Canine visceral leishmaniosis and concurrent infections (hepatozoonosis, toxoplasmosis and canine distemper)

N. TOPLU*, H. AVCI, N. METIN

Department of Pathology, Faculty of Veterinary Medicine, University of Adnan Menderes, 09016 Isikli-Aydin, TURKEY.

*Résumé: Leishmaniose viscérale canine et infections concomitantes (hépatozoonose, toxoplasmose et maladie de Carré)

Cette étude décrit les altérations anatomopathologiques et le diagnostic immunohistochimique obtenus sur 6 chiens atteints de leishmaniose vésicale présentant simultanément une toxoplasmose, une hepatozoonose ou une maladie de Carré. Des lésions inflammatoires intenses de la peau, des reins et des poumons associées à un important infiltrat de cellules mononucléées (lymphocytes, plasmocytes et macrophages) des noeuds lymphatiques, de la rate, de la moelle osseuse, du foie, des reins, des poumons et de la sous-muqueuse intestinale ont conduit à une forte suspicion de leishmaniose dont le diagnostic a été confirmé par la mise en évidence directe des amastigotes dans les macrophages infiltrés par histologie et immunohistochimie. Chez 2 chiens (n°1 et 4), des lésions inflammatoires des méningses de la boîte crânienne ont également été observées et de nombreux tachyzoïdes de Toxoplasma gondii en position extracellaire ou au sein des macrophages ou de kystes tissulaires ont été identifiés par histologie et immunomarquage spécifique. La présence du virus de la maladie de Carré a été confirmée par une immunohistochimie spécifique dans les zones nécrotiques des poumons et du cervelet d’un chiot de 2 mois (n°6). L’identification par examen histologique des différents stades de développement des schizontes de Hepatozoon dans les noeuds lymphatiques, la rate et la moelle osseuse a confirmé le co-diagnostic d’hepatozoonose chez 4 chiens. L’étude de ces cas suggère qu’une infection primaire par les Leishmania peut promouvoir l’apartition d’infections secondaires telles que la toxoplasmose, l’hepatozoonose et la maladie de Carré.

Mots clés : Leishmaniose viscèreale, histologie, immunohistochimie, hepatozoonose, toxoplasmose, maladie de Carré, chien.

Introduction

Canine visceral leishmaniosis (CVL), characterized by both cutaneous and visceral lesions, is an endemic disease of the foxes, wild canids, dogs and humans in the Mediterranean countries, the Middle East, some parts of Africa, India and Central and South America [8, 13, 18, 24, 31]. CVL and parapoxvirus co-infection has recently been reported in a Mediterranean monk seal [36]. The protozoan organisms responsible for CVL, Leishmania infantum (in Mediterranean and in Europe) and Leishmania chagasi (in America), are transmitted through blood-sucking sandflies of the genus Lutzomyia in the New World and Phlebotomus in the Old World [23, 31] and infest macrophages which are the principal host cells.

Clinical features of leishmaniosis can widely vary in sick dogs. Sick animals with CVL show chronic wasting disease with anaemia, intermittent pyrexia, mainly local or generalized lymphadenopathy, hepatitis, chronic nephritis, chronic colitis, epistaxis and skin lesions consisted of exfoliative dermatitis and alopecia, and ulcerative, nodular and pustular dermatitis [8, 18, 30, 31]. Keratoconjunctivitis, arthritis and colitis as an atypical form of CVL are other rare findings in endemic areas. The immunosuppressive effect of CVL may increase susceptibility of co-infections such as Neospora sp., Hepatozoon sp., Rickettsia sp., Ehrlichia sp., Bartonella sp., Babesia sp., Dirofilaria sp., Sarcoptes sp., Candida sp. and Demodex sp. [8, 9, 11, 15, 22, 26, 27, 29, 33, 34].
The main histological lesions are hypertrophy and hyperplasia of cells of the mononuclear phagocyte system, granulomatous inflammatory reactions of the spleen, lymph nodes, bone marrow and liver, and chronic inflammation of the skin [25]. The aims of the study were to describe histopathological and immunohistochemical diagnosis of CVL, and to investigate the occurrence of co-infections in dogs.

Materials and Methods

ANIMALS AND CASE HISTORY

Six dead dogs from the Aegean region (Turkey) were presented to the pathology department of the Faculty of Veterinary Medicine, University of Adnan Menderes, Isikli-Aydın, Turkey, for investigating the death cause. Among the 4 females and the 2 males, 2 months to 5 years old, 3 of animals were mongrels and 3 were purebred (2 pointers and 1 Terrier). According to information from practitioners and owners, these animals have generally exhibited a history of anorexia, weakness, anaemia, depression, weight loss, skin lesions and lymphadenomegaly. The Table I stated the age, breed, case history, and histological and immunohistochemical diagnosis of each dog. Before, the diagnosis of leishmaniosis was confirmed in one dog (dog n°1) by microscopic examination of Giemsa stained peripheral blood smears and detection of Leishmania amastigotes and this dog was treated for leishmaniosis (allopurinal, oral, 20 mg/kg once daily for 15 days). The Hepatozoon sp. gametocytes within neutrophils in Giemsa-stained peripheral blood smears were evidenced in 2 animals (dogs n°2 and 5) which were treated for this infection with a combination of toltrazuril (10 mg/kg, orally once daily) and a trimethoprim-sulfamethoxazole (15 mg/kg, intravenously twice daily) for 5 days. The dog n°6 was not vaccinated against canine distemper and presented Respiratory and nervous signs as tremors and incoordination. According to anamnnesis, this puppy’s mother had usual clinical symptoms of leishmaniosis such as severe weight loss, exfoliative and ulcerative dermatitis.

Necropsy was performed on all the animals, and the sampled tissues including skin, stomach, intestines, liver, spleen, pancreas, kidneys, lymph nodes, lungs, heart, brain and medulla spinalis were fixed in 10% buffered formalin and embedded in paraffin. Sections cut at 5 μm thickness were stained with haematoxylin and eosin (H&E). Replicate sections were used for the immunohistochemistry. Additionally, touch-impression smears from lymph nodes, spleen and bone marrow were stained by May-Grunwald-Giemsa.

The avidin-biotin peroxidase complex (ABC) and the immunofluorescence (IF) methods, essentially described by TOPLU [35], were used for evidencing Leishmania. In both methods, mouse anti-Leishmania monoclonal antibodies (Cedarlane Laboratories, Burlington, ON, Canada) were used as primary antibodies. For the amastigote fluorescein immunolabelling, the tissue sections were dewaxed, rehydrated and digested with 0.1% proteinase K for 10 minutes at 37°C. After washing in PBS (phosphate buffered saline, pH 7.3) for 15 minutes, the sections were incubated for 2 h at 37°C with mouse anti-Leishmania antibody (1:1000), and then washed again for 15 minutes in PBS. After addition of goat anti-mouse gamma globulin serum conjugated with fluorescein isothiocyanate (Sigma, Rehovot, Israel), sections were incubated for 30 minutes at 37°C, washed in PBS for 20 minutes and mounted in phosphate-buffered glycerol (pH 9.0). For control purposes, replicate sections were processed, by substituting the mouse anti-Leishmania antibody with normal mouse serum. Finally, the tissue sections were examined with a fluorescence microscope (Leica DMLB). For the peroxidase immunolabelling, the sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase was then blocked with H2O2 3% in 70% methanol. The tissues were digested with 0.1% proteinase K 10 minutes at 37°C and the slides washed for 10 minutes in PBS. Non-specific staining was blocked by treatment with 2% normal horse serum for 30 minutes. The blocking serum was then replaced by mouse anti-Leishmania antibody (1:1000), follo-

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

TABLE I: Epidemiological data, clinical history and histological / immunohistochemical diagnosis of naturally canine visceral leishmaniosis (CVL) and co-infections (hepatozoonosis, toxoplasmosis and canine distemper) in 6 dogs.

<table>
<thead>
<tr>
<th>Dogs</th>
<th>Epidemiological data</th>
<th>Clinical history / Eventual treatment</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>N°1</td>
<td>Terrier M 5 year</td>
<td>WL, LM, SM Treatment for CVL</td>
<td>CVL + HZ + T</td>
</tr>
<tr>
<td>N°2</td>
<td>Mongrel F 4 year</td>
<td>WL, LM, SM, SL Treatment for HZ</td>
<td>CVL + HZ</td>
</tr>
<tr>
<td>N°3</td>
<td>Pointer F 4 year</td>
<td>WL, LM, SM, SL No treatment</td>
<td>CVL + HZ</td>
</tr>
<tr>
<td>N°4</td>
<td>Mongrel F 5 year</td>
<td>WL, LM No treatment</td>
<td>CVL + T</td>
</tr>
<tr>
<td>N°5</td>
<td>Mongrel M 5 year</td>
<td>WL, LM SL Treatment for HZ</td>
<td>CVL + HZ</td>
</tr>
<tr>
<td>N°6</td>
<td>Pointer F 2 month</td>
<td>Respiratory and nervous signs</td>
<td>CVL + CD</td>
</tr>
</tbody>
</table>

M: Male; F: Female; WL: Weight Loss; LM: lymphadenomegaly; SM: Splenomegaly; SL: Skin Lesions; CVL: Canine Visceral Leishmaniosis; HZ: Hepatozoonosis; T: Toxoplasmosis; CD: Canine Distemper.
wed by overnight incubation at 4°C. After washing for 10 minutes, sections were flooded with biotinylated horse antimouse immunoglobulin for 30 minutes. After a further wash, the sections were covered with streptavidin-peroxidase and incubated for 30 minutes and treated for 7 minutes with diaminobenzidine (DAB) containing H2O2 3%. The sections were then counterstained with haematoxylin, washed in tap water, dehydrated in graded alcohols, and mounted. For control purposes, replicate sections of selected infected tissues were processed, by substituting the mouse anti-Leishmania antibody with normal mouse serum. All incubations were performed at room temperature in a humidified chamber.

For the Toxoplasma gondii immunolabelling, an indirect fluorescent method was performed as described above (for Leishmania parasite) using a rabbit anti-Toxoplasma gondii polyclonal antibody (1:2500) as primary antibody. For control purposes, replicate sections of selected infected tissues were processed, by substituting the rabbit anti-Toxoplasma gondii antibody with a rabbit anti-Neospora caninum polyclonal antibody.

An ABC method was used for the canine distemper virus detection as described above. For control purposes, replicate sections of selected infected tissues were processed, by substituting the rabbit anti-canine distemper virus antibody with normal rabbit serum.

**Results**

**CLINICAL AND PATHOLOGICAL FINDINGS**

Case history and clinical findings were presented in Table I. A moderate to severe weight loss coupled to lymphadeno-megaly, especially of popliteal and prescapular lymph nodes, was observed in all presented cases except for the dog n°6. Skin lesions were conspicuous gross findings in animals n° 2, 3 and 5. They consisted in multifocal hypotrichosis-alopecia, brans and skin xerosis, and ulcers localized on the skin of the ears, nose, limbs and hip. Splenomegaly was a marked finding in 3 cases (dogs n° 1, 2 and 3). Hepatomegaly was observed only in one case (dog n°3) whereas kidney lesions were recorded in all cases except for the dog n°6. These organs were sclerotic in dogs n°2, 3 and 4 or contained white-greyish foci (dogs n°1 and 5).

The lungs of the animal n°6 exhibited diffuse consolidation, oedema and emphysema, and cranial lobes included dark red areas of hepatisation. Trachea and bronchi were congested and contained frothy exudate. The dog n°4 also presented pulmonary necrotic brownish-yellow foci of approximately 0.3-0.5 cm. The brain meninges from dogs n°1 and 4 were opaque and congested; these lesions were restricted on the frontal lobes in the dog n°4. Moreover, in the dog n°1, a softened haemorrhagic area was seen in the left frontal lobe and in the cut surfaces of the left hemisphere cortex. Whilst nervous clinical signs with tremors and incoordination were noted in the dog n°6, no macroscopical lesion was observed.

Infiltration with lymphocytes and macrophages coupled to proliferation of granulomatous tissue thickening the lymph node capsules were observed in dogs n°1, 2, 3 and 4. A marked follicular hyperplasia in cortical and medullar areas was noticed in lymph nodes of all dogs with lymphadenomegaly. Plasma cells were predominant especially in medullar cord and proliferation of macrophages especially in the medullar sinuses was intense. Macrophages loaded with amastigotes of Leishmania sp. were frequently observed especially in these areas and in capsule. Marked lymphoid depletion in parafollicular areas as well as reticuloendothelial cell hyperplasia were also frequently noticed in lymph nodes. Moreover, splenic follicular structures were strongly altered and lymphocytes were replaced by plasma cells and macrophages. Plasma cells and macrophages have also proliferated in bone marrow. Additionally, Hepatozoon sp. schizonts located within the cell in a parasitophorous vacuole were found in spleen and lymph nodes from the dogs n°1, 2, 3 and 5 (figure 1). The touch-impression smears of lymph nodes, spleen and bone marrow revealed the presence of Leishmania amastigotes in macrophages (in support of immunohistochemistry in the tissue sections) and Hepatozoon sp. gamontes in neutrophils from the bone marrow (dogs n°2 and 3) (figure 2).

In all dogs, liver was injured: hepatocyte hydropic degeneration was observed in 4 animals (dogs n°1, 2, 4 and 5) and was associated with sinusoid invasion by isolated or clustered lymphocytes and macrophages. Additionally, mixed cell populations constituted of lymphocytes, plasma cells and macrophages coupled to mild fibrosis mildly to markedly invaded the portal areas in 2 dogs (dogs n°1 and 4). Granulomatous foci constituted by macrophages (loaded with Leishmania amastigotes), lymphocytes and plasmocytes were scattered within the hepatic parenchyma in the animals n°3 and 6.

A non supplicative interstitial nephritis located especially in the renal cortex was seen in all dogs except for the animal n°6. The inflammation was characterized by foci of lymphocytes and plasma cells including scarce macrophages, within the periglomerular, peritubular, intertubular, and perivascular areas. A fibrous tissue proliferation especially in cortical areas was additionally observed in the dogs n°2, 3 and 4. Glomerular changes consisted of focal segmental glomerulosclerosis and diffuse mesangial proliferative glomerulonephritis.

The main lesion of the lungs was a chronic interstitial pneumonia; the interalveolar septum was thickened by mixed populations of lymphocyte, macrophage and fibrocytes. In addition, lungs of the animal n°6 showed acute catarrhal bronchopneumonia whereas focal necrotic areas with macrophages including T. gondii tachyzoites in the cytoplasm were recorded in the lungs from the dog n°4.
Acute non suppurative encephalitis with demyelisation was evidenced in the dog n°6. Furthermore, in this animal, cerebellar astrocytes contained eosinophilic intranuclear inclusion bodies. Meninges and cerebral hemispheres of the brain from the dog n°1 showed haemorrhage, necrosis and perivascular macrophage infiltrations and tachyzoites and tissue cysts of *Toxoplasma gondii* were detected in the injured areas. Large numbers of tachyzoites were also observed in endothelial cells of blood vessels (figure 3). In the dog n°4, haemorrhages and the infiltrating macrophages with tachyzoites were restricted only to meninges.

Whereas no alteration was observed in the animal n°6, there was a mild to a moderate mononuclear cell infiltration of the submucosa in small and large intestines, sometimes extended to the muscular layer.

**IMMUNOHISTOCHEMICAL FINDINGS**

Strong immunolabelling of *Leishmania* amastigotes with the 2 both IF and ABC methods were evidenced in the cytoplasm of macrophages from lymph nodes, spleen and bone marrow (figure 4) in all dogs. The infiltrating macrophages of the skin lesions and of the intestine mucosa were also positively stained. In liver, Kupffer cells, hepatocytes and macrophages gave a positive reaction. Few macrophages of the interstitial inflammatory infiltrate in kidneys as well as tubular epithelial cells and glomerular cells were also positive for *Leishmania* antigens. Control slides were negative.

Antigens of the canine distemper virus were markedly evidenced by the specific ABC method in cerebellar astrocytes, in neurons from the cerebral hemispheres (figure 5A) and in alveolar epitheliums in the lungs (figure 5B) in the dog n°6.

The fluorescein labelling of *T. gondii* tachyzoites was observed especially in endothelial cells and infiltrating macrophages from the meninges and cerebral hemispheres (dogs n°1 and 4) (figure 6). Extracellular positive *T. gondii* immunostaining was also evidenced. A few tissue cysts showed slight positive reaction, as well. In lungs, the immunostaining was recognized in macrophages and alveoli especially in areas surrounding necrotic foci. The staining with *Neospora caninum* antiserum of replicate sections was negative.

**Discussion**

Clinical diagnosis of CVL may be difficult because clinical signs are not specific and may vary depending on the
Figure 2: Touch impression smear of bone marrow: macrophages loaded with Leishmania amastigotes (arrows) and Hepatozoon sp. gamontes in neutrophil granulocyte (arrowhead), May-Grunwald-Giemsa, bar: 20 µm.

Figure 3: Meninges. Infiltrating macrophages (blue arrowheads), endothelial cells (arrow) with Toxoplasma gondii tachyzoites in the vessel (VL), and tissue cysts (black arrowheads) with protozoa on the vessel wall, Haematoxylin - Eosin, bar: 50 µm.

Figure 4: Lymph nodes. Fluorescein immunolabelling (IF method) (A) and peroxidase immunolabelling (ABC method) (B) of Leishmania amastigotes (arrow) in the cytoplasm of infiltrating macrophages into capsule, bar: 30 µm.
organism- or host-specific factors and the state of immunity and previous specific therapies. It is also possible that the degree and type of manifestations associated with leishmaniosis vary according to individuals, depending on presence of concomitant vector-borne organisms [11, 27, 29, 32]. In such situations, numerous CVL cases, as reported in this study, may be not diagnosed, misdiagnosed or unreported, particularly when leishmaniosis diagnostic methods are not available [8, 18, 31].

The main histological lesions of CVL, as described in the dogs of the present study, are the hypertrophy and hyperplasia of the mononuclear phagocytic cell system coupled to granulomatous inflammatory reactions (spleen, lymph nodes, bone marrow and liver) and often to a chronic inflammation in the skin [17, 25]. The presence of *Leishmania* amastigotes in macrophages from the lymph nodes, spleen, bone marrow and liver, also identified by immunohistochemistry, undoubtedly support the CVL diagnostic. Several reports describe a high prevalence of infection by especially *Leishmania infantum* in dogs throughout the Mediterranean basin, as demonstrated by a variety of methods such as serologic investigations, immunohistochemistry and the presence of leishmanial DNA [12, 18, 31, 36]. It is most possible that agent in the cases presented in the present study may be *Leishmania infantum* as before identified in dogs of Aegean region by ÖZBEL *et al.* [23].

In CVL, *Leishmania* ensures its own survival by modulating the host immune system either by inducing immunosuppression or by promoting pro-parasitic host functions. Studies of *Leishmania* infections in mice indicate that resistance or susceptibility

---

**Figure 5:** Immunoreactivity with ABC method for canine distemper viral antigen in cytoplasm and nucleus of neurons (arrow) from cerebral hemisphere (A) (bar: 50 µm) and in alveolar lining epithelial cells (arrows) (B) from the dog n°6.

**Figure 6:** Meninges from the dog n°1. Fluorescein immunolabelling of *Toxoplasma gondii* tachyzoites in the infiltrating macrophages (arrows) in the vessel lumen (VL) and in extracellular position (arrowhead) on the vessel wall, IF method, bar: 20 µm.
to the disease is associated with the development of Th1 or Th2 cell response, respectively [19], and lead to an imbalance between Th1 and Th2 cell responses [5, 14]. A vigorous Th2 immune response, mainly characterized in humans by increased IL-4 expression, polyclonal B cell activation, intense hypergammaglobulinemia and production of anti-leishmanial IgE antibodies was present during CVL [1, 7, 10, 20]. Prolonged latent infection with Leishmania results in cell-mediated immunosuppression that could be related to the expression of IL-10 [28]. On the other hand, phagocytic capacities of macrophages in animals with CVL are temporarily altered [2, 6]. Thus, impaired T lymphocyte or macrophage function in CVL predisposed to the development of infections caused by intracellular pathogens such as Neospora caninum [3, 9], and Toxoplasma gondii as evidenced in the present study. Toxoplasmosis is usually subclinical in dogs, and has been reported as a concurrent infection with canine distemper or some other underlying immunosuppressive agent or condition [4]. Similarly, concurrent Toxoplasma gondii and Hepatozoon canis infections were also reported in a dog [15]. In the present study, toxoplasmosis co-existed with CVL in 2 dogs (animals n°1 and 4); besides, the dog n°4 was also infected with Hepatozoon. It was probable that the severe necrotic lesions in the brain and lungs caused by toxoplasmosis have lead to the fatal outcome. According to our knowledge, the CVL/toxoplasmosis and CVL/hepatozoonosis/toxoplasmosis associations described in these 2 cases have not been previously documented in the literature.

It is emphasized that possibility of co-infections should be increased in dogs living in areas that are highly endemic for several vector-borne organisms, in dogs that are maintained predominantly outdoors (enhanced vector transmission), and in dogs that are not routinely treated with acaricides or other ectoparacidaïcides [11, 27]. Likewise, canine hepatozoonosis (a protozoan disease transmitted by the tick Rhipicephalus sanguineus) was diagnosed based solely on morphologic characters during endogenous developmental stages in the lymph nodes, spleen and bone marrow in four dogs with leishmaniosis of the present study. The protozoan species would be Hepatozoon canis in these cases, which has been recently reported in an epidemiological study, Aegean region, Turkey [16].

Previous epidemiological studies indicate that CVL mainly affects adult (from 9 month old to 15 year old, with a median for 5 years of age) and that the incubation period ranges from 3 months to 7 years [18, 30]. Nevertheless, CVL has also been recorded in young immature dogs, throughout vertical transmission [21]. In the present study, the 2 month old puppy with canine distemper (CD) (dog n°6) would probably have been infected by its mother since it exhibited clinical signs compatible with CVL. It is the first description, to our knowledge, of a CVL and CD co-infection in a puppy.

As a conclusion, this study demonstrates concurrent infections with toxoplasmosis, hepatozoonosis and even canine distemper in CVL-affected dogs from the Aegean region of Turkey. In a clinical perspective, practitioners should be aware of such disease association and should include their screening in the routine diagnostic panel.

References


Acknowledgments

This research was supported by Research Fund of Adnan Menderes University Research Council, Project VTF-07-024. Authors wish to thank Dr. Nalan Kabakci, Dr. Tolga Guvenc and A. Hemphill supplying canine distemper virus, Toxoplasma gondii and Neospora caninum polyclonal antibodies, respectively.