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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, erhlichiosis 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 ehrlichiosis 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, ehrlichiosis 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[19], pointed out as one of the most important zoonotic agents in several countries[1]. 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[10,19,26]. *T. gondii* comprises different clonal lineages, called Type I, II and III, what may influence the progression and severity of the disease in both animals and human beings[22]. Several studies have described the molecular epidemiology of *T. gondii* isolated from humans, based on strain genotyping[18,21]. However, few reports have focused on the role of *T. gondii* strain genotyping in the epidemiology of the disease in dogs[10].

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[29,33]. 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[16,25]. 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[19]. Infection of dogs with canine distemper virus (CDV) has long been considered an important immunosuppressant factor linked to the infection by opportunistic agents[32]. Similarly, it is widely known that severe clinical manifestation of toxoplamosis and ehrlichiosis occurs in debilitated/immunosuppressed dogs[17,19].

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[19]. 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*[20].

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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, oculonasal 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[17,20]. In the chronic phase, immunosuppression often occurs, with severe thrombocytopenia, leukopenia and anemia[19].

Different authors[6,19,20,26,32,34] 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 by 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 in 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 dehydratation, linfadenopathy, signs of pneumonia, splenomegaly and mucopurulent 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 hyperextension 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[5]. 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[12]. Brain material was macerated with five volumes (w/v) of aqueous 146 mM NaCl mixed 0.5% acid pepsin, as described elsewhere[12]. The mixture was incubated under continuous homogenization (one h at 37 ºC), 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 cysts 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[13]. 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: *T. gondii* 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-chlorophorm and precipitation with ethanol[9]. DNA samples were submitted to nested PCRs using SAG-2 gene[21] and restriction enzymes *Sau* 3AI e *Hha*. Fragments were identified in 1.5% agarose gel electrophoresis stained with ethidium bromide. RH, ME-49 and M7741 strains of *T. gondii* were used as positive controls for genotypes I, II and III, respectively.

Table 1
Hematological parameters of the dog sent to the UNESP Veterinary Hospital, Botucatu, SP, 2002

Erythrogram		Reference	Leukogram		Reference
Erythrocytes (X10^6/µL)	3.85	5.5-8.5	Leukocytes (X10^3/µL)	9.29	6-17
Hemoglobin (g/dL)	9.2	12-18	Neutrophils (X10^3/µL)	7.07	3-11.5
PCV (%)	27	37-55	Lymphocytes (X10^3/µL)	0.093	1-4.8
MCV (fl)	70.13	60-77	Monocytes (X10^3/µL)	1.67	0.15-1.35
MCHC (%)	34.07	32-36	Eosinophils (X10^3/µL)	0.46	0.1-1.25

Hematological reference parameters for normal dogs[23].

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 inclusion[19] or Lentz inclusion bodies, which may be found in animals infected with the distemper virus[8]. 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 leukocytes[31], 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 died in a 60-day period of study, nor cysts in brain nor antibodies were detected in these negative control animals. *T. gondii* strain isolated from the neurologically impaired dog brain killed all mice between five and ten days after inoculation of pepsin digested brain material. Simultaneously, *T. gondii* was isolated from the ascitic fluid of inoculated mice, and provided DNA material for genotype characterization. Using nested PCR and specific restriction enzymes, *T. gondii* strain fragments were classified as Type I.

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 dogs[4,26]. Due to neurological picture, epidemiological data and its highly contagious feature, distemper was considered to be the primary disease suspected[4,14,32]. 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. infections [19,32]. It could not be the case indeed, because ehrlichiosis itself causes both specific and nonspecific immunosuppression, what may lead to severe toxoplasmosis manifestations[6,14]. Other authors, however, claim a secondary role for the rickettsiosis. In fact, normal dogs tend to recover naturally from the subclinical phase[20], but the disease can be clinically and pathologically enhanced by concurrent disturbances[6].

Likewise, toxoplasmosis has a known participation on health problems of debilitated patients, and both distemper and ehrlichiosis may cause clinical signs that resemble toxoplasmosis[14,26,30]. It is not known whether another immunologic suppressor took place in the present case. 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 animals[11,15].

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 organs[19]. 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) in lung lymphocytes, were compatible with canine distemper virus infection.

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. Toxoplamosis 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 sediment[32], by means of fluorescent antibody techniques. Usefulness of serology in diagnosis is limited, but CSF antibody detection is probably the most reliable indicator of infection[31]. Such tests would have been necessary if no Lentz inclusion bodies were found.

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, hematological evaluation and identification of morulae in mononuclear leukocytes[19]. 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[19]. *Toxoplasma* 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[4,26]. 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[2,26]. 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[13]. 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[27]. 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[22]. 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 neurological signs[10], and has been related to combined infections with distemper[4,26]. 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[10]. 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.

Neospora caninum infection is described in animals affected with toxoplasmosis. MINEO *et al.* (2001)[24] conducted a study for detection of IgG antibodies to *N. caninum* in dogs presenting neuromuscular, respiratory and/or gastrointestinal disorders at one veterinary hospital in Southeastern Brazil. From 163 dogs, 59 (36%) were seronegative and 104 (64%) seropositive to *T. gondii*. Anti-*Neospora* antibodies were detected in 11 (6.7%) out of 163, whereas five among these positive samples showed strong reactivity to *Toxoplasma* antigens. *Neospora caninum* serological screening was not performed in the case presented here. However, the findings of MINEO *et al.* (2001)[24] points to the parasite presence in Brazil and that it should be considered in the diferential diagnosis or with concurrent detection in cases like the present one.

In the present report, a *T. gondii* strain characterized as Type I was isolated from a dog with neurological signs presenting mixed infection with distemper virus and *Ehrlichia* sp. Genotype characterization of the strain as Type I is in an agreement with previous report[10], and probably indicates a trend in genotype I involvement on neurological disorders caused by *T. gondii* in dogs in Brazil.

Besides zoonotic characteristic of toxoplamosis, distemper has been implicated in Paget's disease in humans[3] and *E. canis* may occasionally cause clinical disease in human beings[28,34]. In order to decrease distemper participation in Paget's disease, animal distemper should be prevented by means of vaccination, what can be achieved more easily in educated communities familiarized with responsible ownership criteria[25], but may be difficult to achieve in wild distemper reservoirs[7]. Canine's ehrlichiosis is not a contagious disease and so human infection depends on the contact with infected ticks. The presence of dogs in human environments represents a potential contact with infective ticks. Thus, tick control remains the most reliable means of ehrlichial control[20].

RESUMO

Genotipagem de *Toxoplasma gondii* em cão co-infectado com o vírus da cinomose e a rickettsia da erliquiose

Este artigo relata a co-infecção tripla pelos agentes da cinomose, erliquiose e toxoplasmose em um cão com acentuado quadro clínico neuropático. Discute-se a doença primária baseando-se em dados clínicos, epidemiológicos, no protocolo imunoprofilático inadequado e no papel daquelas doenças na imunossupressão. A cinomose e a erliquiose foram diagnosticadas mediante a situação epidemiológica da região e sinais clínicos compatíveis, aliados aos achados de hemograma e citologia. Utilizando-se a análise de restrição (RFLP) com os genes SAG-2, caracterizou-se geneticamente a linhagem de *Toxoplasma gondii* isolada, como pertencente ao Tipo I. Discutem-se aspectos de imunossupressão, tanto em cães quanto em seres humanos, bem como suas implicações em saúde pública e animal. Este é o primeiro relato de infecção tripla pelos agentes da toxoplasmose, erliquiose e cinomose no Brasil, associado com a genotipificação da estirpe de *T. gondii* envolvida.

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