EMERGENCE OF CANINE HEPATOZOONOSIS IN WESTERN ROMANIA SUPPORTS THE GEOGRAPHICAL EXPANSION OF THE DISEASE

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Abstract

Hepatozoonosis is an arthropod-borne disease caused by Apicomplexan protozoa from the family Hepatozoidae, genus Hepatozoon described in amphibians, reptiles, birds, marsupials and mammals. Transmission of Hepatozoon spp. in dogs occurs by ingestion of ticks that contains mature oocysts. The aim of this study was to investigate the prevalence of H. canis in dogs from the west and south-west Romania by using non-molecular and molecular techniques and the relationship between infestation and some epidemiological factors. During study 260 symptomatic and asymptomatic dogs from eleven west and south-western Romania counties were investigated by Diff Quik stain and PCR for presence of Hepatozoon spp. Molecular surveillance of blood samples from dogs in the western and south-western Romania, showed a 9.3% prevalence of canine hepatozoonosis. No statistical differences were observed between prevalence reported in age, gender, race, habitat and provenance. Following amplification of 18S rDNA gene sequence Hepatozoon canis species was identified. The results demonstrate the expansion of this disease transmitted by vectors in non-endemic regions and is first screening in the canine population in Romania.

Keywords: *Hepatozoon canis*, dogs, prevalence, Romania.

Introduction

Hepatozoonosis is an arthropod-borne disease caused by over 300 different species of protozoa of the family *Hepatozoidae*, suborder *Adeleorina*, *Hepatozoon* genus described in amphibians, reptiles, birds, marsupials and mammals. Of these, more than 120 species are found in reptiles and about 50 have been reported in mammals (Baneth, 2001, 2011, Vincent Johnson, 2014).

The genus name is due to the merogonic development of type strain *Hepatozoon muris* in the rat liver. The species from amphibians, reptiles and birds are red blood cells parasites, while in mammals *Hepatozoon* spp. gamonts are found mainly in leukocytes. There is a variety of haematophagous arthropod vectors that serve as the definitive host for different species of *Hepatozoon*. These hosts include ticks, mites, mosquitoes, sand flies, tsetse flies, fleas, lice and reduviidae (Baneth 2006, Macintire and Vincent-Johnson, 2006).

Transmission of *Hepatozoon* spp. occurs by ingestion of the definitive host, an invertebrate that contains mature oocysts, by the intermediate host, a vertebrate. There have been identified two species, in domestic dogs as intermediate host, namely *Hepatozoon canis* and *Hepatozoon americanum* (Baneth 2001, 2004, 2011, Vincent-Johnson, 2014 Ivanov and Tsachev, 2008).

The general lack of information on the spread and prevalence of canine hepatozoonosis in Europe, the lack of molecular studies to determine the species and the lack of information on *Hepatozoon* infection in the canine population of Romania led to the present study on 260 dogs, apparently asymptomatic and symptomatic, from different counties of Romania.

The purpose of this study was to investigate the prevalence of *H. canis* in dogs from the west and south-west Romania by using non-molecular and molecular techniques and the relationship between infestation and some epidemiological factors.

Materials and methods

Area and animals studied

During 2011-2015, in order to identify of some pathogens with blood localizations, including etiological agents from *Hepatozoon* genus, a total of 260 symptomatic and asymptomatic dogs were investigated.

The research was conducted at the Parasitology and parasitic diseases clinic of Faculty of Veterinary Medicine Timisoara. Samples representing of dogs blood from various localities in western and south western Romania were collected.

The study was conducted in both rural and urban areas, in eleven west and south-western Romania counties (Arad, Bihor, Caras-Severin, Dolj, Hunedoara, Gorj, Mehedinti, Olt, Satu-Mare, Timis, Valcea) with the support of veterinarians from private veterinary clinics and owners.

Dogs were cases of University Veterinary Clinics, private clinics, shelters or dog households. Animal's age ranged from 2 months to 16 years and there were several purebreds and crossbreds. Symptomatic animals had at least one clinical sign characteristic of a morbid entities, the most common clinical signs recorded were fever, hemoglobinuria, jaundice, dyspnea, arthritis, lameness, resistance to antibiotics related to previous contact with a tick, anorexia progressive weakening.

Working methods

After general examination of each animal whole blood sample were collected in sterile vacutainer with anticoagulant EDTA by puncture of the cephalic vein. In the day of collection or at a later date the samples were processed by classical techniques or molecular biology to highlight the presence of blood parasites concerned. The smears were stained by Diff-Quik method and then microscopically examined.

The first stage in the molecular analysis was the isolation of genomic parasitic DNA from the blood sample analyzed. This extraction was performed using PureLink®Genomic DNA Mini Kit kit (INVITROGEN®). The purified DNA product obtained was kept in a freezer at - 20° C until further processed.

The extracted DNA was subjected to polymerase chain reaction (PCR) of the 18S rRNA gene fragment (about 666 base pairs section) using specific primer set HepF (forward) (5'-ATACATGAGCAAAATCTCAAC-3') and HepR (reverse) (5'-CTTATTATTCCATGCTGCAG-3') and amplification conditions described by INOKUMA et al., 2002.

Positive and negative controls were also included in the reactions. In addition, to confirm the results of PCR 9 PCR products of *H. canis*, randomly selected, were purified and sequenced (Macrogen Europe®, Amsterdam, The Netherlands) using the same primers.

Control of the amplicons was performed by electrophoresis in a system horizontal submerged electrophoresis in 1.5% agarose gel at 120 V and 90 mA for 60 minutes.

After migrating samples in agarose gel, migrated DNA fragments in gel image was captured using a UV photodocumentation system (Molecular Imager® Gel Documentation System DocTM XR + Bio Rad®). Acquisition was performed using image analysis program Quantity One ver. 4.6.5., and using the computer program to calculate the amount of USI Vilber Lourmat amplified fragments.

Statistical analysis of the results was performed using GraphPad Software QuickCalcs to evaluate possible differences between epidemiological data of the dogs in the study. A value of $p<0.05$ was considered statistically significant.

Results and discussions

After examining blood samples (Table 1) different results depending on the method used were obtained. Thus, examination of stained Diff-Quik smears method revealed gamonts in 10 of the 260 dogs examined, which is a prevalence of 3.8%.

Table 1

Sinoptic of positive samples resulting from epidemiological investigation of *Hepatozoon canis* infection in 260 dogs studied, in the western and south-western Romania

Epidemiological data	*Hepatozoon canis*	
	***Diff-Quik* (%)**	***PCR* (%)**
Age		
≤ 2 years (*n*=70)	2 (2,9)	4 (5,7)
> 2 to 6 years (*n*=110)	3 (2,8)	8 (7,3)
≥ 6 years (n= 80)	5 (6,3)	12 (15,0)
Gender		
Female (*n*=149)	6 (4,0)	14 (9,4)
Male (*n*=111)	4 (3,6)	10 (9,0)
Breed		
Pure (*n*=210)	6 (2,9)	16 (7,6)
Mixed (*n*=50)	4 (8,0)	8 (16,0)
Habitat		
Urban (*n*=190)	6 (3,2)	15 (7,9)
Rural (*n*=70)	4 (5,7)	9 (12,9)
Owner		
With (*n*=230)	5 (2.2)	18 (7,8)
Without (*n*=30)	5 (16,7)	6 (20,0)
Total	**10/260 (3,8%)**	**24/260 (9,3%)**

n – dogs examined; % - prevalence obtained

Diff-Quik method is sensitive and specific, gamonts (fig. 1) are observed based on the morphological appearance in leukocytes in mammals, and the disadvantage of this method is that only a small percentage of infection can be detected by this method.

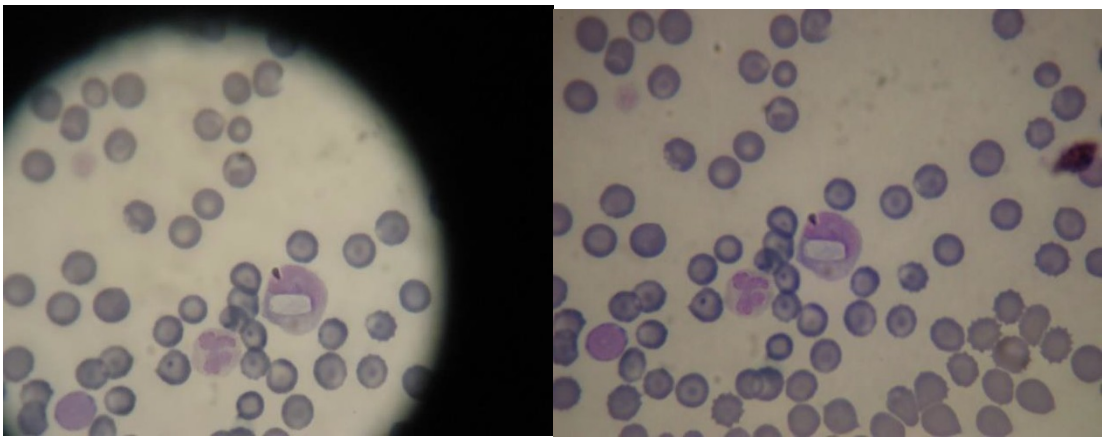

Fig. 1. *Hepatozoon* spp. (original)

Molecular biology analysis of 260 blood samples from dogs originating from 11 counties of western and south-western Romania revealed the presence of *Hepatozoon* spp etiologic agents.

Molecular screening of the 260 blood samples from dogs in the western and south-western Romania, registered a prevalence of 9.3% (24/260) for canine hepatozoonosis. Thus, based on amplification of the 18S rDNA sequence of the gene *Hepatozoon canis* species was identified.

The products of migration in 1.5% agarose gel of the PCR revealed consistent thickness and apparent bright strips at about 666 bp (Fig. 2)

Sequencing was done successfully for all the selected samples (n = 9), and confirmed the results of the conventional PCR. Genetic sequences of 18S rRNA isolated from *H. canis* in dogs, were identical to each other, and indicated the presence of a single genotype in our country.

The presence of this parasite in dogs is not surprising, given that tick *R. sanguineus* is wide distributed in the studied area (Imre et al., 2012, 2015).

During the sampling medical history and data about every animal we obtained and also additional data on the origin (232 dogs from urban areas and 28 dogs from rural areas), breed (50 mongrels and 210 pure-bred), age (≤2 years 70 dogs, 146 dogs from 2 to ≤ 6; 44 dogs aged over 6 years), gender (149 females and 111 males) (Table 1).

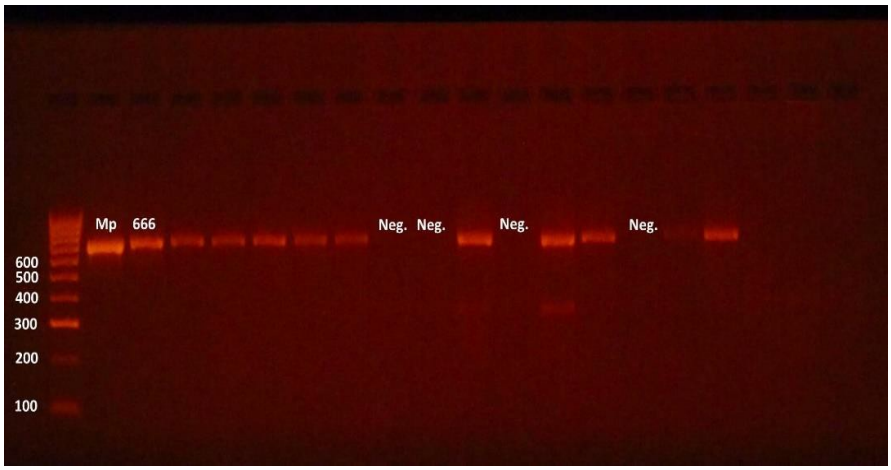

Fig. 2. Migration of PCR products in molecular diagnosis of *Hepatozoon canis* (Mp-positive control; Neg. – negative samples, 666 – positive samples) (original)

It was observed that the prevalence increased with age and were affected both females and males (9.4% - and 9.0 F - M), both pure breed dogs (7.6%) and cross breed (16%). The infestation was present in dogs in urban areas (7.9%) and those in rural areas (12.9%) in those with owners (7.8%) and without owner (20%). No statistical differences were observed between prevalence reported in age, gender, race, habitat and provenance.

The prevalence of hepatozoonosis has different values in the world. In the United States, Grenada, Yabsley et al., 2008, have identified a prevalence of 7%, by PCR in the dogs included in the study. In the south-eastern USA, LI et al. (20) examined blood samples of 614 dogs using the same technique and have found a prevalence of 27.2% for *H. americanum*, 2.3% *H. canis* and a value of 2.3% for mixed infections (*H. americanum* + *H. canis*). In the southern US, Allen et al., 2008, identified a prevalence of *H. americanum* of 1.83% and 98.8% for *H. canis*.

By examining blood smears, Mundim et al., 2008, established in dogs of Minas Gerais, Brazil, a seroprevalence of 77.39% for *H. canis*, while O'DWYER et al., 2001, by the same technique, reported a 39.2% seroprevalence in dogs in Rio de Janeiro, Brazil. In 2008, Metzer et al., 2008, examined samples from wild cats from different zoos by PCR and identified a prevalence

of 17.24% to 3.44% *H. canis* and *H. americanum*. Also in Brazil, Lima de Miranda et al., 2011, found in a tick (*Rhipicephalus microplus*) from a dog *H. canis* oocysts.

Mylonakis et al., 2005, tested by ELISA serological method, 69 samples from dogs in Greece and obtained 65.2% seropositivity for *H. canis*.

On the Aegean coast of Turkey, Karagenc et al. 2006, studied samples from 349 dogs and obtained a prevalence of 10.6% through blood smears method, 36.8% by indirect immunofluorescence and 25.8% by PCR (for *H. canis*).

In France, Criado-Fomelio et al., 2009, examined by PCR serological samples to identify hepatozoonosis in dogs and cats. The authors identified a prevalence of 0.9% and 1.7% for *H. canis*.

In Croatia, Vojta et al. 2009, have obtained a prevalence of 11.6% in dogs.

Conclusions

Molecular surveillance of blood samples from dogs in the western and south-western Romania, showed a 9.3% prevalence of canine hepatozoonosis.

Following amplification of 18S rDNA gene sequence *Hepatozoon canis* species was identified.

The results demonstrate the expansion of this disease transmitted by vectors in non-endemic regions and is first screening in the canine population in Romania.

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