CASE REPORT

Toxoplasma gondii GENOTYPING IN A DOG CO-INFECTED WITH DISTEMPER VIRUS AND EHRLICHIOSIS RICKETTSIA

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SUMMARY

This paper reports a toxoplasmosis, erlichiosis and distemper co-infection in a dog with an exuberant neuropathological clinical picture. Primary involvement was discussed based on information collected in the analysis of the clinical case, such as neurological impairment, epidemiological data, poor immunoprophylactic scheme of the dog affected and the role of these diseases on immunosuppression. Canine distemper and erlichiosis were diagnosed based on epidemiologic data, clinical signs, hematological and cytological evaluation. Toxoplasma gondii was isolated and genetically characterized as Type I using restriction analysis (RFLP) with SAG-2 genes. Immunosuppression features of both dogs and human beings are discussed, as well as implications on animal and public health. This is the first report on toxoplasmosis, erlichiosis and distemper co-infection in a dog in Brazil, associated with genotyping determination of the T. gondii strain involved.

KEYWORDS: Toxoplasmosis; Ehrlichiosis; Distemper; Co-infection; Dogs; Immunosuppression; Genotyping; Restriction fragment length polymorphism (RFLP).

INTRODUCTION

Toxoplasma gondii is an obligate intracellular coccidian parasite related to infection of a wide range of warm-blooded species, pointed out as one of the most important zoonotic agents in several countries. Toxoplasmosis is recognized as an opportunistic disease in dogs, characterized by neuromuscular, respiratory and gastrointestinal signs, or by generalized infection, besides its most-common neurological impairments, such as ataxia, behavioral changes, circling, seizures, paralysis, paraplegia, twitching and tremors. T. gondii comprises different clonal lineages, called Type I, II and III, which may influence the progression and severity of the disease in both animals and human beings. Several studies have described the molecular epidemiology of T. gondii isolated from humans, based on strain genotyping. However, few reports have focused on the role of T. gondii strain genotyping in the epidemiology of the disease in dogs.

Canine distemper is a highly contagious viral disease with worldwide distribution, of utmost importance to the dog population due to high mortality rate and neurological complications. Despite claimed to be controlled in many countries, the disease has reappeared in several European locations since 1980s, remains enzootic and is a current animal health problem in countries where there are no systematic dog vaccination programs. Clinical signs of canine distemper may vary according to the virulence of the strain, environmental conditions, host age and immune status. Different or no specific clinical manifestations have been described in canine distemper, like listlessness, fever, anorexia, bilateral oculonasal discharge, pustular dermatitis, hyperkeratosis of nose and footpads, enamel hypoplasia and diarrhea. Neurological complications are the most important factors concerning prognosis in canine distemper. These neurological disorders are represented by development of hyperesthesia, cervical rigidity, myoclonus, ataxia, seizures, paraparesis, paraplegia and tetraparesis, which are similar to clinical signs observed in dogs affected by toxoplasmosis. Infection of dogs with canine distemper virus (CDV) has long been considered an important immunosuppressant factor linked to the infection by opportunistic agents. Similarly, it is widely known that severe clinical manifestation of toxoplasmosis and erlichiosis occurs in debilitated/immunosuppressed dogs.

Ehrlichiosis is recognized as a tick-borne disease in dogs caused by intracellular microorganisms in the family Rickettsiaceae, genus Ehrlichia, that parasite circulating leukocytes, especially monocytes. In domestic dogs, clinical ehrlichiosis has been related to several species of the genus Ehrlichia, while a distinct, recently recognized disease, canine monocytic ehrlichiosis, is specifically caused by Ehrlichia canis.

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Ehrlichiosis is characterized by a wide range of clinical signs in both acute and chronic phases. The most common clinical signs during the acute phase are represented by depression, lethargy, weight loss, anorexia, fever, lymphadenomegaly, splenomegaly, petechiae, ecchymoses of the skin and mucous membranes, occasional epistaxis, besides vomiting, ocuonalosal discharge, lameness, dyspnea and ataxia. Acute phase signs increase in chronic cases, besides the occurrence of emaciation, pale mucous membranes, peripheral edema, pneumonia, glomerulonephritis, renal failure and arthritis. In the chronic phase, immunosuppression often occurs, with severe thrombocytopenia, leukopenia and anemia.

Different authors have discussed the occurrence of combined infections by *Toxoplasma gondii* and distemper virus and concurrent toxoplasmosis plus ehrlichiosis in dogs, and emphasized that clinical signs are more severe and prognosis poorer in animals suffering with co-infections. The purpose of this report was to describe the first time in Brazil, toxoplasmosis, distemper and ehrlichiosis co-infection in a dog presenting neurological signs, as well the genotype determination of the *T. gondii* strain involved.

**MATERIAL AND METHODS**

**Case report:** A three-year-old female cocker spaniel was admitted to the Infectious Diseases Ambulatory Service/Animal Infectious Diseases at Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, Brazil, with mucopurulent ocular discharge, bloody diarrhea, polyuria, and neurological signs including circling and tetraparesis, that began around seven days before the dog was sent to the hospital. The owner reported presence of ectoparasites. The dog ate commercial food, boiled milk, and raw meat / bones. Vaccination status was unknown, and the animal had free access to the street, besides a history of contact with an adult symptomless cat. In the clinical evaluation, unconsciousness, slight dehydration, lipoadenopathy, signs of pneumonia, splenomegaly and mucopolulent ocular discharge were observed. Neurological abnormalities, such as behavioral change (severe depression to coma), myoclonus of the left forelimb, opisthotonus, circling, paddling movements and left hindlimb hyperreflexia were also observed.

The animal was submitted to complete hematological and biochemical renal/liver function analyses. Due to the poor prognosis in face of the neurological signs, the animal was euthanized. Cerebral congestion, splenomegaly, lymphadenomegaly, diffuse pneumonia associated with exuberant areas of petechial subpleural hemorrhage of the lungs and bloody diarrhea, were the main lesions observed at post-mortem examination.

**Distemper diagnosis:** Distemper was the primary clinical suspicion based on epidemiological data, such as street access and unknown vaccination status, and on clinical evaluation due to compatible neuromuscular, respiratory and gastrointestinal signs. Distemper inclusion bodies were investigated in peripheral blood smear stained by Wright-Leishmann. After post-mortem examination, lung impression smears were stained by Giemsa and analyzed for *distemper* inclusion bodies.

**Ehrlichiosis diagnosis:** There was no initial clinical suspicion of ehrlichiosis because the main expected disturbances, such as pale mucous membranes and bleeding, were absent. The only clue for ehrlichiosis was the presence of ectoparasites in the animal and its environment. This possible co-participation on the disease process was suspected due to exuberant areas of petechial subpleural hemorrhages on the lungs, splenomegaly and lymphadenomegaly, observed in the post-mortem examination. Slides obtained from lung impression were submitted to Giemsa stain for morulae investigation.

**Toxoplasmosis diagnosis**

**a. Serological examination:** Blood samples were obtained from the dog before post-mortem examination and centrifuged (1,650 x g for 15 min) to separate the serum. Serum was 4-fold diluted in phosphate buffered solution to produce dilutions between 1:16 and 1:16,384. These dilutions were immediately submitted to indirect fluorescent antibody test (IFAT) for anti-*T. gondii* antibody investigation. Tachyzoites from RH strain propagated in Swiss mice and fixed in formalin were used as antigen, and rabbit anti-dog IgG was used as immunofluorescent marker. Complete parasite fluorescence in dilutions ≥ 1:64 was considered to be positive. Positive and negative control sera were supplied by the Serviço de Diagnóstico de Zoonoses, Universidade Estadual Paulista, Botucatu, SP, Brazil.

**b. *T. gondii* isolation:** After post-mortem examination, brain samples from the dog were collected for *T. gondii* isolation, using the bioassay method. Brain material was macerated with five volumes (w/v) of aqueous 146 mM NaCl mixed 0.5% acid pepsin, as described elsewhere. The mixture was incubated under continuous homogenization (one h at 37 °C), followed by centrifugation, neutralization and resuspension in antibiotic solution before subcutaneous inoculation in four Swiss albino female mice (30-day-old), obtained from Central Laboratory Animal Facility, Universidade Estadual Paulista, Botucatu, SP, Brazil, where natural *T. gondii* infection has not been reported. Mice that died after inoculation were investigated for the presence of *T. gondii* tachyzoites in peritoneal fluid. Blood was collected from surviving animals by retro-orbital route at 60 days post-inoculation, followed by IFAT in sera (1:16, 1:64, 1:128 and 1:256 dilutions), in order to evaluate the presence of anti-*Toxoplasma* antibodies using anti-mouse IgG as fluorescent conjugate. Simultaneously, brain samples from surviving mice were ground and 50 µL of fresh samples examined under microscope on a slide and coverslip, in order to observe parasite compatible with *Toxoplasma gondii*. Mice were considered *T. gondii* positive when showing positive serology in IFAT, observation of the parasite in peritoneal fluid, or presence of *T. gondii* cysts in examination of fresh brain samples. Brain tissue from a dog seronegative to *T. gondii* was provided by the Serviço de Diagnóstico de Zoonoses, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, SP, Brazil and was used as negative control in mice inoculation.

**c. Toxoplasma gondii genotyping:** Tachyzoites isolated from mice were the source for the DNA samples used for genotype characterization. DNA was extracted by digestion with proteinase K and SDS (sodium dodecyl sulphate) followed by phenol-chloroform and precipitation with ethanol. DNA samples were submitted to nested PCRs using SAG-2 gene and restriction enzymes *Sau 3A* and *Hpa*.

Fragments were identified in 1.5% agarose gel electrophoresis stained with ethidium bromide. RH, ME-49 and M7741 strains of *T. gondii* was provided by the *Rev. Inst. Med. trop. S. Paulo*, 48(6): 359-363, 2006.
inoculated mice, and provided DNA material for genotype and has been associated with toxoplasmosis co-infection in dogs4,26. In Brazil, distemper is considered enzootic in certain regions and problems in dogs from countries without systematic vaccination attempt to perform differential diagnosis and concurrent detection of toxoplasmosis co-infection in dogs4,26. It is not known whether another immunologic suppressor took place in the case presented here, concurrent toxoplasmosis and ehrlichiosis that may lead from mild to severe immunosuppression, as occurs with distemper virus, increased the severity of the clinical signs, especially neurological ones, leading to poorer prognosis. These data are in agreement with other reports that have also described the occurrence of combined infection by these agents in domestic dogs or wildlife animals4,15.

Likewise, toxoplasmosis has a known participation on health problems of debilitated patients, and both distemper and ehrlichiosis may cause clinical signs that resemble toxoplasmosis14,26,30. It is known that another immunologic suppressor took place in the present case. In the case presented here, concurrent toxoplasmosis and ehrlichiosis that may lead to severe immunosuppression, as occurs with distemper virus, increased the severity of the clinical signs, especially neurological ones, leading to poorer prognosis. These data are in agreement with other reports that have also described the occurrence of combined infection by these agents in domestic dogs or wildlife animals4,15.

Routine diagnosis of canine distemper is performed based on epidemiological data, clinical signs and hematological tests, allied to demonstration of typical distemper inclusion bodies in leukocytes from blood and organs4. In the present report, gastroenteric, respiratory and neurological signs, absolute lymphopenia in hematological evaluation, besides observation of characteristic intracytoplasmatic distemper inclusion bodies (singular, oval, and gray to magenta structures), called distemper inclusion19 or Lentz inclusion bodies, which may be found in animals infected with the distemper virus8. Similar structures were not observed in previous hematological tests, using Wright-Leishmann stain. Several pinpoint basophilic structures on round-shaped morulae were found in mononuclear leukocytes32, in lung imprints submitted to Giemsa stain, characteristic of the genus *Ehrlichia*.

A single titer of 1,024 was observed for *T. gondii* antibodies using IFAT in dog sera obtained before the animal was euthanized. Neither mice inoculated with the brain from the *T. gondii* seronegative dog strain isolated increased diagnostic suspicion for distemper. Hematological data were poor immunoprophylactic scheme and physical signs of neuropathy indeed, because ehrlichiosis itself causes both specific and nonspecific immunosuppression, what may lead to severe immunosuppression manifestations5,14. Other authors, however, claim a secondary role for the rickettsiosis. In fact, normal dogs tend to recover naturally from the subclinical phase20, but the disease can be clinically and pathologically enhanced by concurrent disturbances4.

Hematological parameters for normal dogs20.

### RESULTS

Anemia, absolute lymphopenia and monocytosis were observed in hematological tests, which were compatible with distemper, mainly due to severe lymphopenia (Table 1).

Manual platelet estimation showed to be normal. Renal and liver biochemical parameters were inside the normal range for the species.

Lung impression smears submitted to Giemsa stain showed intracytoplasmatic inclusion bodies in lymphocytes (singular, oval, and gray to magenta structures), called distemper inclusion15 or Lentz inclusion bodies, which may be found in animals infected with the distemper virus8. Similar structures were not observed in previous hematological tests, using Wright-Leishmann stain.

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### DISCUSSION

Canine distemper remains as one of the most-common health problems in dogs from countries without systematic vaccination programs. In Brazil, distemper is considered enzootic in certain regions and has been associated with toxoplasmosis co-infection in dogs4,15. Due to neurological picture, epidemiological data and its highly contagious feature, distemper was considered to be the primary disease suspected4,14-15. This presumptive clinical diagnosis was supported by the owner’s report of contact with other dogs, free street access, lack of vaccination, besides compatible clinical signs and the frequency of the disease in Brazil. Additionally, immunosuppression induced by distemper virus probably collaborated for the opportunistic features of *T. gondii* and *Ehrlichia* sp. infections15,32. It could not be the case indeed, because ehrlichiosis itself causes both specific and nonspecific immunosuppression, what may lead to severe immunosuppression manifestations8,34. Other authors, however, claim a secondary role for the disease in Brazil. Additionally, immunosuppression induced by distemper virus probably collaborated for the opportunistic features of *T. gondii* and *Ehrlichia* sp. infections15,32. It could not be the case indeed, because ehrlichiosis itself causes both specific and nonspecific immunosuppression, what may lead to severe immunosuppression manifestations8,34. Other authors, however, claim a secondary role for the rickettsiosis. In fact, normal dogs tend to recover naturally from the subclinical phase20, but the disease can be clinically and pathologically enhanced by concurrent disturbances4.

Enzootic distemper status at Botucatu region, in addition to the poor immunoprophylactic scheme and physical signs of neuropathy increased diagnostic suspicion for distemper. Hematological data were also compatible with the disease. No Lentz inclusion bodies were found on blood smear evaluation, but they were found in lung impression smears stained by Giemsa. Toxoplasmosis serology was used in an attempt to perform differential diagnosis and concurrent detection of distemper encephalitis.

Other techniques are available for distemper diagnosis, such as antigen detection on cells of cerebrospinal fluid (CSF), conjunctival smears, tracheal washings or urine sediment32, by means of fluorescent antibody techniques. Usefulness of serology in diagnosis is limited, but CSF antibody detection is probably the most reliable indicator of infection31. Such tests would have been necessary if no Lentz inclusion bodies were found.

### Table 1

<table>
<thead>
<tr>
<th>Erythrogram</th>
<th>Reference</th>
<th>Leukogram</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes (X10⁶/µL)</td>
<td>3.85</td>
<td>5.5-8.5</td>
<td>Leukocytes (X10⁶/µL)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.2</td>
<td>12-18</td>
<td>Neutrophils (X10⁶/µL)</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>27</td>
<td>37-55</td>
<td>Lymphocytes (X10⁶/µL)</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>70.13</td>
<td>60-77</td>
<td>Monocytes (X10⁶/µL)</td>
</tr>
<tr>
<td>MCH (fl)</td>
<td>34.07</td>
<td>32-36</td>
<td>Eosinophils (X10⁶/µL)</td>
</tr>
</tbody>
</table>

Hematological reference parameters for normal dogs20.
No specific signs led to initial ehrlichiosis suspicion. Similarly to distemper, routine diagnosis of ehrlichiosis is based on the association of clinical signs, epidemiologic data, hematologic evaluation and identification of morulae in mononuclear leukocytes. The presence of anemia and areas of petechial subpleural hemorrhages in lungs, splenomegaly and lymphadenomegaly found during post-mortem examination, associated with the detection of morulae in mononuclear leukocytes compatible with genus *Ehrlichia*, led to the diagnosis of ehrlichia infection in the dog. Thrombocytopenia and other peculiar clinical signs of ehrlichiosis were not evident in the dog, probably due to the initial acute phase of the disease. Similarly, biochemical renal parameters were within the normal range for the species, 25.3 and 0.7 (mg/dL) for urea and creatinine, respectively. No morulae were found on blood smear evaluation. Although other methods have been used for distemper and/or ehrlichiosis diagnosis, such as cell culture, enzyme linked immunosorbent assay, indirect immunofluorescent test, immunoblotting and PCR, these tests were not here considered for the confirmation of the diagnosis or for the species classification of genus *Ehrlichia* in the present study.

Toxoplasmosis is considered to be a relevant infectious disease in dogs with neurological disorders. *Toxoplasma gondii* serology is routinely required in dogs with nervous symptoms even when there is a strong distemper suspicion, because the neural syndromes linked to any of the two diseases are quite indistinguishable. The demonstration of a single titer equal to 1,024, no matter the acute picture, suggests that *T. gondii* enhanced neurological impairment in the disease process. However, the parasite isolation was not enough to confirm active infection on neural disease. This purpose requires tests for tachyzoite demonstration, like peroxidase-immunoperoxidase methods. These tests were not issued in the present case.

*T. gondii* presents a highly clonal populational structure, classified in Types I, II and III. Molecular epidemiological studies with *T. gondii* strains isolated from humans have been performed in order to evaluate the distribution and virulence of different clones of the parasite. The influence of genotype in the severity and evolution of disease in humans is supported by differences of virulence in animal models, due to the more consistent findings of Types II and III in chronic infection with production of cysts in mice tissue, while Type I have shown high virulence and parasitemia, and presents more risks of transplacentary dissemination and fetus infection. However, restrict investigation have been conducted in genotyping of *T. gondii* strains isolated from animals, especially dogs.

In Brazil, toxoplasmosis is recognized as one of the most common diseases in dogs with neuromuscular signs, and has been related to combined infections with distemper. Recently, it was performed in Brazil the first study on the molecular genotype characterization of *T. gondii* strains isolated from 111 dogs with neurological signs. From these animals, 34 brain samples were inoculated in mice, and nine of them led to *T. gondii* isolation. From these, four were classified as Type I and five as Type III using restriction (RFLP) and SAG-2 gene analysis. The authors wish to thank the Zoonosis Diagnostic Laboratory (LDZ) staff for their aid in the laboratorial procedures.

**REFERENCES**
