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PREVALENCE AND PHYLOGENETIC LINEAGES OF *HEPATOZOON CANIS* FOUND IN STRAY DOGS IN MUMBAI, INDIA

A HEPATOZOON CANIS ELŐFORDULÁSA ÉS FILOGENETIKAI VIZSGÁLATA MUMBAI-BAN (INDIA) ÉLŐ KÓBOR KUTYÁKBAN

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Abstract

With the warm weather and large number of hosts, it is not a surprise to hear that tick infestations of dogs are most common in Mumbai. Moreover, the dogs in Mumbai are at the greatest risk of tick-borne pathogens such as *Ehrlichia canis*, *Babesia gibsoni* and others.

The primary objective of this study was to obtain information about the prevalence of *H. canis*, a protozoan species of stray dogs in Mumbai, which was first described in India in 1905. The other aim of this study was to compare the gene sequences of *H. canis* detected in Mumbai with those reported from different parts of India and other countries.

The blood samples were taken from 90 dogs in Mumbai, in the Animal Wellness and Rehabilitation Centre (AWRC) clinic. They were stray dogs which either had no obvious clinical signs or mild non-specific clinical signs such as lethargy, seldom vomiting and mild fever. No information was recorded about sex, age breed etc.

In total 44 of 90 stray dogs were positive for *H. canis*. The prevalence of this protozoan infection was 48.9%. Ten positive samples were sequenced, aligned, and compared to GenBank sequences by the nucleotide BLASTn program. All sequences retrieved from GenBank and included in the phylogenetic analysis had 97-100% coverage. (i.e. aligned with a near-identical length and starting position) as sequences from this study. This study shows that *H. canis* from Mumbai are related to other Indian lineages.

Összefoglaló

A Mumbai-ban élő kutyák között gyakori a kullancsok okozta fertőzöttség és az ezek terjesztette kórokozók, így pl. *Ehrlichia canis* és *Babesia gibsoni* és más kórokozók előfordulása.

A tanulmány elsődleges célja az volt, hogy információt gyűjtsek a *H. canis* előfordulásáról, amelyet először 1905-ben írtak le Indiában, a Mumbaiban élő kóbor kutyák között. A másik cél az volt, hogy az ott talált *H. canis* génszekvenciákat összehasonlítsam az Indiában és más országokban talált szekvenciákkal.

Kilencven, olyan kutyából történt vérvétel az ottani Állatjóléti és Rehabilitációs Központ (AWRC), amelyek tünetmentesek vagy nem jellegzetes enyhébb-súlyosabb klinikai tüneteket (pl. levertség, hányás, láz) mutattak. A mintavételkor nem rögzítették az állatok fajtáját, ivarát, életkorát és egyéb adatokat.

Molekuláris biológiai vizsgálattal összesen 44 (48,9) mintában fordult elő *H. canis*. Ezek közül 10 pozitív minta volt szekvenálva, és ezt követően összehasonlítva a GenBank adatbázisból származó szekvenciákkal a nukleotid BLAST program segítségével. A vizsgált mintáink 97-100%-os azonosságot mutattak. Ezek többsége azonos vagy közel hasonló volt a Mumbai-ban vagy India más területén találtakkal.

Contents

1 Abbreviation list

PCR: Polymerase Chain Reaction.

TP: Total Protein

ALP: Alkaline Phosphatase

CK: Creatine Kinase

AST: Aspartate Transferase

PO: Per os

IM: Intramuscularly

IV Intravenous

NSAID: Non-Steroid Anti-inflammatory Drug

DNA: Deoxyribonucleic acid

rRNA: Ribosomal ribonucleic acid

IFA: Indirect Immunofluorescence Assay

IgM: Immunoglobulin M

IgG: Immunoglobulin G

Spp: Species

ELISA: Enzyme-Linked Immunosorbent Assay

2 Introduction

India is the 7th largest country in the world, situated in the south of Asia. The country has many different types of environments ranging from mountain and semi-arid regions to wet tropics. This variety of weather allows for a diverse range of arthropod vectors and pathogens of animals. There are approximately 28 million dogs in the country, which can be divided into four categories: pets, family dogs, community dogs and feral dogs. Majority, about 80% of dogs fall into the last three mentioned (Rani, et al., 2010). Seventeen percent of homes in India have a dog as a companion animal (Sudarshan et al., 2006).

Mumbai where the dogs were sampled is the economic hub and financial center of India as such it is no surprise that it is also the most populous city in India and most densely populated urban areas in the world. It was reported in 2014 by the BMC that there is in total 95, 172 stray dogs on the streets of Mumbai. This number increases each year (Municipal Corporation of Greater Mumbai, 2022). With the warm weather and large number of hosts it is not a surprise to hear that tick infestations of dogs are most common in Mumbai (Rani et al., 2011). Moreover, the dogs in Mumbai are at the greatest risk of tick-borne pathogens such as *Ehrlichia canis*, *Babesia gibsoni* and others. The coinfections caused by different tick-borne pathogens were detected in the dogs examined in Mumbai (Rani et al., 2011).

Figure 1: Location of the city of Mumbai in India and a closer topographical view of Mumbai (Google Maps)

Very little data were available about the occurrence of *H. canis* in the stray dogs living in Mumbai. Therefore, the primary objective of this study was to obtain information about the prevalence of this protozoan species using molecular biological methods. The other aim of this study was to compare the gene sequences of *H. canis* detected in Mumbai with those ones reported from different parts of India and some other countries.

3 Literature review

3.1 Taxonomy, morphology, and lifecycle

There are more than 300 species in *Hepatozoon* genus which belongs to the Apicomplexan phylum. They are characterized by their lifecycle in which their sexual development occurs in haematophagous arthropods and their asexual development occurs in visceral tissues or leukocytes of vertebrate hosts (Vincent-Johnson, 2014).

There are two main species, *Hepatozoon canis* which was first described in India in 1905 and has then been detected in many parts of Asia, Africa, Southern Europe, and Middle East (Vincent-Johnson, 2014) and *Hepatozoon americanum* which occurs in America. Both species cause canine hepatozoonosis though being close taxonomically, in fact are different in several ways, including final host, clinical signs and treatment. Canine hepatozoonosis could be considered a misnomer for it is not a zoonotic disease and rarely affects the liver (Vincent-Johnson, 2014).

The life cycle involves an intermediate such as a dog but can also include foxes, wolves, jackals, African wild dogs, and coyotes as well as a final host which is *Rhipicephalus sanguineus* tick species (Vincent-Johnson et al., 2021). This tick species is the most widespread and identifiable tick in the world. It is also adapted alongside humans and our dwellings and as such are prominent in urban and rural areas (Dantas-Torres, 2010). The life cycle of *H. canis* (Figure 2) starts uniquely when the dog eats a specimen of this tick species infested with *H. canis* mature oocysts containing many sporocysts each having 10-26 sporozoites. After being ingested, the sporozoites are released from the oocysts. They penetrate the intestinal wall and move towards specific tissues and organs (the liver, spleen, lymph nodes and bone marrow) via the blood and lymph (Taylor et al., 2013). In the intermediate host the asexual development of *H. canis* takes place, which is named merogony. The merogony happens in these tissues at least 13 days after infection. The developed meront forms are around 30x30µm in size has a cartwheel or wheel-spoke appearance. They are round to oval shaped and contain elongated micromeronts forming the recognizable appearance. There are two or four macromeronts and twenty micromeronts in a meront. The meront contains merozoites that penetrate neutrophils developing further into gamonts, which can be detected in the leucocytes of dogs. The gamont (Figure 3) is about 11x4µm in size, elongated, sausage-shaped and surrounded by a thick membrane. Its nucleus is elongated as well and can be horseshoe-shaped

shaped. It takes 28 days for the appearance of gamonts from when a dog is infected (Taylor et al., 2013; Baneth, 2007).

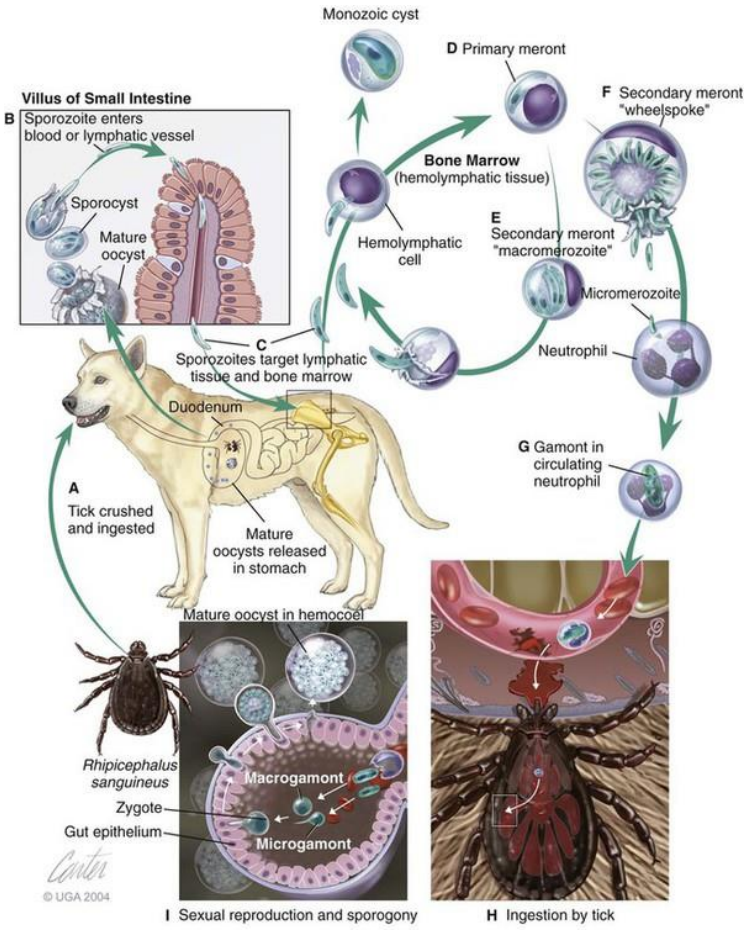

Figure 2: The life cycle of *H. canis*

https://veteriankey.com/hepatozoonosis

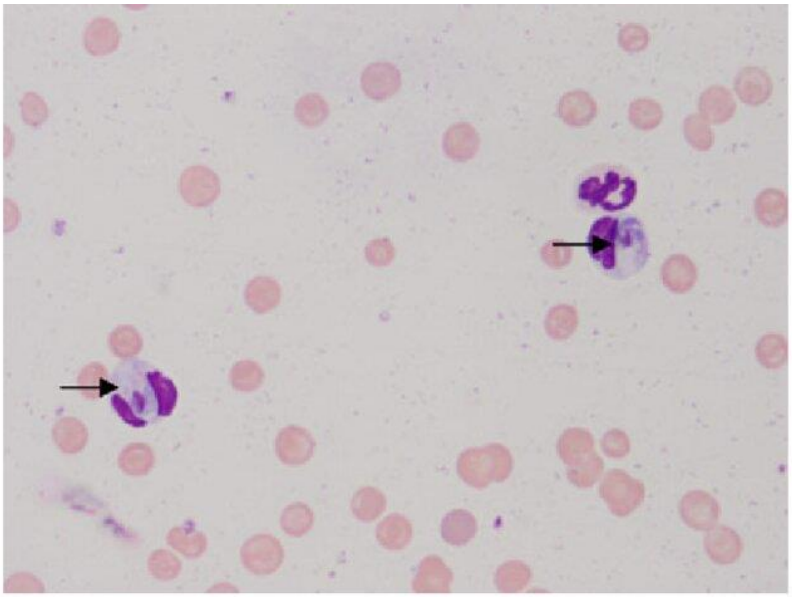

Figure 3: Blood smear of a dog exhibiting the gamonts (arrows) of *H. canis* in neutrophils (Kaur et al., 2012)

A nymphal stage of the vector species will ingest the leucocytes which contain gamonts during feeding on the infected animal. In the final host the sexual development takes place (Figure 2) where the gametogony and sporogony of the life cycle happen (Rani et al., 2011; Schäfer et al., 2022). The gamonts are freed from the leucocytes associate into pairs and turn into male and female gametes. This leads then to the formation of a zygote and oocyte found in the haemocoele of the tick where the gamonts divide after 24 hours post infection, and macro- and microgametes are formed and zygotes and young oocysts can be detected after 8 days (Ivanov and Tsachev, 2008). The life cycle from nymphal attachment till to parasitaemia in the animals is about 81 days (Baneth et al., 2007).

3.2 Pathogenesis and clinical signs of canine hepatozoonosis

The dogs become infected when they consume infected ticks. Very rarely the vertical infection may occur from bitch to puppy (Schäfer et al., 2022**).** A study on 3 families of dogs showed that vertical transmission is possible, possibly through the placenta. The gamonts were found in 14 of 21 puppies 31 days after birth and meronts were found in the spleen of 3 puppies which died (Murata et al., 1993). These dogs should not breed (Pacifico et al., 2020; Schäfer et al., 2022**).**

Most dogs having low parasitaemia may show signs of mild infection with a low inflammatory reaction. In cases of high parasitaemia direct injury to affected tissues and immune system may occur. In these cases, extreme loss of weight and cachexia regardless of good appetite were observed (Taylor et al., 2013). When an animal either has an underdeveloped immune status or has immunosuppressive drugs like prednisolone there is a greater impact on the pathogenesis (Ivanov and Tsachev, 2008). Three of the twelve dogs which did not show parasitaemia were treated with immunosuppressive doses of prednisolone. In two of these animals, parasitaemia developed in 48 hours while in the third one it also developed ten days after the immunosuppressive treatment (Baneth et al., 2001). In addition, it is common to find concurrent infections with *Ehrlichia canis* (Pasa et al., 2009) or *Toxoplasma gondii*. These pathogens worsen the pathogenesis and clinical signs. For example, in Israel, it was documented that two Dalmatian pups were seen to have parvoviral enteritis along with *H. canis*. It was thought to be due to the immunosuppressive nature of Parvo virus (Baneth et al., 1997).

There are three forms of clinical signs of hepatozoonosis, subclinical, acute and chronic (Ivanov and Tsachev, 2008). The subclinical form is the most common. Most infected dogs do not show any clinical signs. A low level of *H. canis* can be considered as an infection where less than 5% of neutrophils are found with gamonts. This is the most common being about 85% of cases where infection is subclinical or mild. While 15% can have what would be considered high parasitaemia where the number of gamonts in neutrophils is closer to 100%. In addition, with cases of high levels of *H. canis* extreme leukocytosis is seen as high as 150,000 leukocytes per microliter of blood is seen (Ivanov and Tsachev, 2008; Farkas et al., 2014; Schäfer et al., 2022). Only at high titers of *H. canis* does the infected dog show serious clinical signs such as fever, weight loss, lethargy as well as anaemia, lower platelet counts and enlarged spleen. The acute form of the disease is seen one week before the death of the animals (Hasani et al., 2024). In another case study done in Karnataka, India, the topic of which surrounds two young dogs between the ages of 2-3 months. The cause of the disease was determined to be acute hepatozoonosis. The dogs arrived at the clinic with a history of anorexia, lethargy, weakness, and deteriorating body condition (Roopali et al., 2017).

In addition, a study performed on ten dogs all from the Aegean region in Turkey showed many clinical signs like other researchers' findings. All dogs showed signs of weight loss, anorexia,

depression, lymphadenopathy, and fever. Eight of them showed skin problems, mucous membranes of six animals were pale and three dogs had ocular discharge. Eight out of the ten dogs were coinfected with other pathogens: either *Ehrlichia canis, Anaplasma phagocytophilum* or *Anaplasma platys*, or a combination of them. Two dogs were not coinfected but also showed all these clinical signs mentioned above except for one which did not have ocular discharge (Pasa et al., 2009).

In another study, three dogs were infected with *H. canis* but only one of them showed clinical signs: stiffness, paralysis, depression, anorexia but no fever. This dog died shortly after its examination (Inokuma et al., 2002).

Moreover, in a study conducted in Sardinia, Italy, nine dogs were positive for *H. canis* diagnosed using PCR. Out of these animals, six showed clinically unspecific symptoms. The other three dogs showed subclinical or mild clinical signs. In this study, the symptomatic dogs showed signs of fever and muscle pain along with more common symptoms such as anorexia and eye discharge (Chisu et al., 2023).

A study performed on a dog that had arrived in a kennel at the age of 2-5 months with heavy tick and flea infestation showed atypical clinical signs of hepatozoonosis due to coinfections. The dog had severe depression, pale mucous membranes, diarrhea, and ascites on day 0. The diarrhea and ascites resolved after one week. However, a cough and slight fever developed unexpectedly. On day 16 anemia and thrombocytopenia were still present. The ascites was concluded to be due to hypoproteinaemia but was noted to be an atypical sign of babesiosis and ehrlichiosis. The respiratory signs had been described as an atypical form of babesiosis and ehrlichiosis. The moderate hyperbilirubinaemia was supposed due to liver damage caused by *E. canis* and *H. canis*. The thrombocytopenia is a recurring finding of ehrlichiosis and babesiosis. Non-regenerative anaemia has been documented in cases with ehrlichiosis (Sasanelli et al., 2009).

3.3 Diagnosis

There are several ways used to help diagnose canine hepatozoonosis. The first step in making a diagnosis should be taking a detailed history and performing a proper clinical examination. The diagnosis starts from a history where veterinarians need to ask the dog owners about geographic location of their animals and recent tick exposure for example.

3.3.1 Clinical examination

Clinical examination should entail checking of temperature, mucous membranes, size and reactivity of submandibular, suprascapular and popliteal lymph nodes and a detailed search for ectoparasites, especially ticks. On clinical examination of two young dogs with confirmed hepatozoonosis severe tick infestation was observed along with enlarged submandibular and popliteal lymph nodes as well as fever and pale mucous membranes (Roopali et al., 2017). Since anaemia is common during a physical examination one would find pale mucous membranes. Blood samples can be taken from dogs for haematological, biochemical, microscopy, serological and PCR examinations to diagnose *H. canis* infection.

3.3.2 Haematology

Once haematological and biochemical tests are performed and analyzed commonly mild normochromic anaemia next along with lymphocytic monocytosis eosinophilia and thrombocytopenia are detected. Biochemically hyperglobulinaemia and elevated blood urea and nitrogen as well as low serum glucose, hypo-albuminaemia and increased serum alkaline phosphatase activity (Voyvoda et al., 2004; Rani et al., 2010; Otranto et al., 2011; Schäfer et al., 2022).

The difference in concentration of parasitaemia can also be seen when performing blood tests, haemoglobin concentration, haematocrit and total neutrophil count are altered. Anaemia hyperglobulinaemia, hypoalbuminaemia and increased serum alkaline phosphatase and creatine kinase activity (Ivanov and Tsachev, 2008). In one study, 28 dogs younger than 18 months naturally infected with *H. canis* confirmed by PCR testing and cytology did not show any clinical signs, but haematological abnormalities were present in 26 out of the 28 dogs (Schäfer et al. 2022).

A study of two Japanese dogs showed nonregenerative anaemia at 312 x 10^4/ml, packed cell volume 19%, haemoglobin concentration 6.9 g/dl, reticulocytes 0.7%, mild leukocytosis (21,500/µl), thrombocytopenia (21,000 µl), high activities of serum alkaline phosphatase (1083 IU/l) and creatine kinase (834 IU/l), hypoalbuminaemia (2.1 g/dl) and a high concentration of serum C-reactive protein (CRP, 8.4 mg/dl) (Inokuma et al., 2002).

In addition, it was seen in a study of 10 dogs, some with and without co-infections, that all dogs had microcytic normochromic anemia. Seven of 10 animals had neutrophilia, monocytosis was seen in two, eosinophilia and lymphopenia in one and thrombocytopenia. In 9 of the 10 dogs. The biochemical analysis showed a high concentration of TP in 7 out of these 10 dogs. Hypoalbuminaemia and hyperglobulinaemia were found in 9 and 7 dogs, respectively. Increased serum ALP activity was detected in 2 animals. Serum CK activity was high in 8 (Pasa et al., 2009).

When PCR is not available cytology on buffy coat is the next best option (Otranto et al., 2011; Singh et al., 2017; Schäfer et al., 2022). The traditional diagnostic method of *H. canis* is via microscopy. This is less sensitive but is a good option to evaluate the presence of the pathogen, examination of a buffy coat or blood smears is recommended. Sausage-shaped gamonts can be found inside the leukocytes when using Wright or Giemsa staining. The gamonts are typically 8-12 x 3-6 micrometers in size (Ivanov and Tsachev, 2008; Otranto et al., 2011). It is 3.8 times more sensitive and 2.5 times more sensitive than bone marrow cytology. Even when a combination of blood, buffy coat and bone marrow smears are done an increase of 7.5% was detected (Otranto et al., 2011).

A study on experimentally infected dogs showed that gametocytes can be detected in blood smears of peripheral blood neutrophils as early as 28 to 43 days post-inoculation. The mean time was 34 days post-inoculation. However, in one of the 5 dogs, parasitaemia was not detected via microscopy of the peripheral blood. Schizonts were seen by cytological examination of spleen and bone marrow touch preparations made at necropsy at day 53 post-inoculation (Baneth et al., 1998).

In a study performed in Egypt, 208 blood samples from dogs were taken. These samples were checked microscopically and then PCR was performed subsequently. The team found no signs of *H. canis* infection in the blood smears; the PCR performed confirmed the results also showing no trace of *H. canis* DNA present in the blood of the dogs (Hegab et al., 2022).

Microscopy is also very important to perform for it allows the veterinarian to check for coinfections. An article shows two cases where the doctor once examining the blood smear under the microscope was able to see neutrophils and monocytes containing *H. canis* gamonts, several monocytes with intracellular inclusions presenting like *E. canis* morulae as well as some monocytes co-infected with both *H. canis* gamonts and intracellular *Ehrlichia* inclusions (Baneth et al., 2015).

From blood smears, one can also determine the level of parasitaemia by counting 500 neutrophils and determining how many are infected. This was done in a study using ten dogs. They were able to determine parasitaemia levels between 1% and 23% for each of the dogs (Pasa et al., 2009). Blood smears were performed in Punjab, India on a mongrel dog. They found approximately 37% of neutrophils positive for H. *canis* gamonts. Capsule-shaped gamonts in the cytoplasm either surrounding the segmented nuclear lobes or along the periphery of the cells (Kaur et al., 2012).

3.3.3 PCR

In recent years confirmation has been received primarily via PCR. The target gene used to detect *H. canis* can be 18S rRNA gene. It is much more sensitive and specific when compared to other methods such as microscopic evaluation of blood smears. A PCR may be performed on 4 samples from one patient, these being blood, buffy coat, bone marrow and skin. Buffy coat, blood and bone marrow samples when examined using PCR are shown to be more sensitive than using a skin sample. The most ideal circumstance is using PCR on both blood and buffy coat from the same dog this method has a sensitivity of 98% (Otranto et al., 2011).

PCR was performed using EDTA anticoagulated peripheral blood on two Japanese dogs. The sequence analysis of the two DNA showed 100% identical to each other. Additionally, the DNA was 99% like the DNA of *H. canis* found in Israel and distantly related to *H. americanum* at 94% (Inokuma et al., 2002).

A recent study in Israel found *H. canis* in ticks of dogs. PCR tests were performed on these samples by crushing 5 ticks of each dog and then from this pool the DNA was extracted. The DNA extracted showed a relatively high homology with the DNA of *H. canis* found in Pakistan and India (Hegab et al., 2022).

PCR was conducted on blood samples from two Indian dogs with hepatozoonosis from Punjab. The team found 100% genetic match with *H. canis* found in Saint Kitts and Nevis and 99% match with *H. canis* found in Brazil (Singla et al., 2017).

3.3.4 Serology

Serological testing can be used as well. Indirect Fluorescent antibody test and ELISA can be used for diagnosis of canine hepatozoonosis. The antigen from gamonts is used. However, the serology

methods are mainly used for epidemiological studies or in dogs with chronic infections. Antibodies of IgM and IgG are also detectable in experimentally infected dogs as early as 16 and 22 post-inoculation (Singh et al., 2017; Schäfer et al., 2022).

In a study using 29 sera and 12 laboratory colony-bred dogs without ticks, 10 dogs had gametocytes from blood smears and 7 dogs did not have gametocytes but had confirmed diagnosis of *H. canis* in the last 4 months. The 12-laboratory colony-bred dogs were not positive with IFA test. However, 9 of the 10 dogs positive, with ranging titers from 1:64 to 1:4096, 6 of the 7 dogs were also found to be positive with ranging titers from 1:32 to 1:256 (Shkap et al., 1994).

Additionally, another study used IFA testing in cases of dogs with hepatozoonosis. In this study, IFA was used to track the antibody production post incubation of IgM and IgG towards *H. canis*. IFA was accurate in determining titer concentrations of the antibodies post incubation. It was able to find the starting day of antibody production as well as peaks of concentration and when the level of concentration was undetectable. The IgM antibody was first observed on day 16 post-inoculation for one of the dogs but the mean time to first IgM detection was 26 days post-inoculation. While the IgG antibody was first observed 22 days post-inoculation for one of the dogs while the mean time to first IgG detection was 29 days post-inoculation. IFA was determined to be very useful diagnostically, especially in cases which are mild or are chronic (Baneth et al., 1998).

Moreover, it is common to find concurrent infections with other tick-borne pathogens such as *E. canis* and *Babesia canis*. As well as viruses such as canine parvovirus, canine distemper virus and others like *Toxoplasma gondii* and *Leishmania infantum* (Baneth et al., 1997). These co-infections are important to consider when diagnosing patients correctly, due to the multiple manifestations of diseases in one patient. In addition, *H. canis* may increase the chance of coinfection, with organisms such as *Leishmania infantum*, *E. canis*, and *Mycoplasma haemocanis* (Pacifico et al. 2020). Coinfection was found when leukocytes were infected with *H. canis* and 8.2% of them also contained *E. canis* (Otranto et al., 2011). The prevalence of coinfection was identical for *H. canis* mixed with *Babesia* spp. and *H. canis* mixed with *E. canis* (Eamudomkarn, 2022).

3.3.5 Necropsy

The principal gross pathologic findings associated with hepatozoonosis are splenic necrosis, in both red and white pulp regions, especially in the lymphoid follicles. Hepatitis is also seen where Kupffer cell hyperplasia and mononuclear and neutrophil infiltration occurs. Interstitial pneumonia and thickened alveolar septa as well as glomerulonephritis and interstitial nephritis with multifocal necrosis can be seen in the kidneys (Vincent-Johnson et al., 2021).

In Egypt, postmortem examination conducted on dogs infected with *H. canis* showed, haemorrhagic in the liver and enlarged spleen. Histopathologically, there was a substantial loss of parenchymal tissue in the liver with hepatocellular degeneration and necrosis. The spleen showed lymphoid depletion and significant expansion of red pulp with active macrophages. Meronts were also found in histological samples from the liver (Mahdy et al., 2024).

A study diagnosing ehrlichiosis and hepatozoonosis in a dog in Bulgaria, many gross pathological findings were found. The body condition was good but with icteric mucous membranes were seen. On opening the abdominal cavity, multiple ecchymoses were seen on the serous coats of the pectoral and abdominal cavities along with the organs within. The cut surface of mesenteric lymph nodes showed a marble-like appearance. More haemorrhages were seen on the pancreas and gastrointestinal mucosa. The spleen and liver were enlarged (Tsachev et al., 2008).

3.4 Treatment and Prevention

The primary drugs used for the treatment of hepatozoonosis are imidocarb diproprionate and doxycycline. Imidocarb dipropionate at 5-6 mg/kg given every day can kill gamonts. Oral doxycycline at 10 mg/kg daily for 21 days along with the imidocarb dipropionate is also possible (Taylor et al., 2013).

In addition to drugs, supportive care is needed. When one should use anti-inflammatories primarily NSAIDS such as aspirin, phenylbutazone or flunixin meglumine to reduce pain and fever, as well as IV fluids if not eating or drinking. Glucocorticoids are prohibited from use because they exacerbate the disease (Voyvoda et al., 2004; Urquhart et al., 2007). In a case report about acute hepatozoonosis, two dogs were diagnosed to be infected with *H. canis*. The animals were treated per os with doxycycline at 10 mg/kg body weight. PO. for 24 days as Amitraz was

used against ticks. For case number 1, the doxycycline alone was sufficient, no pathogen was found in the blood smear on day 21. For case number 2, meloxicam (0.5 mg/kg BW, IM for 3 days) and haemetinics (Dexorange syrup, 5 ml, PO, twice a day) were used along with the same dosage of doxycycline. In this animal, *H. canis* was no longer detected at day 28 post-treatment (Roopali et al., 2017).

When imidocarb is not available a combination of toltrazuril and trimethoprim-sulfamethoxazole was used and proven to be successful (Voyvoda et al., 2004).

The prognosis of the disease is typically good if there is low parasitaemia and responds well to treatment. However, if the opposite, the prognosis can be poor (Taylor et al., 2013).

There are no commercial vaccines available for canine hepatozoonosis. As such prevention is very important. Like with any vector-borne pathogens like *H. canis*; the use of prophylactic acaricides should be used to prevent tick infestation of the dogs. There is no evidence that *H. canis* is transferable via blood transfusions. The infective stage of the parasite is as a sporozoite within the tick. However, it is not clear whether the tissue stages that may be infectious may circulate in dogs' blood, as such it is best to exclude infected dogs as blood donors. Finally, owners should prevent ingestion of ticks while grooming or scavenging. The infected ticks might be in the fur of wild animals as such should be avoided (Taylor et al., 2013**).**

3.5 Geographical occurrence of canine hepatozoonosis

This pathogen may be considered to be widespread worldwide, in Southern Europe, Middle East, Africa, South America and Southeast Asia (Taylor et al., 2013).

In areas of southern Italy *H. canis* infection of 1433 dogs was examined with PCR The prevalence of this protozoon was 14%, and 200 dogs were found to be positive (Pacifico et al., 2020).

Blood samples were taken from 300 dogs living in the south of Romania. After PCR was performed it was found that 54% percent of the animals (163/300) were H. *canis* positive (Cimpan, 2020).

In areas of southern Hungary, a study was performed where blood samples and ticks were taken from 100 shepherd dogs, 12 hunting dogs and 14 stray dogs. The total prevalence of *H. canis* was 26% (33 of the 126 blood samples). The prevalence in shepherd dogs was 31% (31 out of the 100

dogs), in the hunting dogs 8% (1 out of the 12 dogs) and 7% (1 out of the 14 dogs) were found (Hornok et al., 2013).

In Germany, 1050 dog blood samples were taken over a period of a year. When PCR was used for detecting *H. canis* infection 4.4% of the animals were found to be infected (Helm et al., 2020).

In Lisbon, Portugal, a study was performed on 2 groups of 142 dogs using PCR. The prevalence of *H. canis* was 20.4% (Dordio et al., 2021).

In 5 different areas around Sivas, Turkey 150 blood samples were taken from healthy owned dogs. Sixty-seven out of them (44.67%) were PCR positive for *H. canis* (Erol et al., 2021).

Another study was carried out with 694 blood samples collected from local stray and pet dogs in shelters in nine provinces in Turkey. Direct microscopy revealed *Hepatozoon* gamonts in the peripheral blood of three of 285 samples 155 animals were found to be positive with PCR for the presence of *H. canis* (Aktas et al., 2015).

In the northeastern region of Iran blood was collected from 150 stray dogs without any clinical signs. Five dogs out of them were deemed positive when using microscopy however, when using PCR, 12 dogs were confirmed to be positive for *H. canis* (Barati and Razmi, 2018).

In a study from Tunisia, blood samples were taken from 99 dogs showing clinical signs such as weight loss, apathy, dehydration pale mucous membranes, fever and anorexia. Only 2 of them were infected with *H. canis* (Bouattour et al., 2021).

In four different parts of Costa Rica, PCR was done on 146 dogs of which 7.5% were found to have *H. canis* (Rojas et al., 2014).

In Brazil, blood samples of 346 dogs (212 of the dogs from an urban environment and 134 from a rural one) were tested with PCR. The study states that 161 of the urban samples were positive while 113 of the rural samples were positive for *H. canis*. The prevalence of the disease in urban areas was 75.9% and 84.3%. in the rural areas, the overall prevalence was 79.2% (Miranda et al., 2014).

In the central western region of Colombia, 91 blood samples were taken from the cephalic vein of dogs. These dogs were from different cities in this area, some pets and some from shelters. Twenty-nine were positive for *H. canis*, of which 25 were positive only with PCR while one was positive

with examining the blood smear and 3 samples became positive with PCR and blood smear examination (Vargas et al., 2011).

In Punjab Pakistan, a study was performed on farm dogs using conventional PCR targeting of the 18S rRNA. Out of the 341 blood samples tested 155 were confirmed positive (Ahmad et al., 2018).

In Khon Kaen province, Thailand PCR detection of tick-borne pathogens in 70 ticks collected from 70 sick dogs 55 (78.57%) were positive. The most common pathogens were *H. canis* (65.71%) followed by *Babesia* spp. (31.43%) and *E. canis* (30.00%). Coinfection was observed in 14 ticks (20.00%), and coinfection with *Babesia* spp. and *E. canis* was the most prevalent double infection (n = 6). The prevalence of coinfection was identical for *H. canis* mixed with *Babesia* spp. and *H. canis* mixed with *E. canis* (n = 4) (Eamudomkarn, 2022).

In Xi'an and Hanzhong cities of Shaanxi province, China a study was done where 196 blood samples from dogs were collected. The prevalence of *H. canis* using PCR was 2.04% meaning that only 4 dogs were infected (Guo et al., 2020).

A study was conducted across nine Japanese islands and peninsulas. In this study, blood samples were taken from 196 hunting dogs. Using microscopy of Giemsa-stained blood smears, gametocytes of *H. canis* were found in 45 dogs' peripheral blood. Using PCR of the 18S rRNA gene 84 dogs were deemed to be positive and using PCR with a specific primer 81 dogs were positive (El-Dakhly et al., 2013).

In Kyrgyzstan, a total of 170 blood samples were applied to PCR looking for DNA from *H. canis*. The results showed the prevalence of *H. canis* to be 28.8% meaning 49/170 dogs were infected (Altay et al., 2018).

In Punjab, India a polymerase chain reaction was used on blood smear positive cases. Out of 778 blood samples, the prevalence of *H. canis* was 0.26%, meaning 2 of the total of 778 were positive for the parasite (Singla, 2016).

In Ludhiana district, Punjab (India) examination of Giemsa-stained peripheral thin blood smears revealed 5.78% (13/225) positivity for the gamonts of *H. canis*. The parasitaemia levels in the positive samples ranged from 1.5% to 10.5%. Of the 13 dogs, positive for *H. canis*, 12 were classified to be having low parasitaemia levels (Singh et al., 2017).

Blood samples collected from 130 dogs, 80 from Mizoram and 50 from Tripura, India were examined using PCR techniques. *H. canis* was found in 50/130 (38%) dogs, 34 samples were taken from Mizoram and 16 in Tripura (Sarma et al., 2019).

Blood samples and ticks were collected from stray dogs in Tamil Nadu, South India to assess *Anaplasma s*pp., *Babesia* spp., *Ehrlichia* spp., *Hepatozoon* spp., filaroids and *Leishmania* spp. infections. Of the 230 dogs examined, 229 (99.6%) were infested with ticks. Overall, 67.8% (n = 156) of dogs were positive for at least one pathogen, *H. canis* was the most prevalent (37.8%). Out of 295 ticks analysed, *H. canis* was found to be the most prevalent at (42.5%), (Manoj et al., 2020).

On microscopy examination, *Hepatozoon* gamonts were observed in twelve out of 525 blood smears collected in many areas of India. Using polymerase chain reaction, *H. canis* prevalence of 38.3% was in Delhi out of the 162 samples taken, 43.8% in Mumbai out of 162, and 24% in Ladakh out of the 100 samples (Rani et al., 2011).

4 Method and materials

4.1 Study population and sampling

The blood samples were taken from dogs in Mumbai, India in the Animal Wellness and Rehabilitation Centre (AWRC) clinic. These animals were stray dogs which either had no obvious clinical signs or mild non-specific clinical signs such as lethargy, seldom vomiting and mild fever. No information was recorded about sex, age breed etc.

An approximately 2ml of blood per animal was taken in EDTA tubes sampling. The blood samples were then sent to Hungary in appropriate packaging to the university. The samples were then kept at the appropriate temperature of -80°C.

4.2 Diagnostic method

The detection of *H. canis* in each sample was conducted in the Department of Parasitology and Zoology at the University of Veterinary Medicine, Budapest. The DNA of the samples was extracted using the QIAamp Tissue Kit procedure (QIAGEN GmbH, Hilden, Germany). Then each DNA sample was eluted for 200µl of TE buffer. The modified conventional PCR (Inokuma et al., 2002) was applied using the primers

HepF 5' – ATA CAT GAG CAA AAT CTC AAC – 3' and HepR 5' – CTT ATT ATT CCA TGC TGC AG – 3' to amplify a 650 bp long fragment of the *Hepatozoon* 18S rRNA gene.

The protocol was as follows. 2.5 µl of extracted DNA were added to 22.5 µl of reaction mixture containing 1.0 U HotStar Taq Plus DNA Polymerase (5U/µl) (QIAGEN GmbH, Hilden Germany), 0.5 µl dNTP Mix (10mM), 0.2 µl of each primer (50µM), 2.5 µl of 10x Coral Load PCR buffer (15mM $MgCl_2$ included), 1µl $MgCl_2$ (25mM) and 17.9 µl DW. An initial denaturation step at 95^0C for 5 min was followed by 35 cycles of denaturation at 95^0C for 40 s, annealing at 57^0C for 40 s and extension at 72^0C for 60 s. Final extension was performed at 72^0C for 7 min. then hold at 4^0C. DNA of a rodent *Hepatozoon* sp. served as positive control. PCR products were electrophoresed in 1.5% agarose gel (100V, 50 min), stained with ethidium-bromide, and visualized under ultra-violet light.

Some positive samples were then purified and sequenced by Biomi Ltd. (Gödöllő, Hungary). The sequences of each sample were then submitted to GenBank.

4.3 Phylogenetic analyses

The gene-tree was constructed (Shuangbin et al., 2022) based on multiple sequence alignment by MAFFT (Katoh and Standley, 2013). The best substitution model (HKY) was selected by functions of phangorn (v2.11.1) package (Schliep et al., 2017) based on the Bayesian information criterion (BIC). The generated neighbour-joining tree was optimized by the maximum likelihood method. Bootstrap values were produced by 100 iterations. All data processing and plotting were done in the R-environment (R Core Team R, 2024).

5 Results

5.1 *Hepatozoon canis* infection of the sampled dogs

The 90 dogs sampled were either clinically healthy or had very mild non-specific clinical signs. The breed, age, sex, or any other characteristics of the dogs were not recorded due to the inability of the people bringing in the stray dogs to provide sufficient information.

The blood after being confirmed positive for *H. canis* some of them were sequenced. The final ID submitted into the GenBank are PQ339814.1, PQ339815.1, PQ339816.1, PQ339817.1, PQ339818.1, PQ339819.1, PQ339820.1, PQ339821.1.

In total 44 of 90 stray dogs were positive for *H. canis*. Of the positive samples, 10 were weakly positive while 34 were confidently positive. Based on this result the prevalence of this protozoon infection was 48.9% in the stray dogs sampled in Mumbai.

5.2 Phylogenetic tree of *Hepatozoon canis* sequenced

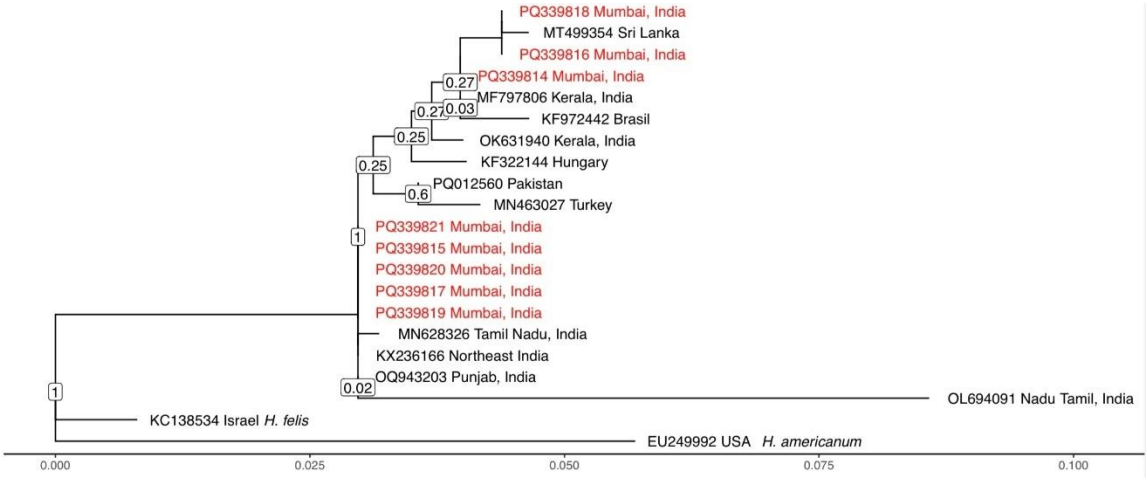

Figure 4: Phylogenetic tree showing genetic similarities between *H. canis* sampled for this study and some countries

In Figure 4, it can be determined that many of the *H. canis* detected in this study come from similar lineages and are like other lineages found In India. PQ339821, PQ339815, PQ339820. PQ339817, and PQ339819 are very similar to lineages found in Tamil Nadu and Northeast India. While PQ339814, PQ339816 and PQ339818 are very distant in relation to the previously mentioned lineages. In fact, PQ339814 is more like lines found in Kerela and Brazil, while PQ339816 and PQ339818 are more like a line found in Sri Lanka.

6 Discussion

Hepatozoon canis is a protozoon causing a disease called hepatozoonosis. This is a tick-born disease vectored by the tick *Rhipicephalus sanguineus* (Vincent-Johnson et al., 2021). The dog or other susceptible species such as foxes, wolves, and jackals can become infected with *H. canis* when eating the infected tick. After this sporozoites develop and spread via the bloodstream and lymph to many organs where meronts develop and undergo cycles releasing merozoites which invade white blood cells (Baneth et al., 2007; Vincent-Johnson et al., 2021). After invading the white blood cells, they become gamonts which are then consumed by the nymphal form of the vector where sexual reproduction occurs, and the life cycle completes (Taylor et al., 2013; Baneth et al., 2007).

Hepatozoon canis may be found as an accidental finding and frequently causes mild or subclinical clinical symptoms (Sant et al., 2017; Tołkacz et al., 2023). However, when an animal either has an underdeveloped immune status or using immunosuppressive drugs like prednisolone there is a greater impact on the pathogenesis (Ivanov and Tsachev, 2008). Acute forms of the disease are seen one week before the death of the animals (Hasani et al., 2024). The prevalence of the disease may differ from country to country, however, *H. canis* may be considered a worldwide disease. As such, should be on the minds of veterinarians consistently when dealing with patients.

Rhipicephalus sanguineus was found to be the most common and widespread tick species in India and was found to be the only vector tick species found in Mumbai (Rani et al., 2011; Balakrishnan et al., 2019). Mumbai is a major city in India. Due to its tropical climate, compared to other parts of India which may be considered more temperate. This climate is suitable for ticks, especially for the vector species, *R. sanguineus.* Mumbai is the city with the highest concentration of tick infection of dogs (Rani et al., 2011). Upon visual inspection, out of the 162 dogs, 80% were found to be infested with ticks. Delhi had a prevalence of 75.2%, Jodhpur showed a prevalence of 68%. while Sikkim and Ladakh had lower concentrations of dogs with tick infestations at 17% and 11% respectively (Rani et al., 2011; Totton et al., 2011).

The prevalence of *R. sanguineus* is similar in other different areas in India, where 100% of the ticks found on infested dogs were of *Rhipicephalus* spp. in Delhi, Ladakh, Kolkata and

Maharashtra. However, in Sikkim, only 44.4% were of *Rhipicephalus* spp., and the rest were of *Haemaphysalis* spp. (Raut et al., 2006; Rani et al., 2011; Jena et al., 2021).

From this study, the prevalence of *H. canis* in Mumbai was determined to be 48.9%. This result is in accordance with the result from Rani et al. (2011) where they determined the prevalence of hepatozoonosis in Mumbai to be 43.8% (Rani et al., 2011). However, in another earlier study, the prevalence of canine hepatozoonosis in Mumbai was determined to be only 9.64% (Pawar et al., 2005).

In the state of Punjab, the northwest region of India, *H. canis* caused subclinical infections in the local dogs with 3 to 9 prevalence (Rani et al., 2011). This protozoon was also transmitted due to ingestion of the tick *R. sanguineus.* As per this study, *H. canis* is the most found tick-borne pathogen in dogs in India (Rani et al., 2011).

The prevalence of *H. canis* differs in India significantly. As seen in the literature review, the prevalence varies from 0.26% (in Punjab) to 43.8% (in Mumbai) (Rani et al., 2011; Singla, 2016). The results of this study show the highest prevalence of *H. canis* recorded compared to available literature from India. This may be due to the sample taken from stray dogs which could be infested with the vector more often than the owned dogs.

This study also shows that the *H. canis* from Mumbai are related to other Indian lineages. This is different from a study where two samples from Punjab were 100% and 99% related to lineages found in Saint Kitts and Nevis (X112783.1) and Brazil (KF692040.1) respectively. Another study with phylogenetic analysis of *H. canis* in Kerala showed very close genetic similarity with the West Indian lineage (Singla et al., 2017; Lakshmanan et al., 2018).

In a study with confirmed *H. canis* found in an Indian jackal in Maharashtra, India. The phylogenetic analysis of this *H. canis* gave 99.68% similarity with other previously reported *H. canis* of wild dogs from India (Kolangath, et al., 2022).

This field of research requires more information, as gaps in knowledge are apparent. To add to this survey, I would like to include characteristics of the dogs in the survey, for example, age, gender, sex, type of housing, use of anti-ectoparasites, whether dogs live with other animals in the household and so on. This would allow a researcher the ability to provide results showing trends in groups. In addition, I would like to see research performed on dogs which have many concurrent

clinical illnesses, for example, diabetes, congestive heart failure, hepatitis and so on. There is no information in this field. In addition, concurrent tick-borne diseases are common findings in dogs. These concurrent diseases contribute to the clinical manifestations of the disease, leading to complex interconnected clinical signs in one patient. Much research has been done to show the relationship between *H. canis* infection and other tick-borne pathogens like *Ehrlichia canis,* and *Babesia canis* (Rani et al., 2011; Eamudomkarn, 2022). These 3 pathogens have close ties due to being vectored by the same tick species. As such, more information gathered on this topic will further allow veterinarians to better treat patients, by being able to distinctly target specific symptoms and form exact diagnoses. Research is also present, showing the relationship between *H. canis* and other non-tick-borne pathogens such as canine parvovirus, canine distemper virus, *Toxoplasma gondii* and *Leishmania infantum* (Baneth et al., 1997). There is less information, however, seen on the relationship between these pathogens. Therefore, I would suggest that researchers investigate this aspect of hepatozoonosis as well, to better help veterinarians treat patients.

As seen earlier, 80% of dogs in Mumbai are found to be infested with ticks (Rani et al., 2011). As such it is clear as to why the prevalence of the disease in this report is very high compared to other countries and areas in India. To prevent such high prevalence, I would encourage veterinarians to actively promote the use of effective control measures against tick infestation. In addition, I would encourage owners to prevent allowing contact between their dogs and other owned or stray dogs. As stated previously, a large proportion of stray dogs are already infested with ticks and as such transmission of ticks between stray and pet dogs is possible. Likewise, one may not know the anti-parasitic regimen of other pet owners; as such, keeping a safe distance between pets is also important to not allow transmission of ticks.

There are a few limitations in relation to this study. The first is that the sample size taken is not large enough to represent the scale of stray dogs in Mumbai. The next limitation is that no information regarding the sex, age, reproductive status, lifestyle etc. of the dog was recorded.

A larger sample size would allow the results to be more representative of Mumbai. With more information about the dog, the characteristics of dogs could have been grouped and analyzed showing trends between characteristics of dogs and infection of *H. canis*.

7 Conclusion

In conclusion, stray dogs in Mumbai are at relatively high risk of infection caused by *H. canis*. The results gathered in this study state that 44 of 90 stray dogs were positive for *H. canis*. The prevalence of this protozoan infection was 48.9%. This should indicate the need of veterinarians in the region to actively promote the use of tick prevention methods to pet owners. In addition, veterinarians should keep these results in mind, as the identification of *H. canis* may change the treatment plans of patient.

Moreover, all sequences retrieved from GenBank and included in the phylogenetic analysis had 97-100% coverage (i.e. aligned with a near-identical length and starting position) as sequences from this study.

This study also shows that *H. canis* from Mumbai are related to other Indian lineages.

8 Summary

In summation, *Hepatozoon canis* is a protozoa and vector born pathogen which typically does not cause clinical signs to dogs (Schäfer et al., 2022). However, when in high concentration in or decreased immune status of the dog may cause serious symptoms even death (Hasani et al., 2024). *H. canis* infects dogs orally by consuming the infected vector *Rhipicephalus sanguineus* (Schäfer et al., 2022**).** Once entering the intestines *H. canis* spreads and multiplies within many organs. Finally, it reaches the white blood cells where the iconic wheel-spoked appearance is seen. This form of *H. canis* is the consumed by the tick to continue its lifecycle (Taylor et al., 2013). The main symptoms seen in dogs with hepatozoonosis is weakness, lethargy and weight loss, more symptoms can be seen as well, especially if concurrent infection occurs which is common (Pasa et al., 2009; Hasani et al., 2024). *Ehrlichia canis, Toxoplasma gondii* as well as viruses such as parvovirus and canine distemper virus are known to cause symptoms alongside *H. canis* (Pacifico et al., 2020). Treatment of hepatozoonosis is usually with imidocarb diproprionate and/or doxycycline (Taylor et al., 2013). *H. canis* is a worldwide pathogen being found in countries Southern Europe, Middle East, Africa, South America and Southeast Asia (Taylor et al., 2013).

In this study the prevalence of *H. canis* was determined to be 48.9% this is the highest finding in similar parts of the region. This may be due to the sample taken from stray dogs. In addition, all sequences retrieved from GenBank and included in the phylogenetic analysis had 97-100% coverage. (i.e. aligned with a near-identical length and starting position) as sequences from this study. This study shows that *H. canis* from Mumbai are related to other Indian lineages.

These results show the need for tick prevention in owned dogs, in order limit the transfer of ticks and as such disease transmission. In addition, these results should resonate with veterinarians, who when testing and diagnosing should keep *H. canis* in mind.

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