

Prevalence of Antibodies to *Trypanosoma cruzi*, *Toxoplasma gondii*, *Encephalitozoon cuniculi*, *Sarcocystis neurona*, *Besnoitia darlingi*, and *Neospora caninum* in North American Opossums, *Didelphis virginiana*, from Southern Louisiana

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PREVALENCE OF ANTIBODIES TO *TRYPANOSOMA CRUZI*, *TOXOPLASMA GONDII*, *ENCEPHALITOOZON CUNICULI*, *SARCOCYSTIS NEURONA*, *BESNOITIA DARLINGI*, AND *NEOSPORA CANINUM* IN NORTH AMERICAN OPOSSUMS, *DIDELPHIS VIRGINIANA*, FROM SOUTHERN LOUISIANA

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ABSTRACT: We examined the prevalence of antibodies to zoonotic protozoan parasites (*Trypanosoma cruzi*, *Toxoplasma gondii*, and *Encephalitozoon cuniculi*) and protozoans of veterinary importance (*Neospora caninum*, *Sarcocystis neurona*, and *Besnoitia darlingi*) in a population of North American opossums (*Didelphis virginiana*) from Louisiana. Samples from 30 opossums were collected as part of a survey for *T. cruzi* in Louisiana. Frozen sera from these 30 opossums were examined using an indirect immunofluorescent antibody test (IFAT) against in vitro-produced antigenic stages of these protozoans. Additionally, 24 of the 30 samples were examined using hemoculture, and all 30 were examined in the modified direct agglutination test (MAT) for antibodies to *To. gondii*. The prevalences of reactive IFAT samples were as follows: 60% for *T. cruzi*, 27% for *To. gondii*, 23% for *E. cuniculi*, 17% for *S. neurona*, 47% for *B. darlingi*, and 0% for *N. caninum*. Hemoculture revealed that 16 (67%) of 24 samples were positive for *T. cruzi*, compared to 18 of 30 (60%) by IFAT. The sensitivity and specificity for the IFAT compared to hemoculture was 100% for each. The modified direct agglutination test revealed that 9 (30%) of the 30 samples from opossums had antibodies to *To. gondii*, compared to 8 (27%) using the IFAT. The sensitivity and specificity of the IFAT compared to the MAT was 100% and 72%, respectively.

Little is known of the prevalence of antibodies to protozoans of zoonotic and veterinary importance in North American opossums, *Didelphis virginiana*. In the present study, we examined sera from opossums for antibodies to the important human parasites *Trypanosoma cruzi*, *Toxoplasma gondii*, and *Encephalitozoon cuniculi*. Additionally, we examined the sera from these animals for antibodies to *Sarcocystis neurona*, *Besnoitia darlingi*, and *Neospora caninum*, which are parasites of importance in veterinary medicine.

MATERIALS AND METHODS

Opossum samples

Sera from 30 opossums from southern Louisiana (Barr et al., 1991) were examined in the present study. Frozen samples were sent by overnight carrier from the Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York to the Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, Virginia (CMMID). Samples were stored at -20°C until examined.

Parasite culture and antigen production

Epimastigotes of the Brazil strain of *T. cruzi* were grown in Grace's insect medium containing 30% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (antibiotics). Tachyzoites of the RH strain (Sabin, 1941) of *To. gondii*, tachyzoites of *B. darlingi* (strain of Dubey et al., 2002), and spores of *E. cuniculi* (ATCC 50502 "canine subtype," American Type Culture Collection, Manassas, Virginia) were grown in human foreskin fibroblasts (Hs68, ATCC CRL1635) that had grown to confluence in 75-mm² tissue culture flasks. Growth media consisted of 10% FBS (v/v) in RPMI 1640 medium supplemented with antibiotics. After monolayers had reached confluence, the growth medium was removed and replaced with a maintenance medium that

was identical to the former, except that the volume of FBS was 2% (v/v). Flasks were incubated at 37°C in a humidified incubator containing 5% CO_2 and 95% air. Merozoites of the SN-37R isolate (Sofaly et al., 2002) of *S. neurona* and tachyzoites of the NC-1 strain (Dubey et al., 1988) of *N. caninum* were grown and maintained in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1, ATCC CCL-70, American Type Culture Collection) using techniques identical to those described for Hs68 cells.

Indirect fluorescent antibody tests

Approximately 0.5 to 1×10^4 stages of *T. cruzi*, *To. gondii*, *S. neurona*, *B. darlingi*, and *N. caninum*, or 5×10^5 spores of *E. cuniculi* in 30 μl phosphate-buffered saline (PBS), were separately air-dried onto 12-well Teflon-coated indirect fluorescent antibody test (IFAT) slides (Fisher Scientific, Pittsburgh, Pennsylvania). Antigen-containing slides were then dried in air for 4–12 hr. Antigen-coated IFAT slides were stored at -20°C until used.

The IFAT described by Roellig et al. (2009) for the detection of IgG antibodies in opossum sera against *T. cruzi* was modified for use in the present study. Opossum sera were diluted 1:100 in PBS and 25 μl was added to a test well. Opossum test sera were incubated with antigens for 30 min and the slides were washed in PBS. Next, rabbit anti-opossum IgG sera (Bethyl Laboratories, Montgomery, Texas) were incubated with the antigens on IFAT slides for 30 min and the slides were washed in PBS. Finally, fluorescence-labeled goat anti-rabbit IgG (Bethyl Laboratories) sera were incubated with the antigens on IFAT slides for 30 min and slides were washed in PBS. Slides were washed twice in PBS, mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, Alabama), and viewed with an Olympus BX60 epifluorescent microscope equipped with differential contrast optics (Olympus America Inc., Center Valley, Pennsylvania). Only samples that exhibited fluorescence of the entire parasite surface were considered positive.

Modified direct agglutination test for *Toxoplasma gondii*

Samples were shipped by overnight carrier from CMMID to the Animal Parasitic Diseases Laboratory, Agricultural Research Service, United States Department of Agriculture, Animal and Natural Resources Institute, Beltsville Agricultural Research Center, Beltsville, Maryland for examination in the modified direct agglutination test (MAT; Dubey and Desmonts, 1987). Sera were examined at a 1:25 dilution in the MAT.

Sensitivity and specificity of IFAT

The sensitivity and specificity of the IFAT for *T. cruzi* was examined using parasite isolation from the blood (Barr et al., 1991) as the gold standard, and the sensitivity and specificity of the IFAT for *To. gondii* was

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TABLE I. Prevalence of antibodies to *Trypanosoma cruzi*, *Toxoplasma gondii*, *Encephalitozoon cuniculi*, *Sarcocystis neurona*, and *Besnoitia darlingi* in opossums (*Didelphis virginiana*) from Louisiana.

Opossum no.	<i>Trypanosoma cruzi</i>	<i>Toxoplasma gondii</i>	<i>Encephalitozoon cuniculi</i>	<i>Sarcocystis neurona</i>	<i>Besnoitia darlingi</i>
	IFAT/Culture*	IFAT/MAT†	IFAT	IFAT	IFAT
9-1	P/P‡	P/P	N	N	N
9-2	N/C	N/N	N	N	N
10-1	P/P	N/N	N	N	N
10-2	P/P	N/N	N	N	N
11-1	P/P	N/P	N	N	N
11-2	P/C	P/P	N	N	P
11-3	P/P	N/N	P	N	P
12-1	P/P	N/N	P	N	P
12-2	P/P	P/P	N	N	P
13-1	P/P	N/N	N	N	N
13-2	N/N	P/P	N	P	P
14-1	P/P	N/N	N	N	N
15-1	P/P	P/P	P	N	P
16-1	N/C	N/N	P	N	P
16-2	N/N	N/N	N	P	N
17-1	N/N	N/N	N	N	N
18-1	N/N	N/N	N	N	P
19-1	P/P	N/N	N	N	N
19-2	N/C	N/N	P	P	N
20-1	P/P	N/N	P	N	P
20-2	P/P	N/N	P	N	P
20-3	P/P	P/P	N	N	N
21-1	N/N	P/P	P	N	N
21-2	N/N	N/N	P	P	N
21-3	N/C	N/N	N	N	N
21-4	P/C	N/N	N	N	P
22-1	N/N	N/N	N	N	P
23-1	P/P	N/N	N	N	N
23-2	P/P	N/N	N	N	P
24-1	N/N	P/P	N	P	P

* IFAT = indirect immunofluorescent antibody test, culture = hemoculture.
 † MAT = modified direct agglutination test.
 ‡ P = positive, C = contaminated unusable, N = negative.

examined using the MAT (Dubey and Desmonts, 1987) as the gold standard. The sensitivity was equal to the number of true positive (gold standard positive) samples, divided by the number of true positive samples plus the number of false negative samples, multiplied by 100. The specificity was equal to the number of true negative (gold standard) samples, divided by the number of true negative samples plus the number of false positive samples, multiplied by 100.

RESULTS

The results from individual opossums are presented in Table I. Using the IFAT and a cutoff titer of 1:100, antibodies to *T. cruzi* were found in 18 (60%), *To. gondii* in 8 (27%), *E. cuniculi* in 7 (23%), *S. neurona* in 5 (17%), *B. darlingi* in 14 (47%), and *N. caninum* in 0 of the 30 opossums from southern Louisiana. Six of the 30 hemocultures became contaminated with bacteria. Hemoculture demonstrated that 16 (67%) of the 24 opossums were positive (Table I) for *T. cruzi*. The sensitivity and specificity of the IFAT for *T. cruzi* was 100% each when the 30 IFAT samples were compared to the 24 hemoculture samples as the gold standard. The MAT indicated that 9 (30%) of the 30 opossums were positive for agglutinating antibodies (Table I) to *To. gondii*. The sensitivity and specificity of the IFAT for *To. gondii* was 100%

and 72%, respectively, when compared to the MAT as the gold standard.

Three (10%) of the 30 samples were negative for antibodies—culture for all parasites, 9 (30%) were positive for only 1 parasite, and 18 (60%) samples had evidence of exposure to 2 or more of the protozoans (Table I).

DISCUSSION

All but 1 of the previous studies on the prevalence of *T. cruzi* in North American opossums has been conducted by hemoculture (McKeever et al., 1958; Olsen et al., 1964; Barr et al., 1991; Karsten et al., 1992; Pung et al., 1995; Brown et al., 2010). Hemoculture studies have demonstrated that 17 (14%) of 126 opossums from east-central Alabama (Olsen et al., 1964), 88 (16%) of 552 opossums from southwestern Georgia and northwestern Florida (McKeever et al., 1958), 16 (33%) of 48 opossums from Louisiana (Barr et al., 1992), 6 (15%) of 39 opossums from southeast Georgia (Pung et al., 1995), and 1 (8%) of 12 opossums from North Carolina (Karsten et al., 1992) were infected with *T. cruzi*. Brown et al. (2010) used an IFAT and reported that antibodies were present in 14 (52%) of 27 opossums from Florida, 118 (28%) of opossums from Georgia, and

1 (17%) of 6 opossums from Virginia. When a subset of 83 opossums from Florida and Georgia were examined by hemoculture and IFAT, significantly ($P < 0.05$) more positive samples were identified using IFAT (Brown et al., 2010). Our results using the IFAT, and those of Brown et al. (2010), indicate that the IFAT is an effective alternative for hemoculture when examining the seroprevalence of *T. cruzi* infections in opossums.

Seroprevalence studies on antibodies to *To. gondii* in opossums have used several different serological tests. Burrige et al. (1979) reported that 37 (11%) of 349 opossums from Florida had antibodies to *To. gondii* when examined using an indirect hemagglutination test. Antibodies to *To. gondii* were found in 1 (3%) of 34 (Smith et al., 1992) and 12 (23%) of 53 (Hill et al., 1998) opossums from Iowa using the MAT. Smith and Frenkel (1995) reported that 6 (16%) of 38 opossums collected from Kansas and Missouri were positive for antibodies to *To. gondii* using the Sabin–Feldman dye test. Antibodies to *To. gondii* were demonstrated in 11 (17%) of 66 (Dubey et al., 2009) and in 3 (10%) of 29 (Suzán and Ceballos, 2005) *D. virginiana* from Mexico, using the MAT and complement fixation, respectively. The results of our sensitivity and specificity studies indicate that the MAT and IFAT produce similar results.

We were unable to find previous studies examining the prevalence of *E. cuniculi* or other microsporidian parasites in *D. virginiana*. The lack of IFAT reagents and the difficulty in demonstrating spores in urine or feces probably accounts for the lack of prevalence studies on *E. cuniculi* in these animals.

Sarcocystis neurona is the cause of equine protozoal myeloencephalitis, an important neurological disease of horses in the Americas for which opossums are the definitive host (see Dubey, Lindsay et al., 2001). The epidemiology of *S. neurona* infections in *D. virginiana* is not well understood. Molecular or animal feeding–inoculation studies are needed to accurately identify the species of *Sarcocystis* present in opossum feces (Dubey, 2000; Cheadle et al., 2001); this has limited the number of prevalence studies conducted to date. Sporocysts of *S. neurona* were found in 19 (26%) of the 72 opossums from Mississippi using animal inoculation (Dubey, Black et al., 2001), and 17 of the 19 isolates were consistent with *S. neurona* using molecular methods (Rickard et al., 2001). Sporocysts identified by molecular techniques as *S. neurona* were found in intestinal scrapings or feces from 31 (15%) of 206 opossums from Michigan (Elsheikha, Murphy et al., 2004) and in 17 (6%) of 288 opossums sampled from California (Rejmane et al., 2009). Serological examination of opossums for antibodies to *S. neurona* is complicated by the fact that they are definitive hosts for 4 species and intermediate host for 2 species of *Sarcocystis* (Cheadle, 2001; Elsheikha, Fitzgerald et al., 2003). Concurrent infection of the intestine with *S. neurona*, and in muscle with *Sarcocystis inghami*, has been reported (Elsheikha, Fitzgerald et al., 2004). Opossums experimentally fed tissues containing sarcocysts of *S. neurona* did not seroconvert (Cheadle et al., 2006) in the modified direct agglutination test (SAT) using *S. neurona* merozoites as antigen (Lindsay and Dubey, 2001). None of the sera from 7 opossums from Connecticut was positive for antibodies to *S. neurona* when examined using the SAT (Mitchell et al., 2002). The potential for cross-reactivity of opossum antibodies to *Sarcocystis greineri* and *S. inghami* with merozoites of *S. neurona* needs further evaluation before the utility of the IFAT test for *S. neurona* can be fully validated in opossums.

This is the first report about using the IFAT to examine the prevalence of exposure to *B. darlingi* in opossums. In the United States, *B. darlingi* tissue cysts have been reported from 1 (100%) of 1 opossums from Illinois (Flatt et al., 1971), 6 (100%) of 6 from Indiana (Jack et al., 1989), 2 (40%) of 5 from Kansas (Smith and Frenkel, 1977), 1 (20%) of 5 from Kentucky (Conti-Diaz et al., 1970), 1 (100%) of 1 from Louisiana (Shaw et al., 2009), 15 (11%) of 137 from Michigan (Elsheikha, Mansfield et al., 2003), 2 (100%) of 2 from Mississippi (Dubey et al., 2002), 7 (58%) of 12 from Missouri (Flatt et al., 1971), and 1 (100%) of 1 in Texas (Stabler and Welch, 1961).

There are no reports of *N. caninum* infection in *D. virginiana*. Yai et al. (2003) reported that 84 (21%) of 396 South American opossums (*Didelphis marsupialis*) from São Paulo, Brazil had antibodies when examined using an IFAT. The lack of antibodies to *N. caninum* suggests that opossums do not play a significant role in the sylvatic cycle of this protozoan (Rosypal and Lindsay, 2005).

The present study demonstrates that opossums can be frequently exposed to zoonotic protozoan parasites. They are reservoir hosts for *T. cruzi* and *To. gondii* and could potentially be involved in the direct transmission of *E. cuniculi* by spores in their urine or feces. The results of the present study represent the prevalence of these parasites in opossums from Louisiana found approximately 20 yr previously in 1991. They provide valuable baseline data to examine the changes in prevalence of these zoonotic parasites associated with changes in populations of hosts or vectors due to environmental changes induced by hurricanes, other natural disasters, and man-made disruptions in the environment.

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