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Author(s): David S. Lindsay , David G. Goodwin , Anne M. Zajac , J. A. Cortés-Vecino , S. M. Gennari , Alexa C. Rosypal , and J. P. Dubey

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Serological Survey for Antibodies to *Encephalitozoon cuniculi* in Ownerless Dogs From Urban Areas of Brazil and Colombia

David S. Lindsay, David G. Goodwin, Anne M. Zajac, J. A. Cortés-Vecino*, S. M. Gennari†, Alexa C. Rosypal‡, and J. P. Dubey§, Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathology, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, Virginia 24061; *Laboratorio de Parasitología, Facultad de Medicina Veterinaria y de Zootecnia, Universidad Nacional de Colombia-Sede Bogotá, Bogotá, Colombia 11001; †Faculdade de Medicina Veterinária e Zootecnia, Departamento de Medicina Veterinária Preventiva e Saúde Animal, USP, São Paulo, SP, Brazil; CEP 05508-270; ‡Department of Natural Sciences and Mathematics, Johnson C. Smith University, 100 Beatties Ford Road, Charlotte, North Carolina 28216; §United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, Maryland 20705. e-mail: lindsayd@vt.edu

ABSTRACT: There are 3 strains of *Encephalitozoon cuniculi* that occur in mammals. Strain III is associated with clinical disease in dogs, although some can be asymptomatic carriers and excrete spores in their urine. Several cases of human *E. cuniculi* infection caused by strain III have been observed in immunocompromised patients, indicating that *E. cuniculi* should be considered a zoonotic agent. *Encephalitozoon cuniculi* can cause fatal disease in maternally-infected or young dogs. Clinical signs in these animals included blindness, encephalitis, retarded growth rate, and nephritis. *Encephalitozoon cuniculi* has also been associated with primary renal failure in adult dogs. The present study used the direct agglutination test (DAT, cut-off 1:50) and the indirect fluorescent antibody test (IFAT, cut-off 1:10) to examine the prevalence of antibodies to *E. cuniculi* in dogs from Brazil and Colombia. Using the DAG, 31 (27.4%) of 113 dogs from Brazil and 47 (18.5%) of 254 dogs from Colombia were seropositive. Nine (14.3%) of 63 dogs from Brazil and 18 (35.3%) of the 51 dogs from Colombia were seropositive by indirect immunofluorescent antibody test. These results indicate that dogs from Brazil and Colombia are exposed to *E. cuniculi*.

Encephalitozoon cuniculi is a microsporidian parasite of zoonotic importance. Little is known about its prevalence in animals from South America. A survey of 559 horses from Brazil demonstrated that 79 (14.1%) were positive in an indirect fluorescent antibody test and 70 (12.5%) were positive in a direct agglutination test (Goodwin et al., 2006). Nothing is known about the prevalence of this parasite in dogs from Brazil or Colombia. Canine encephalitozoonosis causes severe disease in transplacentally infected puppies (Plowright, 1952; Shaddock et al., 1978; Cole et al., 1982; McInnes and Stewart, 1991). Clinical signs in naturally infected dogs include encephalitis, vasculitis, blindness, and renal disease (Botha et al., 1979; Botha, Stewart, and van Dellen, 1986; Stewart et al., 1988). Dogs have their own genotype (referred to as genotype III) of *E. cuniculi* (Snowden et al., 1999). Knowledge of this genotype has helped in understanding the epidemiology of *E. cuniculi* in humans and other animals. The purpose of the present study was to determine the prevalence of antibodies to *E. cuniculi* in 2 urban canine populations from São Paulo, Brazil and Bogotá, Colombia.

Samples used in the present study had previously been examined for antibodies to *Trypanosoma cruzi*, *Leishmania infantum* (Rosypal et al., 2007), and *Toxoplasma gondii* (Dubey, Gennari et al., 2007; Dubey, Cortés Vecino et al., 2007). Briefly, samples were obtained from 367 domestic dogs from Brazil and Colombia, South America. One hundred and thirteen unwanted dogs (1 yr or older) were killed between December 2005 and April 2006 from São Paulo, Brazil. The dogs were ownerless pets or had been caught by the Municipality of São Paulo. They were taken to the Center for Zoonosis, São Paulo, where the dogs were killed by overdose injection of phenobarbital. A total of 254 unclaimed ownerless dogs (6 mo or older) was collected between February and May 2006 from Bogotá, Colombia. After efforts to place the dogs as pets failed, they were killed by intravenous injection (Euthanex®, Invet, S.A. Bogotá, Colombia) by Centro Distrital de Zoonosis, Bogotá.

Blood samples were collected at necropsy. Serum was separated by centrifugation, and refrigerated sera were initially sent by air to the United States Department of Agriculture Animal Parasitic Diseases Laboratory, Beltsville, Maryland as part of a *T. gondii* prevalence and ge-

netic characterization study (Dubey, Cortés Vecino et al., 2007; Dubey, Gennari et al., 2007). Serum samples were stored at -20 C; frozen sera were subsequently sent to the Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland College of Veterinary Medicine, Blacksburg, Virginia for serologic testing. The dogs were greater than 6 mo of age, or older, but precise ages were unavailable. The sex and breeds of the dogs were also not available.

Two serologic tests were conducted to detect antibodies to *E. cuniculi*. *Encephalitozoon cuniculi* (ATCC 50502 "canine subtype," Manassas, Virginia) was grown in human foreskin fibroblasts (Hs68, ATCC CRL1635, Manassas, Virginia) and the spores were harvested from the supernatant. Dog samples from Colombia were screened by the direct agglutination test (DAG) (Jordan et al., 2006) at a dilution of 1:50 in phosphate-buffered saline (PBS) using positive and negative control samples from a previous experiment as controls for the DAG. When a positive dog was identified, it was re-examined by the immunofluorescent assay test (IFAT) at a concentration of 1:50. After confirmation that it was positive by indirect immunofluorescent antibody test (IFAT) and DAG, the sample was used as a positive control for the duration of the experiment. All positive and negative dog samples used for sensitivity and specificity analysis were titrated out to 1:400; however, a 1:50 cut-off was used to identify a sample as positive.

In the DAG, we examined 113 samples from Brazil and 254 samples from Colombia. Subsamples, consisting of sera from 63 dogs from Brazil and 51 dogs from Colombia, were examined in the IFAT. Using the IFAT as the "gold standard," these samples were used to calculate the sensitivity and specificity of the DAG for canine serum.

The DAG test was conducted as previously described (Jordan et al., 2006). Briefly, spores of *E. cuniculi* were collected and fixed in 2 ml of 37% formaldehyde solution for 10-15 sec in a 15-ml conical centrifuge tube and then diluted with PBS (pH 7.4) up to 15 ml and stored at 4 C. The antigen solution was prepared by washing the fixed spores twice in PBS and then resuspending them in alkaline buffer-eosin solution (7.02 g NaCl; 3.09 g H₃BO₃; 24 ml 1 N NaOH; 4 g horse serum albumin factor V; 50 mg eosin Y; 1.0 g sodium azide; distilled H₂O to make 1 L; pH 8.7). Eosin was added to increase visualization of the agglutination reaction. Next, 0.5 ml of 0.2 M 2-mercaptoethanol was added to each 1 ml of the spore buffer solution to destroy IgM antibodies that might be present in the test serum and to prevent non-specific agglutination caused by IgM molecules. The DAG test was conducted in 96-well round-bottom plates. Test sera were diluted with PBS; 25 µl of serial test dilutions were combined with 75 µl of antigen solution and mixed thoroughly by pipetting up and down several times. The plates were covered with parafilm and incubated overnight at 37 C in a CO₂ incubator. Positive- and negative-control sera were separately examined on each plate. The agglutination reactions were read the next morning. Diffuse opacity across the entire diameter of the well was considered a positive agglutination reaction. A central discrete opaque dot or button was considered a negative reaction.

For the IFAT, 5 × 10⁵ spores were air-dried onto 12-well Teflon-coated IFAT slides (Fisher Scientific, Pittsburgh, Pennsylvania). The air-dried spores were fixed in 100% acetone for 30 sec and then stored at -20 C until use. Dilutions of dog sera were made at 1:50 in PBS and incubated with the antigen-containing slides for 30 min in a humidified box. Unbound antibodies were then washed off in 2 changes of PBS in a Coplan jar. A 1:2 dilution of fluorescence, labeled anti-dog IgG (KPL,

TABLE I. Prevalence of antibodies to *Encephalitozoon cuniculi* in dogs.

Location	Status of dogs	Number examined/% positive	Reference
South Africa	Owned	220/18*	Stewart, Botha, and van Dellen (1979)
Eastern Slovakia	Not stated	193/39*	Halánová et al. (2003)
Great Britain	Ownerless	248/13†	Hollister et al. (1989)
Norway	Owned	237/0*	Akerstedt (2003a)
Norway	Owned	1104/0†	Akerstedt (2003a)
Brazil	Ownerless	113/27‡	Present study
Brazil	Ownerless	63/14*	Present study
Colombia	Ownerless	254/19‡	Present study
Colombia	Ownerless	51/35*	Present study

* Indirect fluorescent antibody test.

† Enzyme-linked immunosorbent assay.

‡ Direct agglutination test.

Gaithersburg, Maryland), was then added, and the slides were incubated for 30 min in a humidified box. Unbound, labeled anti-dog antibodies were then washed off in 2 changes of PBS in a Copland jar. Slides were mounted using Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, Alabama). Positive and negative dog sera were used as controls in each test. Slides were viewed with an Olympus BX60 microscope equipped with reflected light epifluorescent optics (Olympus America, Inc., Center Valley, Pennsylvania).

Sera from 31 (27.4%) of 113 dogs from Brazil and 47 (18.5%) of 254 dogs from Colombia were positive in the DAG. Sera from 9 (14.3%) of 63 dogs from Brazil and 18 (35.3%) of 51 dogs from Colombia were positive for IgG antibodies to *E. cuniculi* by IFAT. The sensitivity was 68% and the specificity was 89% when the DAG was compared to the IFAT.

We investigated the prevalence of antibodies to *E. cuniculi* in ownerless dogs from urban areas of Brazil and Colombia to provide epidemiological information on infection of these animals with this zoonotic parasite. Few other studies on the prevalence of *E. cuniculi* in dogs have been reported (Table I). Molecular methods are needed to determine what strain of *E. cuniculi* is present (Didier et al., 1995) because we can only say that the dogs in our study were positive for antibodies to *E. cuniculi*. Stewart, Botha, and van Dellen (1979) developed an IFAT for *E. cuniculi*. They demonstrated that experimentally infected dogs seroconverted 32 to 39 days after infection. When the sera from 50 dogs from 2 kennels with confirmed *E. cuniculi* infection were examined in the IFAT, the prevalence of positive samples was 70% (Stewart, Botha, and van Dellen, 1979). Stewart et al. (1986) later demonstrated that experimentally infected dogs seroconvert to *E. cuniculi* in the IFAT by 7 days, and that antibodies persisted for 370 days.

Our studies on the prevalence of antibodies to *E. cuniculi* in ownerless dogs from Brazil and Colombia are similar to those reported from owned and ownerless dogs from other countries (Table I). The major exception is that no antibodies to *E. cuniculi* were seen in dogs from Norway using an IFAT and an enzyme-linked immunosorbent assay (Akerstedt, 2003a). This is somewhat surprising, because *E. cuniculi* infection is a problem in farmed blue foxes (*Alopex lagopus*) in Scandinavia (Akerstedt et al., 2002). *Encephalitozoon cuniculi* strain II isolated from a blue fox (Akerstedt et al., 2002) was nonpathogenic for experimentally inoculated blue foxes; however, the animals did seroconvert (Akerstedt, 2003b). The lack of *E. cuniculi* antibodies in dogs from Norway may be due to the strain of *E. cuniculi* present in domestic and wildlife species in Norway.

The sensitivity of the DAG in mice experimentally infected with the same strain of *E. cuniculi* used in the present study was 86% and the specificity was 98% (Jordan et al., 2006). These values are higher than the 68% sensitivity and 89% specificity observed in sera from naturally exposed dogs in the DAG in the present study.

Natural cases of *E. cuniculi* infection are usually more severe in maternally-infected or young puppies. All but 1 of a litter of puppies from Texas died from *E. cuniculi* infection (Shaddock et al., 1978). Most lesions were in the brain and kidneys. The pups had antibodies to *E. cuniculi* in an IFAT, but their owners did not (Shaddock et al.,

1978). Botha et al. (1979) reported on 12 cases of *E. cuniculi* infection in dogs from South Africa; all but 1 dog was less than 1 yr old. Clinical signs in these dogs included blindness, retarded growth rate, and nephritis. Two clinically normal Staffordshire bull terrier bitches, each of which had produced a litter that developed disease caused by *E. cuniculi*, were examined for lesions associated with the parasitic infection (McInnes and Stewart, 1991). Mild lesions, similar to those described from puppies, were observed in the tissues of both dogs. In addition, both dogs had antibodies to *E. cuniculi*, as did a 10-yr-old girl that had close contact with 1 of the infected litters (McInnes and Stewart, 1991). No antibodies to *E. cuniculi* were present in the serum of her 2 siblings. An association of chronic renal disease and *E. cuniculi* infection in dogs has been demonstrated by Stewart et al. (1988). They found that 12 of 52 serum samples from dogs with primary renal failure were positive for antibodies to *E. cuniculi*. Only 2 of 42 control dogs were positive for antibodies to *E. cuniculi*. Renal disease leading to renal failure is often a major clinical sign of *E. cuniculi* infections in dogs (Botha, Dormehl, and Goosen, 1986). Stewart, van Dellen, and Botha (1979) examined 3 outbreaks of *E. cuniculi* infection in different kennels in South Africa. They reported that puppies often developed a fading syndrome, with nervous signs arising in some cases. Stewart, van Dellen et al. (1979) were not able to reproduce disease in immunocompetent or immunosuppressed dogs inoculated with *E. cuniculi* spores collected from infected puppies.

Experimental infections in dogs with *E. cuniculi* have been reported with a variable clinical outcome (Stewart, Botha, and van Dellen, 1979; Botha, Stewart, and van Dellen, 1986; Stewart et al., 1986; Szabo and Shaddock, 1987, 1988), but these studies were conducted before there were methods to define which strain of *E. cuniculi* was present. Szabo and Shaddock (1987) experimentally infected 12.5-mo-old beagles intravenously with 1.75×10^9 viable *E. cuniculi* spores and monitored the dogs for 18 mo; only minor histopathologic lesions were found in these dogs. They concluded that adult dogs were able to mount an effective immune response to the parasite that limited tissue destruction and that the dogs eventually cleared the infection. Neonatal dogs are more susceptible to experimental *E. cuniculi* infection (Szabo and Shaddock, 1987).

Captive tamarins appear to be highly susceptible to disease caused by *E. cuniculi* strain III (Guscetti et al., 2003; Reetz et al., 2004; Juan-Sallés et al., 2006). Fatal disease was reported in 2 litters of emperor tamarins (*Saguinus imperator*) infected with *E. cuniculi* strain III (Guscetti et al., 2003). Histologic examination revealed systemic vasculitis and disseminated mixed inflammatory cell infiltration, with and without necrosis, in several organs (Guscetti et al., 2003). Disseminated *E. cuniculi* strain III infection (Reetz et al., 2004) was also observed in 2 captive cotton-top tamarins (*Oedipomidas oedipus*). Additionally, disseminated encephalitozoonosis in captive, juvenile, cotton-top (*S. oedipus*) and neonatal emperor (*S. imperator*) tamarins has been reported from zoos in North America (Juan-Sallés et al., 2006). Two of the emperor tamarins were less than 1 day old, suggesting that they had been infected in utero (Juan-Sallés et al., 2006). Experimental infections of vervet monkeys (*Cercopithecus pygerythrus*) with *E. cuniculi* spores

isolated from dogs have been reported (van Dellen et al., 1989); *E. cuniculi*-associated disease was produced in non-gravid and late-pregnant adults, immunocompetent infants, and in infants that were immunologically compromised by parenteral steroid administration, as well as in one infant that was immunologically immature because of its premature birth (van Dellen et al., 1989).

Human infections with *E. cuniculi* strain III are usually in HIV-infected individuals (Didier et al., 1996; Mertens et al., 1997; del Aguila et al., 2001; Weitzel et al., 2001; Tosoni et al., 2002), or in individuals that are immunosuppressed due to organ transplantation (Orenstein et al., 2005). The infection is often disseminated, and *E. cuniculi* strain III can be found in a variety of tissues.

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