



Retrospective study of canine infectious haemolytic anaemia cases reveals the importance of molecular investigation in accurate postmortal diagnostic protocols

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ABSTRACT

Infectious haemolytic anaemia (IHA) in dogs share similar clinical signs including fever, lethargy, icterus, paleness of mucous membranes and splenomegaly. Postmortal findings are similar and, without additional diagnostic methods, an accurate aetiological diagnosis is difficult to achieve. In order to investigate causes of lethal IHA in Croatian dogs, we performed a retrospective study on archived formalin-fixed, paraffin-embedded tissue blocks (FFPEB) from dogs that died due to haemolytic crisis, using microscopic and molecular diagnostic tools to determine the aetiological cause of disease. Molecular analysis was performed on kidney, lung, myocardium and spleen on FFPEB from all dogs. The originally stated aetiological diagnosis of *B. canis* or leptospirosis was confirmed in only 53% of the dogs. PCR and sequencing revealed that, in addition to the expected pathogens, *B. canis* and *Leptospira interrogans*, the presence of previously undiagnosed “new” pathogens causing anaemia including *Candidatus Neohrlichia mikurensis* and *Anaplasma phagocytophilum*. Furthermore, *Theileria capreoli* was detected for the first time in a dog with postmortal descriptions of lesions. Intensive extravascular hemolysis was noticeable as jaundice of the mucosa, subcutis and fat tissue, green or yellow discoloration of renal parenchyma caused by bilirubin excretion in the renal tubules and bile accumulation within the liver in 90% of the dogs. This work highlights the value of molecular diagnostics to complement traditional ante-mortem and post-mortem diagnostic protocols for the aetiological diagnosis of pathogens associated with IHA.

1. Introduction

Haemolytic anaemia develops as a consequence of red blood cell lysis due to infectious or non-infectious causes [1]. Infectious haemolytic anaemia (IHA) in dogs is the result of production of anti-erythrocyte antibodies, increased phagocytosis and lysis of erythrocytes by the host's phagocytic cells, and a shortened erythrocyte life-span caused by oxidative stress and haemolysins. Canine IHA is the consequence of infection by various pathogens including *Babesia*, *Theileria* and haemotrophic mycoplasma spp. [1–3]. The clinical manifestations of IHA include fever, lethargy, icterus, paleness of mucous membranes, splenomegaly and pigmenturia with haemoglobinuria [1,2]. Postmortal findings correspond to those in an acute haemolytic crisis with haemorrhagic diathesis, multiple organ dysfunction due to hypovolemic

hypoxia or pulmonary oedema development and hypercoagulability [1]. For successful treatment, precise aetiological diagnosis is essential because the diverse causes of IHA respond to different treatment protocols [4]. The overlapping clinical signs, which are independent of the identity of the causative agent of IHA, require the use of additional diagnostic methods like light microscopy, serological testing and PCR [5].

In Croatia, the major pathogens causing IHA in dogs are *Babesia canis* [6] and *Leptospira interrogans* [7]. These pathogens share the same epizootiology and the clinical manifestations found in dogs infected by them are frequently similar [4]. For this reason, the administration of antiprotozoal drugs and antibiotics has been the routine treatment protocol in Croatian clinics for canine IHA for almost a century [8–10]. The current diagnostic protocol for dogs that died of suspected IHA in

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Croatia includes histopathology to determine the cause of death. Additional tests like cytology from organ imprints for piroplasm detection and postmortal microscopic agglutination test (MAT) for serological determination of leptospirosis are rarely used.

In order to investigate causes of lethal IHA in Croatian dogs, we performed a retrospective study on archived formalin-fixed, paraffin-embedded tissue blocks (FFPEB) from dogs that died due to hemolytic crisis, using microscopic and molecular diagnostic tools to determine the aetiological cause of disease.

2. Materials and methods

2.1. Selection of cases

The archive of the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Zagreb was screened for cases of lethal IHA in dogs that were necropsied from January 2009 to December 2012. All cases were required to have an archived necropsy report with descriptions and photographic documentation of gross findings as well as archived histologic slides and FFPEB. Necropsy request forms were evaluated for details on the treatment protocols applied prior to an animal's death. Cases with an incomplete necropsy report or without archived tissue blocks were excluded. Nineteen dogs met all criteria during the study period.

In 17 out of 19 dogs, additional tests were carried out at the time of necropsy. Postmortal imprints of the kidney cut surface ($n = 5$) were evaluated for the presence of piroplasms using light microscopy by parasitologists of the Faculty of Veterinary Medicine. Serum samples extracted from blood clots from the heart at necropsy ($n = 7$) were subjected to MAT for the presence of *L. interrogans* (serovars *icterohaemorrhagiae*, *canicola*, *ballum*, *australis*, *pomona*, *grippityphosa*, *sejroe*, *saxkoebing*, *tarassovi*, *bataviae* and *hardjo*). In five dogs, both cytology and MAT were carried out on postmortal archival samples. In another dog, historical data on the presence of piroplasms in blood smears was available. Unfortunately, re-evaluation of the original cytology could not be performed since the slides were no longer available. The aetiological diagnosis described in the archived necropsy reports was determined after considering all pathological and additional test results available at that time. In six dogs, the determined cause of IHA was *L. interrogans*. In 10 dogs, *B. canis* was diagnosed as the cause of haemolytic crisis, and the aetiological agent of IHA could not be determined in three dogs. The history and test data for each dog are presented in Supplementary Table 1.

Molecular analysis was performed on kidney, lung, myocardium and spleen on FFPEB from all dogs. If some of the organs were missing, they were replaced with other available organs: in Dogs #11 and #18, liver was used instead of myocardium and lungs, respectively, while in Dog #5, liver and skin replaced kidney, myocardium and lung tissue. The spleen of all dogs was tested.

FFPEB were cut on a rotary microtome (Carl Zeiss HYRAX M 25 Rotary Microtome, Carl Zeiss AG, Oberkochen, Germany) at a thickness of 30 μm and placed in sterile 1.5 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany). The blocks were cut from one to six times, depending on the surface area of the tissue, to obtain approximately equal volumes of tissue. Thorough cleaning was performed between sectioning of different organs with an aliphatic hydrocarbon-based xylene substitute (Bioclear, BioGnost, Zagreb, Croatia) and DNA-Off (PureBiotech, Middlesex, NJ, USA) to prevent cross contamination.

2.2. Molecular investigations

Dewaxing before DNA extraction was done according to the protocol described by Chan et al. [11]. Dried tissue was digested with 180 μL ATL buffer (Qiagen, Hilden, Germany) and 20 μL proteinase K (Qiagen) for 18 h at 56 °C in a thermomixer instrument (Eppendorf). DNA was extracted from digested samples using the DNA 'Blood and

tissue kit' (Qiagen) in the automatic extraction system, (Qiacube, Qiagen). A sample of DNase/RNase-Free distilled water was included as control for DNA extraction in each round of extraction.

PCR mixtures of 50 μL were prepared using 25 μL G2 GoTaq mastermix (Promega, Madison, WI, USA), 18 μL DNase/RNase-Free distilled water (Qiagen), 1 μL at 10 pmol/ μL of each primer and 5 μL of sample. As a negative control, 5 μL RNA/DNAase-free water were added to a 45 μL reaction mixture. Positive and negative controls were included in all PCR reactions. To prevent cross contamination, DNA extractions, PCR, reagent preparation and DNA amplifications were carried out using strict precautions. All procedures were performed in separate rooms and disposable pipette tips, gloves and aprons were used throughout.

Samples were initially screened by a PCR assay targeting the mammalian cytochrome C gene to confirm successful DNA extraction and lack of inhibition [12]. Positive samples were further subjected to six different conventional PCRs for detection of *Anaplasma/Ehrlichia* spp. [13], *Babesia/Theileria* spp. [6], *Bartonella* spp. [14], *Hepatozoon* spp. [15], *Leptospira interrogans* [16] and *Rickettsia* spp. [17] DNA. Although *Anaplasma*, *Bartonella*, *Ehrlichia* and *Rickettsia* are not causes of haemolytic anaemia, their presence was investigated as these bacterial organisms are intracellular.

The successful amplification of the PCR product was confirmed by capillary electrophoresis QIAEXEL (Qiagen) using a QIAxcel DNA Fast Analysis kit, alignment markers (DNA QXAlignmentMarker15bp/3 kb) and QX DNA Size Marker 50bp–3000 bp. Amplified PCR products were purified using EXOSAP-it® (USB® Products AffyInc., Ohio, USA) according to manufacturer's instructions and sequenced in both directions (Macrogen, Amsterdam, The Netherlands). The resulting sequences were assembled using the SeqMan Pro software, edited with Edit Seq tools in Lasergene (DNASTAR, Madison WI, USA) and compared with available sequences present in GenBank using BLAST. A dog was considered positive for a pathogen if at least one organ showed PCR positivity that was confirmed by DNA sequencing.

2.3. Pathologic findings

Necropsy findings and photographs of gross lesions were re-evaluated and all lesions noted. Histological slides of kidney, liver, lung, heart and spleen were re-inspected by light microscopy to determine the presence of lesions. Additionally, in Dog #5, slides of skin tissue were investigated as well due to the presence of multifocal necrotic dermatitis. Erythrocytes and leukocytes within the blood vessels of analysed organs were screened for the presence of *Babesia* merozoites and other visible pathogens. A final aetiological diagnosis was determined based on a comparison of the previously determined aetiological diagnosis in the archived necropsy reports, serology and the pathological and molecular results determined in the current investigation.

3. Results

3.1. Archival detection of pathogens and leptospirosis serology

In 6 of 19 (31.6%) investigated dogs, piroplasm merozoites were found in postmortal kidney imprints (Dogs #3, 4, 6, 13, 14, 18), while in Dog #5, piroplasms were observed in clinical blood smears (Table 1). Antibodies against *L. interrogans* were detected by MAT in 4/12 (33.3%) dogs. Dog #8 and #17 were positive for serovar *icterohaemorrhagiae* (antibody titer 1:100 and 1:102400), Dog #11 for serovar *poi* (1:6400), and Dog #19 for serovar *pomona* (1:51200) (Table 1).

3.2. Molecular findings

The mammalian cytochrome C was successfully amplified from all samples (19/19; 100.0%) investigated in this study. Conventional PCR

Table 1

Additional tests results, sequencing outcome and comparison of previously determined diagnosis (initial diagnosis) and the diagnosis revealed in the current study (final diagnosis).

	Cytology	Histology	Serology	Molecular results	Treatment	Initial diagnosis	Final diagnosis
1	–	–	ND	<i>A. phagocytophilum</i>	At, Im	<i>B. canis</i> infection	<i>A. phagocytophilum</i> infection
2	ND	–	ND	<i>B. canis</i>	At, Im	<i>B. canis</i> infection	<i>B. canis</i> infection
3	+	+	ND	<i>B. canis</i>	At, Im	<i>B. canis</i> infection	<i>B. canis</i> infection
4	+	+	ND	<i>B. canis</i>	unknown	<i>B. canis</i> infection	<i>B. canis</i> infection
5	+	–	ND	<i>B. canis</i>	At, Im	<i>B. canis</i> infection	<i>B. canis</i> infection
6	+	–	Neg	<i>Theileria capreoli</i>	At, Im	<i>B. canis</i> infection	Theileriosis
7	–	+	ND	<i>B. canis</i>	At, Im	IHA of unknown cause	<i>B. canis</i> infection
8	–	–	<i>Ich</i>	neg	unknown	Leptospirosis	Leptospirosis
9	ND	–	Neg	<i>Candidatus Neorhlichia mikurensis</i>	At	Leptospirosis	<i>Can. N. mikurensis</i> caused sepsis
10	ND	–	Neg	<i>B. canis</i>	At, Im	Leptospirosis	<i>B. canis</i> infection
11	ND	–	<i>Poi</i>	neg	Im	Leptospirosis	Leptospirosis
12	ND	–	Neg	<i>B. canis</i>	At, Im	<i>B. canis</i> infection	<i>B. canis</i> infection
13	+	+	Neg	<i>A. phagocytophilum</i> and <i>B. canis</i>	No	<i>B. canis</i> infection	<i>A. phagocytophilum</i> and <i>B. canis</i> co-infection
14	+	+	Neg	<i>B. canis</i>	no	<i>B. canis</i> infection	<i>B. canis</i> infection
15	ND	–	Neg	<i>B. canis</i>	At	IHA of unknown cause	<i>B. canis</i> infection
16	ND	–	Neg	<i>B. canis</i>	At, Im	IHA of unknown cause	<i>B. canis</i> infection
17	–	–	<i>Ich</i>	neg	At, Im	Leptospirosis	Leptospirosis
18	+	–	ND	neg	At	<i>B. canis</i> infection	Piroplasmosis
19	ND	–	<i>Pom</i>	neg	At	Leptospirosis	Leptospirosis

The symbol “+” indicates that piroplasms were visible, whereas “–” indicates that piroplasms could not be found on cytologic or histologic slides; ND – not done; * – in this dog, piroplasms were visible on blood smears made before treatment; ** – only piroplasms were found on cytology and histology, while *A. phagocytophilum* was not detected; neg – negative result; *Ich* – *Leptospira interrogans* serovar *icterohaemorrhagiae*; *Poi* – *L. interrogans* serovar *poi*; *Pom* – *L. interrogans* serovar *pomona*; At – antibiotic treatment; Im – Imidocarb dipropionate treatment.

screening for *Bartonella* spp., *Hepatozoon* spp., *Leptospira* spp. and *Rickettsia* spp. DNA failed to detect any positive samples.

At least one organ was positive in 12/19 (63.2%) dogs for the presence of *Babesia/Theileria* DNA (Table 2). Sequencing revealed that amplified DNA fragments in 11/12 dogs (91.7%) were identical to the previously detected 18S rRNA sequence of *B. canis* BBC1 isolate reported from Croatian dogs (GenBank accession FJ209024). Of these *B. canis* positive animals, eight were male and three were female, ranging from 2 months to 10 years of age (mean 4.1 years). Three dogs were mixed-breed, while the other dogs were pure-bred (English Setter, German Shepherd, Šarplaninac, Pinscher, Hungarian vizsla, Chau-chau, Samoyed, Collie). Piroplasms were detected by cytology in 4/5 (80.0%) of the dogs in which postmortal cytology had previously been completed. Histological investigation in the current study revealed that in 5/11 (45.5%) of the *B. canis* positive dogs, piroplasms were found within erythrocytes (Table 1). In Dog #5, piroplasms were detected on ante-mortem blood smear. Further, the distribution of *B. canis* PCR positivity in organs varied across PCR positive animals. *B. canis* was most frequently detected in the myocardium (7/11, 63.6%) and lungs (6/11, 54.6%). A kidney sample was positive in one dog (1/11, 9.1%), and the spleen was positive in three dogs (3/11, 27.3%). One out of four organs was positive in eight dogs, while in the three remaining dogs, three organs were positive.

Amplicon sequencing of the other *Babesia/Theileria* DNA positive animal (1/12; Dog #6), revealed 100% identity to a *Theileria capreoli* (former *Theileria* sp. 3185/02) sequence detected in Croatian wolves and deposited in Genbank (GenBank acc. number MK089570) (Table 1). This animal was a mixed-breed, nine year old male dog treated with imidocarb dipropionate and antibiotics. Postmortal

cytology showed piroplasms while they were not observed on histological slides. The only PCR positive organ for piroplasms was the myocardium and MAT for leptospirosis was negative.

Anaplasma/Ehrlichia DNA was successfully amplified from three dogs (Table 1). Sequences from two dogs were identical to *Anaplasma phagocytophilum* (GenBank acc. Number MH643588), while one sequence was identical to *Candidatus Neorhlichia mikurensis* (GenBank acc. Number MH643587) (Table 1). *A. phagocytophilum* was molecularly confirmed from kidney and lungs without visible morulae in cytology and histology (Tables 1 and 2) in a 13 year old male English Cocker Spaniel (Dog #1) treated with imidocarb dipropionate and antibiotics. MAT was not performed in this case (Table 2). An untreated four year old, female Hungarian Vizsla (Dog #13) was co-infected with *A. phagocytophilum* and *B. canis* based on PCR screening for both pathogens. The myocardium was the only organ positive for *A. phagocytophilum*, while *B. canis* DNA was detected in the kidney, lungs and myocardium. MAT was negative (Table 1). *Can. N. mikurensis* DNA was detected in the kidney of a two months old male Belgian Shepherd puppy (# 9), treated with antibiotics. *Leptospira* infection was excluded due to negative MAT and PCR from organs.

We were able to molecularly detect pathogens in 18 dogs, except in a 12 year old male Collie (Dog #18) treated only with antibiotics, although piroplasms were detected on its archival postmortal imprints. Despite the lack of piroplasm detection by histology in this dog, diagnosis was based on postmortal findings and detection of merozoites in the archival cytology and the final diagnosis was determined as piroplasmosis based on this evidence (Table 1).

The overall results of PCR screening for each pathogen are presented in Table 1 and Supplementary Table 1.

Table 2

Results of organ positivity by PCR to the studied pathogens.

Pathogen/Organ* -N (%)	Kidney	Lungs	Myocardium	Spleen
<i>Babesia canis</i>	1/18 (5.5)	6/17 (35.3)	7/17 (41.2)	3/19 (15.7)
<i>Anaplasma phagocytophilum</i>	1/18 (5.5)	1/17 (5.9)	1/17 (5.9)	0/19 (0)
<i>Candidatus Neorhlichia mikurensis</i>	1/18 (5.5)	0/17 (0)	0/17 (0)	0/19 (0)
<i>Theileria capreoli</i>	0/18 (0)	0/17 (0)	1/17 (5.9)	0/19 (0)
Total	3/18 (16.0)	7/17 (41.1)	9/17 (52.9)	3/19 (15.7)

* Liver and skin samples were negative. Co-infection were counted separately.

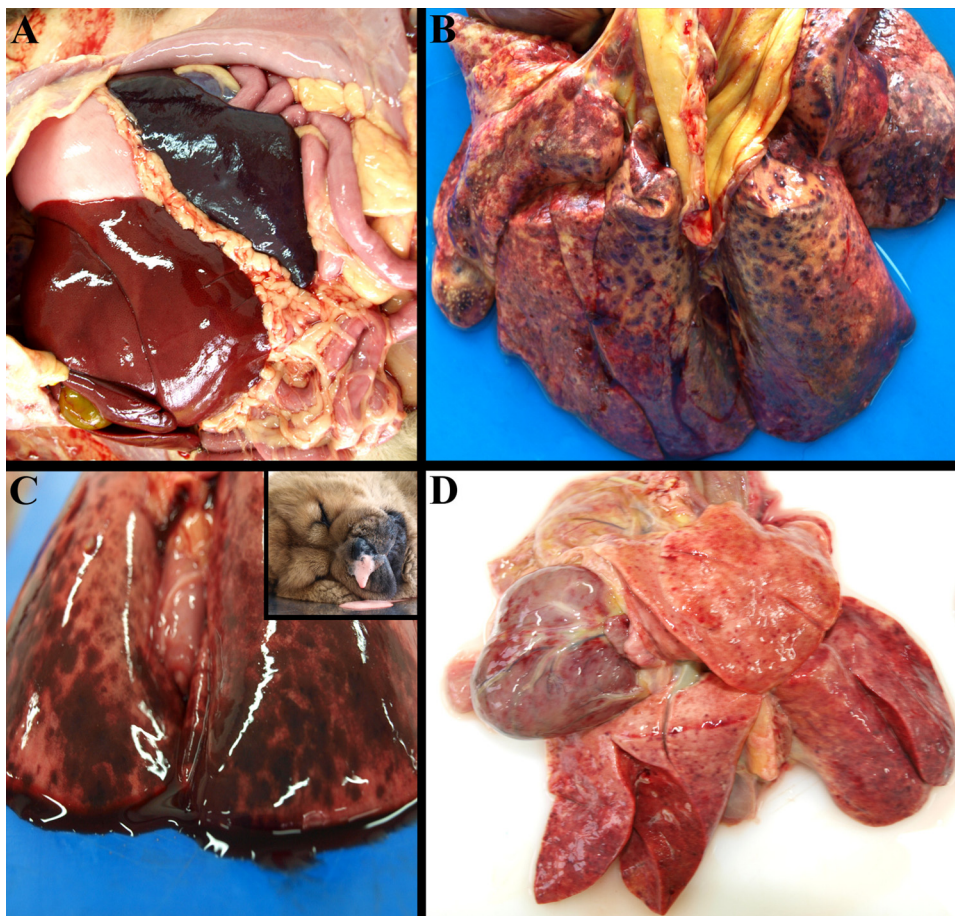


Fig. 1. A. Dog 14, *Babesia canis* infection. Ventral view of the abdominal cavity, after opening through the medial line. The liver and spleen are enlarged with rounded edges, indicating hepato- and splenomegaly. B. Dog 8, leptospirosis. Diffusely enlarged lungs with multifocal to coalescing bleeding within the parenchyma and red foam oozing from cut lung tissue, indicating haemorrhagic pulmonary oedema. C. Dog 14, *B. canis* infection. Haemorrhagic lung oedema. Inset: bloody foam seeping from both nostrils of the dog, indicating haemorrhagic pulmonary oedema. D. Dog 9, *Can. N. mikurensis* infection. Diffuse, moderate enlargement of lungs with multifocal to coalescing pin-point bleeding throughout the pulmonary parenchyma as well as heavy and rubbery lung tissue with foam oozing from the cut surface (pulmonary oedema). Multifocal to coalescing, marked subepicardial bleeding on the left side of the heart.

3.3. Pathological findings

In most of the animals investigated ($n = 19$), gross and microscopic findings mostly overlapped, reflecting severe hemolytic crisis with multiple organ dysfunction. Intensive extravascular haemolysis was noticeable as jaundice of the mucosa, subcutis and fat tissue, green or yellow discoloration of renal parenchyma caused by bilirubin excretion in the renal tubules and bile accumulation within the liver in 90% of the dogs (17/19) (Dogs #2–8, 10–19). Dog #9 showed slight jaundice of the subcutis which was attributed to liver damage but not to haemolysis. Dark brown to black kidneys with haemoglobin excretion indicated intravascular haemolysis in 68% (13/19) of dogs (Dogs # 2–4, 6–8, 11–14, 16–18). Massive acute necrosis of tubular cells caused renal failure in four dogs (Dogs # 1, 7, 11, 16; 21%, 4/19). Hepatomegaly (Fig. 1A) associated with dilation of the capillary network and blood plasma stasis (sinusoidal edema) with atrophy of adjacent hepatocytes and hepatocyte dissociation was observed in 74% (14/19) of dogs (Dogs #. 1–8, 11, 13, 14, 16–18). Splenomegaly (Fig. 1A) was found in 84% (16/19) of dogs with evident histiocyte accumulation in the red pulp (Nos. 1–7, 9, 11–14, 16–19). In 46% (5/11) of *B. canis* PCR positive dogs (Dogs #3, 4, 7, 13, 14), round, blue merozoites measuring 0.7–1.5 μm (Fig. 2A) were evident within erythrocytes on histological slides (Table 1). No evidence of other IHA-associated pathogens could be observed on histological slides.

A detailed description of the pathological findings with the final diagnosis for each dog is presented in Appendix Table A.1. Death was determined to be accompanied in eleven dogs (58%) in which molecular evidence for *A. phagocytophilum* (Dog #1), *B. canis* (Dogs #3, 4, 5, 7, 14, 16), *T. capreoli* (Dog #6) could be detected singularly or as a co-infection (*B. canis* and *A. phagocytophilum*; Dog #16) by septic shock complicated with disseminated intravascular coagulation (DIC) with

thrombosis and subsequent infarction of the kidney, liver (Fig. 2B), myocardium or spleen (Fig. 2C) as well as petechial bleeding in the liver, lungs, heart, or spleen. Similar findings were determined in Dog #18 for which an IHA pathogen could not be detected. Additionally, focally extensive dermal and epidermal necrosis as consequence of fibrin thrombosis of blood vessels associated with DIC was evident in Dog #5 with *B. canis* infection.

All dogs with leptospirosis (Dogs # 8, 11, 17, 19) and three out of 11 (27%) *B. canis* PCR positive dogs (Dogs # 2, 12, 15) showed massive, diffuse hemorrhagic pulmonary edema (Fig. 1B and C) and/or diffuse protein-rich edema of lungs. Severe hemorrhagic and suppurative myocarditis associated with *B. canis* infection was the likely cause of death for Dog #10 (Fig. 2D).

Dog #9 found to be PCR positive for *Can. N. mikurensis* succumbed to sepsis that caused focally-extensive mixed inflammatory pneumonia (Fig. 1D and 2F), multifocal mixed inflammatory perivascular and interstitial hepatitis with dissociation of hepatocytes and multifocal parenchymal bleeding. Furthermore multifocal to coalescing mixed inflammatory myocarditis with necrosis of myocytes (Fig. 2E) and subendocardial and subepicardial bleeding (Fig. 1D), as well as multifocal to coalescing mixed inflammatory glomerulonephritis were detected. The mucosae of the dog were pale, with white subcutis and pale red muscles indicating anaemia, and slightly yellow (icteric) mucosae and subcutis.

3.4. Evaluation of initial diagnosis, current diagnosis and treatment

Diagnostic discrepancies for each dog are summarised in Table 1 and in Appendix Table A.1. The originally stated aetiological diagnosis of *B. canis* or leptospirosis was confirmed in 53% of the dogs (10/19), including six dogs with babesiosis and four with leptospirosis. The

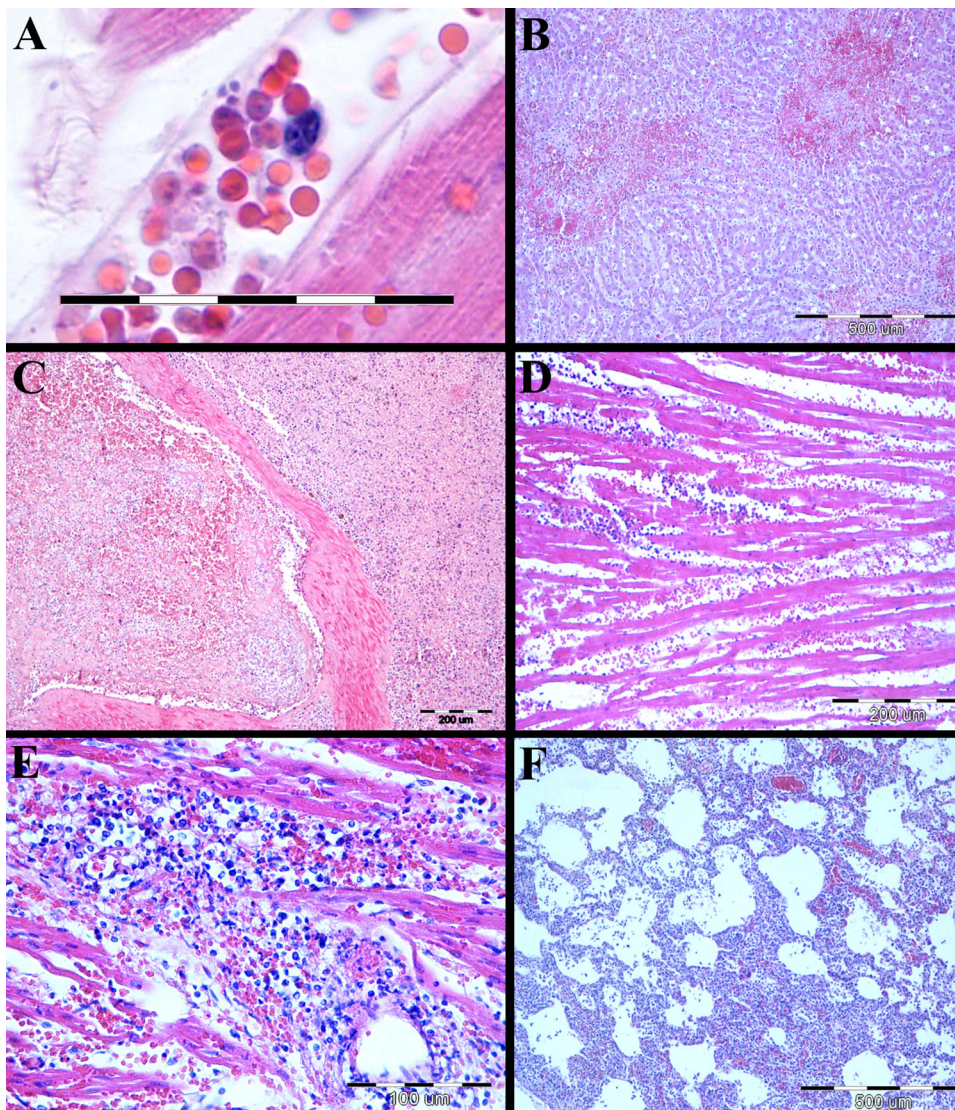


Fig. 2. A: Dog 4, *B. canis* infection. Numerous intraerythrocytic and extracellular round, 0.7 μ m–1.5 μ m, blue merozoites of *B. canis* within a vein in the myocardium. Hematoxylin and eosin stain (HE), objective magnification 100x, bar: 50 μ m. B: Dog 13, *B. canis* and *A. phagocytophilum* co-infection. Multifocal, acute centrilobular necroses of hepatocytes accompanied by bleeding. Surrounding sinusoids are dilated and filled with proteinaceous fluid and erythrocytes. HE, 10x, bar: 500 μ m. C: Dog 16, *B. canis* infection. Thrombosis of splenic artery with infarction of surrounding parenchyma. HE, 10x, bar: 200 μ m. D: Dog 10, *B. canis* infection. Acute purulent myocarditis with necroses of myocytes and endomyocardial edema. HE, 20x, bar: 200 μ m. E: Dog 9, *Can. N. mikurensis* infection. Mixed inflammatory myocarditis with bleeding and necroses of myocytes. HE, 40x, bar: 100 μ m. F: Dog 9, *Can. N. mikurensis* infection. Mixed inflammatory interstitial pneumonia. HE, 10x, bar: 500 μ m.

previous aetiological diagnosis differed, however, from that described in the current study in four dogs (21%): (i) in dog #1, *A. phagocytophilum* infection was confirmed instead of the originally diagnosed *B. canis*; (ii) in Dog #6, *T. capreoli* was detected instead of *B. canis*; and (iii) in Dogs #9 and #10, *Can. N. mikurensis* and *B. canis* were detected instead of *L. interrogans*, respectively. For a fifth dog, Dog #13, *A. phagocytophilum* co-infection was additionally detected beyond the original and confirmed detection in this study of *B. canis*. *B. canis* DNA was amplified in three dogs with no previously known cause of IHA found in the original investigation (Dogs #7, 15 and 16).

Treatment was known for 17/19 dogs examined in this study. Ten dogs (58.8%, 10/17) were treated with both antibiotics and imidocarb dipropionate. Based on the current study results, retrospectively five dogs (29%) were not managed correctly. These include two dogs (Dogs #13 and 14) with *B. canis* infection that were not treated at all, two dogs (#15 and 18) infected with *B. canis* that received only antibiotics; and Dog #11 with leptospirosis which was not treated with antibiotics. Two dogs (12%) infected with *L. interrogans* (#19) and *Can. N. mikurensis* (#9) were treated only with antibiotics.

4. Discussion

The results of the current study highlight the importance of molecular diagnostic tools in routine veterinary clinical and post-mortem

investigations to obtain an accurate diagnostic outcome for veterinary diseases associated with IHA. While most of the detected pathogens in this study are associated with haemolysis and haemolytic crisis with hypoxic damage of parenchymal organs, cases initially considered IHA were disproven as such by our study (Dogs #1 and #9 with *A. phagocytophilum* and *Can. N. mikurensis* infection, respectively). Our retrospective investigation confirmed the original diagnosis in only 53% of cases with alternative newly discovered (16%) or expanded (5%) diagnoses following molecular screening. Differentiation of pathogens responsible for IHA is not possible based only on postmortal lesions, as shown by the results of this study. The most striking pathological findings, including jaundice (95%), splenomegaly (84%), and hepatomegaly (7%), were commonly observed for all confirmed pathogens. Furthermore, diffuse hemorrhagic pulmonary edema was found in all *Leptospira*-infected dogs and 27% of the *B. canis* infected dogs.

B. canis and *L. interrogans* have been traditionally considered as the only causes of IHA in Croatian dogs. In the current study, PCR-based screening confirmed that *B. canis* was the most common pathogen (11/19; 57.9%), followed by *L. interrogans* (4/19; 21.1%), but this analysis also expands this list of potential IHA pathogens in Croatian dogs to include *T. capreoli*. Although the dog infected with *A. phagocytophilum* and the dog with *Can. N. mikurensis* infection showed anaemia, its mechanism was not attributed to haemolysis. Anaemia of chronic disease or immune-mediated mechanisms may explain this finding in

the two dogs, considering that antibodies against erythrocytes were detected in an *A. phagocytophilum* infected dog [18].

T. capreoli has been reported from various wild ruminants [19–22] and recently in foxes and wolves from Croatia [23,24], but not from dogs. Its pathogenicity is still unknown, however, infection with related pathogens including *Theileria equi* and *Theileria* sp. was associated with anemia and hemorrhagic diathesis in Croatian and African dogs [3,6]. Septic shock was the cause of death in the *T. capreoli* infected dog in our current study, based on gross and histologic criteria for septic shock [25], despite imidocarb dipropionate and antibiotic treatment. The archived diagnosis was *B. canis* infection because of merozoite detection on postmortal cytology and histological lesions detected that were consistent with those caused by *B. canis*. The modified diagnosis found in our study was based on DNA detection of *T. capreoli* from the heart of the affected dog. Archival cytology was unavailable to re-evaluate merozoites morphology from this dog and merozoite forms were also not detected by histology.

So far, bacteria from the Anaplasmataceae family have only been found in apparently healthy dogs from Croatia, while deceased dogs were not found to be infected in a previous study [26]. In the current study, the *A. phagocytophilum* infected dog showed anaemia only, while the animal co-infected with *B. canis*, experienced severe haemolytic anaemia, DIC and shock. The previously reported clinical findings attributed to erythrocyte destruction, due to immune-mediated haemolysis, and DIC in *A. phagocytophilum* infected dogs, including hepatosplenomegaly, petechial and ecchymotic bleeding on the surface of most organs and microthrombi in capillaries of kidney and lungs [18,27] are consistent with the postmortal changes recorded in the dog in this study.

To the authors' best knowledge, this is the first report of the detection of infection with the tick-borne pathogen, *Can. Neohrlichia mikurensis* at necropsy in a deceased dog. Infection in ticks, rodents and immunocompromised humans has been reported in Europe and Asia [28–30]. It was also described in an immunosuppressed dog with a mammary gland malignancy from Germany [31] and a splenectomized dog from Switzerland [32]. The petechial bleeding and anemia together with other postmortal lesions described in the current dog are consistent with reported clinical signs and histological findings from humans and the clinical signs from the aforementioned German dog [28,29,31,33]. The two month old puppy was treated with antibiotics because of leptospirosis despite a negative MAT result. The histological lesions observed were not consistent with adenoviral infectious canine hepatitis which causes centrilobular zonal necrosis of hepatocytes and mild, mostly neutrophilic inflammation in the necrotic tissue [1] and, instead, showed mixed inflammatory infiltration around blood vessels similar to inflammation described in one lethal human case [28]. In the absence of the detection of any other pathogens, it is likely that *Can. N. mikurensis* was the cause of disease in this animal.

Pathogens causing IHA are difficult to detect by histology due to their small size and occasional low intermittent parasitemia [5]. As a result, accurate aetiological diagnosis based on pathological examination alone is almost impossible. Clearance of diagnostic life stages soon after treatment can also further complicate the final diagnosis [34]. For example, *A. phagocytophilum* morulae in the current study could not be detected by cytology or histology and neither could piroplasms in treated dogs. In these cases, reliance on microscopy methods leads to the risk of a false negative diagnosis. Successful detection of pathogens from archival samples depends also on the selection of proper tissue. So far, the spleen has been the most commonly used organ for parasite detection [35]. Our results showed that myocardial and lung tissues represented more suitable tissues for molecular detection of *B. canis* in 41% and 36% positive samples compared to the spleen. The detection of *A. phagocytophilum* in the lungs and kidney from one dog, the myocardium from another dog and *Can. N. mikurensis* in the kidney of a positive animal, highlights the importance of analysing several organs from a suspected animal to decrease the potential number of false

negative results.

The current diagnostic protocol, using MAT and cytology, is suitable for diagnosis of leptospirosis and untreated babesiosis but not for treated dogs and for “newly” recognized pathogens. Also, it is not possible to differentiate among canine *Babesia* species just based on morphology [36], and changes in *B. canis* morphology from large to small in dead animals complicate cytologic detection even more [34]. It is perhaps for this reason that *T. capreoli* was misdiagnosed as *B. canis* and treated with imidocarb dipropionate. So far, no data exists on the treatment of *T. capreoli* infection in any host and, here, we report a lack of efficiency of imidocarb dipropionate. In two cases of leptospirosis, PCR testing revealed the presence of *Can. N. mikurensis* or *B. canis* infection. Diagnostic protocols including only cytology, histology and serology, could hence miss “unusual” pathogens.

5. Conclusions

Retrospective studies allow re-evaluation of current diagnostic and treatment protocols based on molecular investigation results as presented in the current study. Using this approach, we not only documented a range of existing pathogens that were misdiagnosed in the original investigations but also detected the presence of several new infectious causes of disease that had not previously been detected in Croatian dogs, including *T. capreoli* as a potential cause of anaemia in dogs. This work highlights the value of molecular diagnostics to complement traditional ante-mortem and post-mortem diagnostic protocols for the aetiological diagnosis of pathogens associated with IHA.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.cimid.2019.05.006>.

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