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Leptospirosis and immune-mediated hemolytic anemia: A lethal association

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Abstract

Immune-mediated hemolytic anemia (IMHA) is a common cause of anemia in dogs. The immune aggression towards erythrocytes can be triggered by many pathological conditions such as infection, inflammatory disease or neoplasia. Upon ruling out any eliciting conditions, a diagnosis of the primary immune-mediated disease can be made. In this particular case of severe anemia (tested positive for circulating antibodies against red blood cells with flow cytometry), vector-borne diseases (which are a common cause of immunopathology in Mediterranean countries) were excluded, leptospirosis was not. This resulted in an unsuccessful immunosuppressive therapy with prednisone, two whole blood transfusions and ultimately death of the patient. Leptospirosis (confirmed positive in two tests, micro-agglutination test for antibodies and PCR for microbial DNA in urine), can mimic a primary IMHA and must be considered in its differential list of causes. A liver involvement, that included elevated serum activity of liver enzymes and increased serum bile acid was observed at the admission and suggested an etiopathogenesis other than a primary IMHA.

Introduction

Leptospirosis is a bacterial infection with a worldwide distribution. It can affect most mammals, but some species seem more susceptible than others such as dogs and humans.[1] Infection with pathogenic leptospires can lead to a wide range of clinical manifestations varying from subclinical to severe and to a potentially lethal disease. The outcome of acute infection depends on the age and immune response of the host, and the virulence and inoculum amount of the pathogen.[2]

Leptospires commonly infect kidneys and the liver and therefore, should be considered as a plausible differential diagnosis in patients presenting with acute icterus, hepatic and/or kidney injury. Leptospires may also infect the lungs, spleen, endothelial cells, eyes, heart, skeletal muscles, meninges, pancreas, and the genital tract. This means that leptospirosis should be considered as a differential diagnosis for patients with disease affecting any of these organs.[1]

The mechanisms by which leptospires cause disease are not well understood, however, the presence of bacterial virulence factors have been considered. The involvement of toxins or toxic factors in the pathogenesis of leptospirosis has long been incriminated. The mechanism by which leptospira activates the immune system has been the main focus of the studies with a special emphasis on the involvement of cytokines[3] and immune responses to leptospires which have been implicated in target organ damage in severe cases of leptospirosis. Several inflammatory mediators were shown to be higher in susceptible animals than in resistant hosts.[4] Secondary immune-mediated diseases such as polyarthritis, IMHA and others have been suspected to occur but the actual incidence of canine cases is unknown.[5] In this case presentation, we will describe a complex and ultimately fatal leptospiral infection that occurred in a canine patient.

Case Description

An eight-year old crossbreed dog was referred to San Marco Veterinary Clinic, Veggiano, Italy, with a history of acute illness presenting a severe hemolytic anemia, and intense icterus most likely immune-mediated condition. Serological tests for the most common vector-borne diseases were negative. Since the dog had been vaccinated

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with a heptavalent vaccine (Canigen CEPPi/L; Virbac, Milan, Italy) a few days before the onset of the anemia, a vaccine-induced IMHA was suspected for which the dog was given one whole blood transfusion and received corticosteroid therapy with prednisone (Bruno Farmaceutici, Rome, Italy) at an immunosuppressive dose of 2.00 mg kg^{-1}, PO, q24hr, without any relief. Upon clinical examination, the dog was depressed, hypothermic and the mucous membranes were pale and icteric. The complete blood count (CBC) test revealed a severe macrocytic hypochromic regenerative anemia and severe leukocytosis with a neutrophil left shift (Table 1 and Fig. 1).

The serum biochemistry profile, amongst other abnormalities, displayed inflammation, increased liver enzymes, hyperbilirubinemia and increased serum bile acids (Table 2). In the coagulation profile, indicators of disseminated intravascular coagulation such as the increase of prothrombin time and activated partial thromboplastin time, fibrin/fibrinogen degradation products and D-dimers were significant ($p < 0.05$). In order to further assess the contribution of the immune system to the regenerative anemia, the presence of antibodies (IgG) against RBCs were detected with flow-cytometry (Beckman Coulter, Brea, USA) and despite the ongoing immunosuppressive therapy the result was positive (10%, normal < 1%). The percentages of erythrocytes binding IgG (EBIgG) were obtained by the Epics MCL 500 (Beckman Coulter, Brea, USA) FCI instrument on a K3 EDTA blood sample, using anti-canine IgG fluorescein isothiocyanate Isomer 1 (AbD Serotec, Oxford, UK).

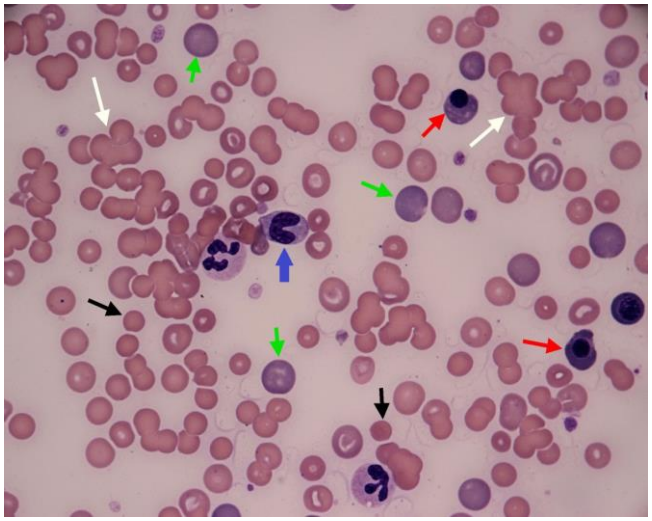

Fig. 1. Blood Smear of the examined dog. White arrows: agglutinates, green arrows: large polycromatophils RBCs, black arrows: spherocytes; red arrow: nucleated RBC, blue arrow: band granulocyte neutrophil. The two-segmented neutrophils visible in the picture display foamy and basophilic cytoplasm, as signs of toxicity, (Diff Quik stain; 100×).

Bearing in mind that a primary IMHA could be resistant to standard immunosuppressive therapy, the accompanying liver involvement was a rather unusual finding and so a PCR test for detecting Leptospira DNA in urine was performed which yielded a positive result. Total DNA from blood and urine was isolated by using the High Pure PCR Template Preparation Kit (Roche Diagnostic SpA, Milan, Italy). Primer LFB-1 (5'-CATTCAT

Table 1. Daily complete blood counts, from the admission (day 1) to day 4.

Parameters	Day 1	Day 2	Day 3	Day 4	Reference range
White blood cells (10^3 μL^{-1})	72.95	85.37	55.70	39.28	5.60-11.35
Metamyelocyte	2652.00	742.00	0.00	0.00	0.00
Bands (μL^{-1})	15912.00	14840.00	5570.00	0.00	0.00
Segmented neutrophil (μL^{-1})	43095.00	48972.00	42889.00	30360.00	3402.00-8025.00
Lymphocyte (μL^{-1})	2652.00	4452.00	4456.00	1380.00	941.00-3032.00
Monocyte (μL^{-1})	1326.00	5194.00	2228.00	2760.00	161.00-616.00
Eosinophil (μL^{-1})	663.00	0.00	557.00	0.00	49.00-997.00
Basophil	0.00	0.00	0.00	0.00	0.00-58.00
Platelets (10^3 μL^{-1})	261.00	318.00	451.00	312.00	196.00-436.00
Red blood cells (10^6 μL^{-1})	1.07	1.17	1.06	0.78	6.06-8.43
Hemoglobin (g dL^{-1})	2.80	4.00	4.20	3.50	14.30-19.80
Hematocrit (%)	9.90	11.40	9.50	6.60	41.80-59.00
MCV (fL)	92.20	97.10	89.80	84.10	64.30-73.50
MCH (pg)	25.80	34.00	39.60	44.90	22.00-25.10
MCHC (g dL^{-1})	28.00	35.00	44.10	53.40	31.60-36.80
CHCM (g dL^{-1})	28.00	28.70	29.50	30.10	32.00-37.60
CH (pg)	25.40	27.20	26.00	25.10	22.00-25.60
NRBC (per 100 WBC)	10.00	15.00	12.00	14.00	0.00
Reticulocytes (%)	25.94	18.96	12.32	12.67	0.11-1.26
Absolute reticulocytes (µL)	277558.00	221832.00	130592.00	98826.00	7645.00-99552.00
CRP (%)	5.71	4.80	2.60	1.86	0.11-1.55
Reticulocytes index	2.07	1.79	0.94	0.64	0.11-4.47

MCV: Mean cellular volume; MCH: Mean cellular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; CHCM: Cell hemoglobin concentration mean; CH: Hemoglobin concentration; NRBC: Nucleated red blood cell; CRP: Corrected reticulocyte percentage.

Table 2. Serum biochemical profile of the examined dog at the admission time.

Parameters	Result	Reference range
PON-1 (U L^{-1})	0.86	2.52-4.31
C reactive protein (mg dL^{-1})	3.24	0.01-0.36
Haptoglobin (mg dL^{-1})	33.00	33.00-165.00
Ferritin (µg dL^{-1})	2360.00	71.00-279.00
Total iron (µg dL^{-1})	155.00	87.00-185.00
UIBC (µg dL^{-1})	199.00	154.00-304.00
TIBC (µg dL^{-1})	354.00	305.00-436.00
Saturation (%)	43.80	23.10-51.50
CK (U L^{-1})	131.00	25.00-203.00
AST (U L^{-1})	92.00	17.00-38.00
ALT (U L^{-1})	1139.00	22.00-128.00
ALP (U L^{-1})	1624.00	8.00-198.00
GGT (U L^{-1})	9.00	0.80-8.80
LDH (U L^{-1})	146.00	7.00-122.00
Cholinesterase (U L^{-1})	5886.00	2935.00-7841.00
Total bilirubin (mg dL^{-1})	10.89	0.13-0.27
Fasted Biliary acids (µmol L^{-1})	64.40	1.00-16.90
Total protein (g dL^{-1})	5.10	5.80-7.40
Albumin (g dL^{-1})	2.10	2.80-3.70
Globulin (g dL^{-1})	3.00	2.70-3.90
A/G ratio	0.70	0.75-1.22
IgG (mg dL^{-1})	406.00	274.00-552.00
IgM (mg dL^{-1})	52.00	40.00-177.00
IgA (mg dL^{-1})	38.47	0.10-25.33
Cholesterol (mg dL^{-1})	244.00	141.00-338.00
Triglycerides (mg dL^{-1})	79.00	24.00-139.00
Amylase (U L^{-1})	503.00	368.00-1130.00
Lipase (U L^{-1})	262.00	64.00-543.00
Urea (mg dL^{-1})	58.00	20.00-48.00
Creatinine (mg dL^{-1})	0.75	0.82-1.31
Glucose (mg dL^{-1})	196.00	84.00-119.00
Calcium (mg dL^{-1})	7.80	9.50-11.00
Phosphorus (mg dL^{-1})	2.80	2.10-4.70
Magnesium (mEq L^{-1})	1.99	1.36-1.86
Sodium (mEq L^{-1})	138.00	143.00-150.00
Potassium (mEq L^{-1})	3.60	3.90-5.00
Na/K ratio	38.30	29.10-37.70
Chlorine (mEq L^{-1})	111.00	109.00-116.00
Lactate (mmol L^{-1})	1.30	0.80-3.80
HCO3 (mmol L^{-1})	17.00	16.70-24.90
Osmolality (mOsm kg^{-1})	289.00	293.00-306.00

PON-1: Paraxonase-1; UIBC: Unbound iron binding capacity; TIBC: Total iron binding capacity; CK: Creatine kinase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transferase; LDH: Lactate dehydrogenase; A/G ratio: Albumin/globulin ratio; Na/K ratio: Sodium/potassium ratio HCO_3: Sodium bicarbonates.

GTTTCGAATCATTTCAAA-3') and primer LFB1-R (5'-GGCCCAAGTTCCTTCTAAAAG-3') chosen in the locus LA0322 of *L. interrogans* Lai sequence were used to amplify a 331-bp product. Primers were synthesized at Eurofins Genomics (Vimodrone, Italy). A real-time PCR using Fast Sybr Green Master Mix (4385612; Applied Biosystems, Foster City, USA,) was performed. Also, serum micro-agglutination test (MAT) displayed positive results for various serovars: 1:800 for *L. interrogans* serogroup Australis serovar Bratislava and for *L. interrogans* serogroup/serovar Pomona, 1:200 for *L. kirshneri* serogroup/serovar Grippothyposa and for *L. interrogans* serogroup/serovar Icterohaemorrhagiae and 1:100 for *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenagheni. Despite quick discontinuation of the corticosteroid therapy, the administration of ampicillin (Ceva Salute Animale, Agrate Brianza, Italy) 20 mg kg^{-1}, IV, q8hr and a subsequent second whole blood transfusion with compatible blood (assessed by blood typing and cross-matching tests), there was a rapid deterioration of the overall clinical condition, with the anemia taking a turn for the worse leading to the death of the patient after three days of hospitalization.

Discussion

The diagnosis of leptospirosis was highly plausible, because of the positive PCR result for leptospiral DNA in urine. The MAT was not fully diagnostic, due to the lack of high titers of agglutinant antibodies and the confounding effect of the recent immunization with a bivalent leptospirosis inactivated bacterin vaccine. A seroconversion/convalescence sampling would have been of great interest[1,2] however, because of the rapid progress of the disease and its outcome, the employment of such a useful and highly significant diagnostic procedure was impeded. Nevertheless, according to the current guidelines, a dog with clinical signs consistent with leptospirosis, vaccinated with a bivalent vaccine against *L. interrogans* serogroup/serovar Canicola and serogroup/serovar Icterohaemorrhagiae, a single titre of at least 1:800 for one or more serogroup(s) is highly suggestive of an active infection.[2] The patient was seropositive for *L. interrogans* serogroup Australis serovar Bratislava and to *L. interrogans* serogroup/ serovar Pomona with a titer 1:800.

We cannot offer any further information regarding the serogroup or serovar of the identified Leptospira because of using universal primers for *L. interrogans*, the PCR that was performed is able to only recognize the genus and species of the bacteria as *L. interrogans*.

Unfortunately, although the authors' institution belongs to a working group which undertaking the molecular identification of Leptospira isolates,[6] in this particular case, as in many others, the amount of bacterial DNA was too low to be characterized further using any other test. In fact, it is believed that different serogroups of *L. interrogans* are able to cause various and unspecific syndromes, depending on the variations in the host's immune response and possibly lateral transfer of the virulence factors between *L. interrogans* serovars.[7]

Approximately 50.00% of the dogs with leptospirosis are presented with anemia which is usually mild to moderate. The causes of anemia can be blood loss via the

respiratory or the gastrointestinal tracts and inflammatory diseases. Hemolysis due to the effect of leptospiral toxins on erythrocytic membranes is rare in dogs in comparison to cattle. [2,8] In this case, the anemia appeared to be a major feature and fitted the classical criteria for the diagnosis of IMHA, namely icterus, marked regeneration and a positive test for antibodies on the surface of RBCs. The IMHA is the most common autoimmune disease in dogs, where the produced antibodies bind to the erythrocytes and cause anemia by a type 2 hypersensitivity mechanism.[9] An association of IMHA with vaccination has been described but in the most recent case studies no significant differences were found in the temporal relationship between vaccination and initiation of IMHA.[10,11]

Within all immune disorders, it is mandatory for the clinician to discriminate between primary (i.e., auto-immune) and secondary (i.e., consequences of neoplasia, infections) causes that are able to elicit, with various mechanisms, intravascular or extravascular destruction of RBCs. In case of secondary immune-mediated conditions, an emergency immunosuppressive therapy is temporarily administered but the root cause must be identified appropriately and thoroughly.[9-11] In this case, the misdiagnosis of primary IMHA resulted in a catastrophe consequence, not only due to the known immune-suppressive effects of corticosteroids facilitating bacterial infection but also the high mortality rate associated with this type of infection despite proper therapy.[1]

In the present case, leptospirosis caused an unusual pattern with severe hepatic involvement and without the typical acute kidney complication. Similar presentations have also been recently described in UK.[12] The icterus, documented by the elevated total hyperbilirubinemia, could also be caused by the hemolysis and is currently considered a relevant prognostic factor in IMHA.[11] In our patient, it is interesting to point out that serum bile acids were also increased. This parameter is not influenced by the indirect bilirubin or the bilirubin resulting from RBCs breakdown and it is a useful marker of hepatic function.[13] The coagulation profile did not indicate a specific diagnosis since disseminated intravascular coagulation is featured in both IMHA[10,11] and leptospirosis.[1,5]

In conclusion, when a clinician is faced with an IMHA, it is strongly advised that an inciting cause other than the autoimmunity should be considered.[10] Obtaining the proof of circulating antibodies against RBCs with flow cytometry or Coombs' tests are recommended for the diagnosis of IMHA since those antibodies are present in both primary and secondary forms of the disease.[14] The former veterinarian had the correct approach in ruling out canine vector-borne diseases since they are a primary cause of hemopathies of immune-mediated origin.[15] Unfortunately, leptospirosis had not been considered. This may be most likely because it is only occasionally mentioned in literature as a possible underlying cause for IMHA. To our knowledge, only one dog has been described with a clinical presentation[8] comparable to our case.

At the time of the occurrence of the case in 2016, two tetravalent anti-leptospira (L4) vaccines were commercially available in Italy, both containing *L. interrogans* serogroup Australis serovar Bratislava. And that in accordance with the MAT test, could be one of the two most likely infectious agents that caused the clinical manifestations. However, it is hardly arguable that a L4, instead of a bivalent vaccine could have spared the dog this lethal disease.

As a final yet important matter to point out is the severe zoonotic risk imposed by an untreated, immunosuppressed dog infected with *L. interrogans* to the environment, pet's owner including family members and attending clinicians and nurses.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Murphy K. Leptospirosis in dogs and cats: New challenges from an old bacteria. In Pract 2018; 40: 218-229.
2. Schuller S, Francey T, Hartmann K et al. European consensus statement on leptospirosis in dogs and cats. J Small Anim Pract 2015; 56(3): 159-179.
3. Marinho M, Cardoso T. Pathogenesis of leptospirosis: Important issues. J Med Microbiol Diagn 2015; e127. doi: 10.4172/2161-0703.1000e127.
4. Chirathaworn C, Kongpan S. Immune responses to Leptospira infection: Roles as biomarkers for disease severity. Braz J Infect Dis 2014; 18(1):77-81.
5. Goldstein RE. Canine leptospirosis. Vet Clin North Am Small Anim Pract 2010; 40(6): 1091-1101.
6. Natale A, Lucchese L, Boniotti A et al. Trying to better understand the epidemiology of leptospirosis in dogs: strain genotyping. In proceedings: The Prato conference on the pathogenesis of bacterial infections of animals, Monash University, Prato, Italy 2016; 11-14.
7. Klaasen E, Adler B. Recent advances in canine leptospirosis: Focus on vaccine development. Vet Med (Auckl) 2015; 6: 245-260.
8. Bovens C, Fews D, Cogan TA. Leptospirosis and immune-mediated hemolytic anemia in a dog. Vet Rec Case Reports 2014; 2 (1): e000065. doi: 10.1136/vetreccr-2014-000065.

9. Gershwin LJ. Current and newly emerging autoimmune diseases. Vet Clin North Am Small Anim Pract 2018; 48(2): 323-338.
10. Piek CJ. Canine idiopathic immune-mediated hemolytic anemia: A review with recommendations for future research. Vet Quart 2011; 31(3): 129-141.
11. Swann J, Skelly B. Canine autoimmune hemolytic anemia: Management challenges. Vet Med (Auckl) 2016; 7: 101-112.
12. McCallum KE, Constantino-Casas F, Cullen JM, et al. Hepatic leptospiral infections in dogs without obvious renal involvement. J Vet Intern Med 2018; 33(1): 141-150.
13. Chapman SE, Hostutler RA. A laboratory diagnostic approach to hepatobiliary disease in small animals. Clin Lab Med 2015; 43(6): 1209-1225.
14. Wardrop KJ. Coombs' testing and its diagnostic significance in dogs and cats. Vet Clin North Am Small Anim Pract 2012; 42(1): 43-51.
15. Day MJ. The immunopathology of canine vector-borne diseases. Parasit Vectors 2011; 4: 48, doi:10.1186/ 1756-3305-4-48.