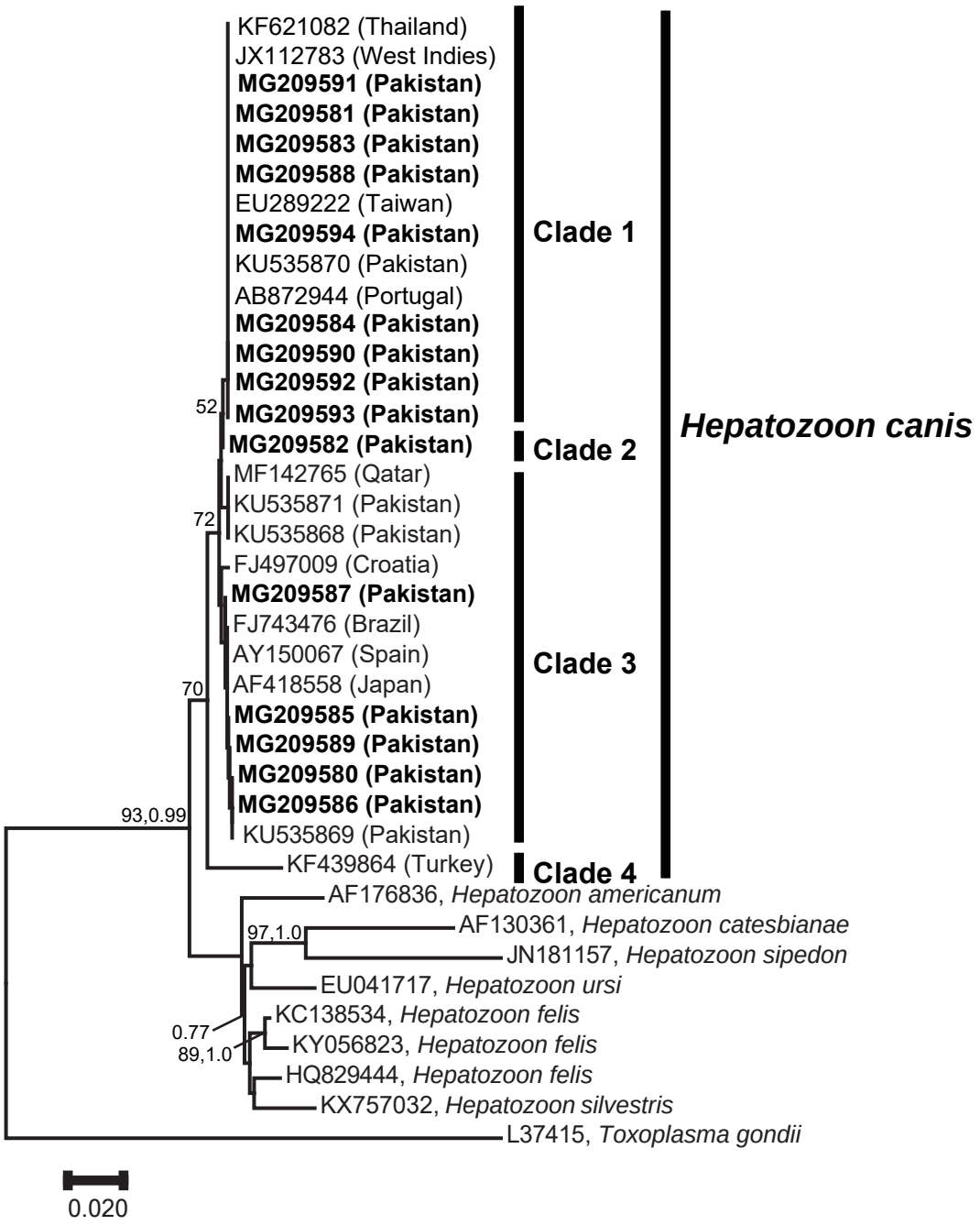
Table 1 Prevalence of *Hepatozoon canis* in farm dogs detected by PCR from three districts of Pakistan.

District	Coordinates	Average temperature ºC (min-max)		Annual rainfall (mm)	No. of samples	PCR test-positive	Prevalence (%)
		Summer	Winter				
Kasur	31° 12′ 21″N, 74° 45′ 81″E	31.6 (22.0-46.0)	15.4 (4.0-26.0)	731.9	119	73	61.3
Rawalpindi	33º 59′ 84″N, 73º 04′ 41″E	30.2 (21.0-42.0)	15.0 (3.0-26.0)	932.1	108	46	42.5
Muzaffargarh	30º 07′ 36″N, 71º 18′ 05″E	33.3 (23.0-46.0)	17.1 (3.0-28.0)	199.2	114	36	31.5
				Total	**341**	**155**	**45.9**

Table 2 Pairwise comparison (similarity) of 18S rRNA nucleotide sequences determined in this study (bold) with the selected reference sequences of *Hepatozoon canis* available from GenBank.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 .K2																						
2. K1	0.982																					
3. K4	0.984	0.988																				
4. K5	0.998	0.982	0.985																			
5. K6	0.992	0.98	0.988	0.993																		
6. K7	0.982	0.998	0.988	0.982	0.98																	
7. K10	0.98	0.998	0.987	0.98	0.979	0.996																
8. K14	0.982	0.996	0.988	0.982	0.98	0.998	0.995															
9. K15	0.995	0.979	0.982	0.996	0.99	0.979	0.977	0.979														
10. K18	0.908	0.921	0.915	0.908	0.907	0.923	0.92	0.921	0.907													
11. K22	0.993	0.984	0.988	0.995	0.992	0.984	0.982	0.984	0.992	0.91												
12. K23	0.995	0.977	0.979	0.993	0.99	0.977	0.976	0.977	0.99	0.904	0.988											
13. K24	0.924	0.926	0.926	0.923	0.923	0.926	0.924	0.926	0.92	0.849	0.923	0.92										
14. K26	0.987	0.985	0.993	0.988	0.985	0.985	0.984	0.985	0.985	0.912	0.99	0.982	0.926									
15. K28	0.948	0.934	0.936	0.947	0.94	0.934	0.932	0.934	0.944	0.86	0.945	0.944	0.974	0.939								
16. MF142765	0.988	0.99	0.985	0.988	0.985	0.99	0.988	0.99	0.985	0.913	0.99	0.984	0.924	0.984	0.94							
17. AF418558	0.985	0.996	0.99	0.985	0.982	0.996	0.995	0.995	0.982	0.921	0.987	0.98	0.926	0.988	0.937	0.99						
18. KF621082	0.996	0.98	0.982	0.995	0.988	0.98	0.979	0.98	0.992	0.907	0.992	0.992	0.923	0.985	0.947	0.987	0.984					
19 .FJ743476	0.984	0.995	0.988	0.984	0.98	0.995	0.993	0.993	0.98	0.923	0.985	0.979	0.924	0.987	0.936	0.988	0.998	0.982				
20. KU535870	0.961	0.947	0.949	0.96	0.954	0.947	0.946	0.947	0.957	0.874	0.958	0.957	0.942	0.952	0.964	0.954	0.95	0.963	0.949			
21. KU535869	0.907	0.921	0.914	0.907	0.906	0.92	0.92	0.918	0.906	0.848	0.909	0.903	0.905	0.901	0.916	0.912	0.92	0.906	0.921	0.874		
22. KU535871	0.933	0.932	0.93	0.933	0.93	0.932	0.93	0.932	0.932	0.842	0.935	0.929	0.967	0.929	0.886	0.941	0.933	0.932	0.935	0.9	0.965	
23. KU535868	0.921	0.922	0.918	0.921	0.918	0.922	0.921	0.922	0.918	0.848	0.922	0.916	0.924	0.916	0.904	0.931	0.922	0.922	0.921	0.854	0.848	0.876


Baltistan
Khyber
Pakhtunkhwa
Punjab
Balochistan
Sindh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AB872944 (Portugal)
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MF142765 (Qatar)
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FJ497009 (Croatia)
MG209587 (Pakistan)
FJ743476 (Brazil)
AY150067 (Spain)
AF418558 (Japan)
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MG209586 (Pakistan)
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KF439864 (Turkey)
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AF130361, Hepatozoon catesbianae
JN181157, Hepatozoon sipedon
EU041717, Hepatozoon ursi
KC138534, Hepatozoon felis
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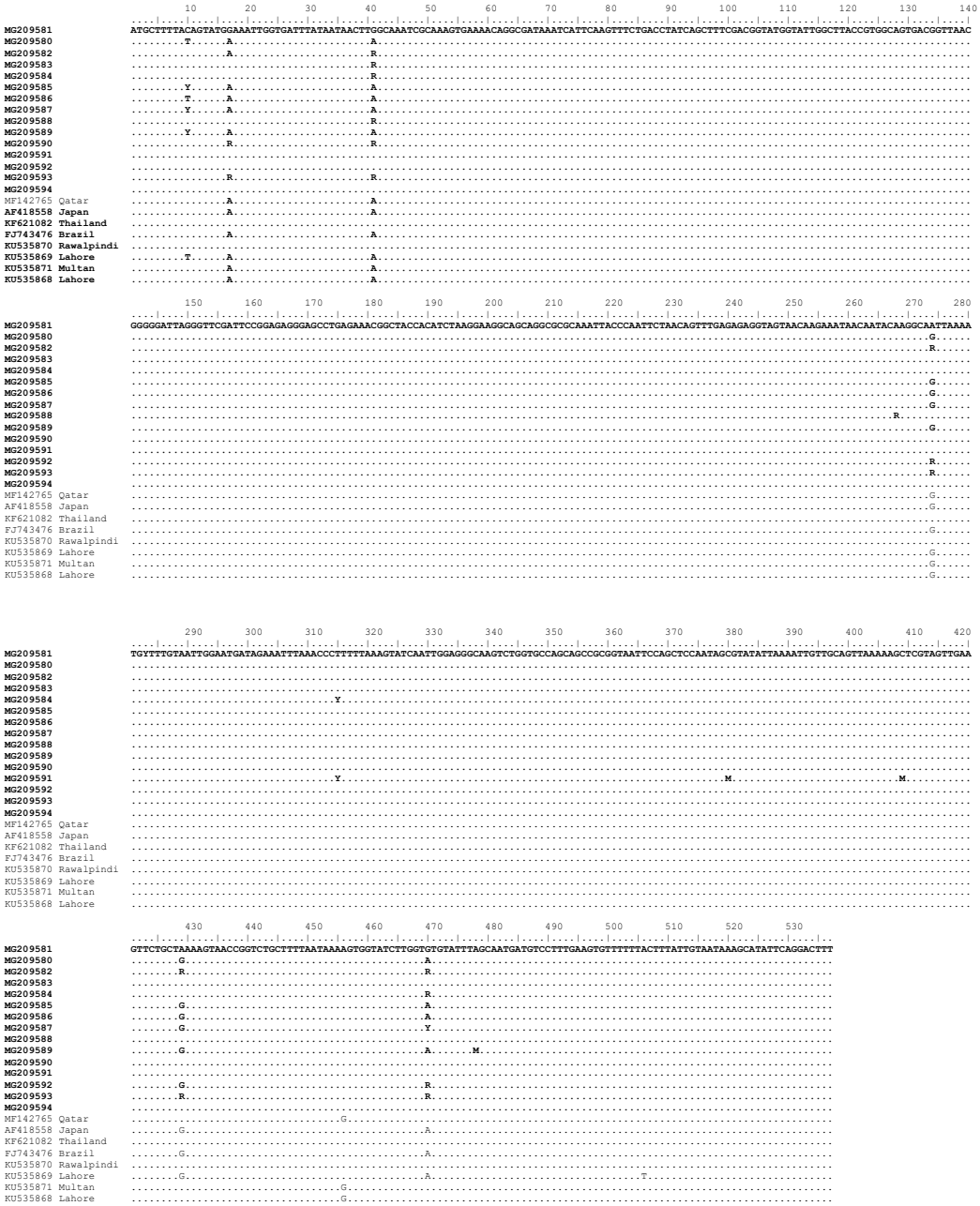
HQ829444, Hepatozoon felis
KX757032, Hepatozoon silvestris
L37415, Toxoplasma gondii
52
72
70
93,0.99
97,1.0
0.77
89,1.0
Clade 1
Clade 2
Clade 3
Clade 4
Hepatozoon canis
0.020

Supplementary Material



Supplementary Fig. 1. Alignment of the partial 18S rRNA for *Hepatozoon canis* sequences obtained in this study (bold) with previously reported sequences. The sequences have been deposited in the GenBank database (accession numbers: MG209580-MG209594). A dot indicates an identical nucleotide with respect to the sequence K2. IUPAC codes indicate polymorphic positions in the sequences.