NEOSPORA CANINUM–SPECIFIC ANTIBODIES IN *FREE-RANGING* WHITE-LIPPED PECCARIES (*TAYASSU PECARI*) FROM THE PERUVIAN AMAZON: DETECTION OF ANTIBODIES IN SERUM AND EVALUATION OF INDIRECT FLUORESCENT ANTIBODY TEST WITH HETEROLOGOUS SECONDARY ANTIBODY

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NEOSPORA CANINUM–SPECIFIC ANTIBODIES IN *FREE-RANGING* WHITE-LIPPED PECCARIES (*TAYASSU PECARI*) FROM THE PERUVIAN AMAZON: DETECTION OF ANTIBODIES IN SERUM AND EVALUATION OF INDIRECT FLUORESCENT ANTIBODY TEST WITH HETEROLOGOUS SECONDARY ANTIBODY

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Abstract: *Neospora caninum* is a coccidian parasite originally reported in dogs and widely prevalent in numerous species of wild and domestic animals and has as definitive hosts some species of canids. The white-lipped peccary (WLP) (*Tayassu pecari*) is a Tayassuidae mammal, found from Mexico to south of Brazil and north of Argentina. It is a game species with great economic importance in the Peruvian Amazon. Blood samples from 101 WLPs were collected from near or within three different conservation reserves located in the southeastern region of the Peruvian Amazon. For the detection of antibodies against *N. caninum*, indirect fluorescent antibody tests (IFAT) were performed using collared peccary (*Pecari tajacu)* and swine (*Sus scrofa domesticus*) heterologous secondary antibodies. For both IFAT tests, the cutoff was 1:50. Positive samples were titrated by a two fold serial dilution. In addition to IFAT, samples were also analyzed using an immunoblotting test (IB) with anti-swine conjugate. To confirm the viability of the anti-swine conjugate, the results of these samples previously tested by a modified agglutination test (MAT) for *Toxoplasma gondii* were used as reference. From the total of 101 samples tested, 5 (4.9%) were *N. caninum* positive by the three tests and an extra sample was positive by both IFATs and negative in the IB. Comparing both IFATs and considering IB as the gold standard, the relative sensitivity of IFATs was 100%, the specificity was 98.9%, the positive predictive value was 83.3%, and the negative predictive value was 100%. The agreement between tests was characterized by a κ value of 0.904 (95% confidence interval, 0.717 to 1.0) and an SE of 0.095. This is the first report of *N. caninum* antibodies in free-ranging *T. pecari*, and swine and collared peccary conjugate can be used as a secondary antibody for detection of antibodies in *Tayassu* species.

Key words: Immunoblotting test, indirect fluorescent antibody test, *Neospora caninum, Pecari tajacu* (collared peccary), *Sus scrofa domesticus* (swine), *Tayassu pecari* (white-lipped peccary).

INTRODUCTION

The peccary species, collared peccary (*Pecari tajacu*), Chacoan peccary (*Catagonus wagneri*), and the ehite-lipped peccary (*Tayassu pecari*), are mammals that belongs to the family Tayassuidae and are all confined to the American continent.[17] The species are phylogenetically closely related, diverging from a common ancestor between the late Miocene and late Pliocene.[15]

The white-lipped peccary (WLP) is found in the Neotropical region, from southeastern Mexico, in the north, through Central and South America, north of Argentina and south of Brazil. The WLP is catalogued as vulnerable in the IUCN Red List of Threatened Species, due to an ongoing decline of its population in many regions as a result of deforestation, intense hunting pressure, competition with livestock, and epidemics.[1,20] Also, in the Peruvian Amazon, subsistence hunting is legally

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permitted, and the WLP is a game species and important for the local rural economy especially for pelt trades.[35,36]

Neospora caninum is a coccidian parasite originally reported in dogs[4] and widely prevalent in numerous species of wild and domestic animals.[11] The definitive hosts are members of the *Canis* genus, including domestic and wild dogs,[24] coyotes,[13] dingoes,[22] and gray wolves.[9] *N. caninum* antibodies and/or DNA have been detected, and clinical disease was also reported in wild animal species.[6]

N. caninum diagnosed with sera samples from domestic animals is generally made using indirect fluorescent antibody tests (IFAT), immunoblotting (IB), and enzyme-linked immunosorbent assay (ELISA).[10] All those techniques use species-specific conjugates. However, for wildlife, due to the difficulty in finding a specific commercial secondary antibody for most wild species, this is not possible. Often a heterologous secondary antibody is used in the diagnostic techniques. Assuming that a particular antigen may have different epitopes that can be recognized by a particular antibody, it is expected that a secondary antibody in a test, like IFAT, can recognize regions of the primary antibody that are common to phylogenetically related species.[27] However, with phylogenetically related rodent species, a study[12] found that IFAT was not effective for some species of rodents, all experimentally infected with *T. gondii*, and the authors recommended caution in the interpretation of tests using heterologous secondary antibody, even among phylogenetically close species.

The aim of this study was to test wild *T. pecari* for *N. caninum* antibodies, thus contributing to knowledge on *N. caninum* in tropical wild animal systems and their ecology. The occurrence of *N. caninum* antibodies was tested by IFAT, using two different heterologous conjugates, and IB as a confirmatory test.

MATERIALS AND METHODS

Study area and sampling

The study was carried out between 2005 and 2009 near or within three conservation areas in the department of Madre de Dios in southeastern Peru: Boca Manu (12°15′S, 70°54′W; 240 to 260 miles above sea level [m.a.s.l.]), Los Amigos Conservation Concession (12°33′S, 70°05′W; 240 to 320 m.a.s.l), and the Reserva Nacional Tambopata, Parque Nacional Bahuaja Sonene (12°30′S, 69°30′W; 200 to 250 m.a.s.l). Blood samples (n = 101) were collected from the jugular and saphenous veins from hunter-killed or live-captured WLPs, respectively. Sera samples were stored at –20°C until analysis.

Fluorescein-conjugated anti–*P. tajacu* antibody

Anti–*P. tajacu* IgG conjugate was produced in the Immunology Section of the Zoonosis Control Center of the municipality of São Paulo, SP (CCZ-SP), Brazil, according to previously described methods.[3,5,18] Briefly, a pool of serum from *P. tajacu* was precipitated with 18% sodium sulfate and dialyzed with a 0.9% sodium chloride solution. Immunoglobulins were obtained by ion-exchange chromatography in a diethylaminoethyl-celulose (DEAE)-cellulose solution (Sigma, St. Louis, MO 63103, USA), and protein fraction purity was checked by agar gel immunoelectrophoresis.[18] Protein concentration was measured by biuret reaction.[16] A sheep was immunized with a series of 10 intramuscular inoculations of *P. tajacu*–purified immunoglobulin (1 mg protein/ml) associated with Freund's complete adjuvant at 15-day intervals. The antiserum was analyzed quantitatively by radial immunodiffusion, and after identification of precipitating line at the 1:128 dilution, the animal was bled through jugular puncture. Then, serum was precipitated with an 18% sodium sulfate solution and purified using DEAE-cellulose resin, and the anti–*P. tajacu* IgG serum was conjugated with fluorescein isothiocyanate in dialysis overnight. The excess of fluorochrome present in the serum was removed by filtration through a Sephadex G-50 column (Sigma, St. Louis, MO 63103, USA). The conjugate was kept at –20°C until use.

Serologic tests

The samples had been previously analyzed for the presence of *T. gondii* antibodies by modified agglutination test (MAT),[7] which is a test that does not require species-specific conjugates. All samples were also tested by IB for *T. gondii* and for *N. caninum* antibodies using the p30 (TgSAG1) and the p38 (NcSRS2) antigen, respectively (details below). In these tests, a peroxidase anti-swine IgG conjugate was used to confirm the viability of the heterologous conjugate against the antibodies of WLP. Further, indirect fluorescent antibody tests (IFATs) were used to determine the presence of IgG antibodies against *N. caninum* and to compare the efficacy of the anti–*P. tajacu* IgG conjugate and commercial anti-swine IgG conjugates in the tests. Immunoblotting using *N.*

caninum total antigen and a commercial anti-swine IgG conjugate was used as a further confirmatory test.

N. caninum and *T. gondii* IB were performed using peroxidase anti-swine IgG conjugate (affinity purified goat anti-swine IgG [H + L], Jackson Immuno Research Laboratories, West Grove, PA 19390, USA) in a dilution of 1:500. RH strain of *T. gondii*[29] and the Nc-1 strain of *N. caninum*[8] were maintained in MARC-145 cells[21] cultures and purified as previously described.[30–32] Cell culture–derived tachyzoites were frozen as a pellet at –20°C until used for IB or antigen purification. The IB test was performed as previously described.[2,33] Purified *N. caninum* and *T. gondii* tachyzoites (i.e., p38 [NcSRS2] and p30 [TgSAG1]) were used as antigen in IB. In the *N. caninum* p38 IB, using purified p38 as antigen, sera that presented a single band of 38-kDa Mr were regarded as positive. As a positive control (i.e., as a control to define the location of specific bands), a serum of a European fox (*Vulpes vulpes*) was experimentally infected with *N. caninum,*[31] and as a negative control, the preinfection serum of this fox was applied. In *T. gondii,* p30 IB reactions to a single band of 30-kDa Mr were regarded as positive.[2] As a positive control (i.e., as a control to define the location of specific bands), a serum of a European fox (*Vulpes vulpes*) was experimentally infected with *T. gondii,*[19] and as a negative control, the preinfection serum of this fox was used. As a secondary antibody for fox sera, a commercial anti-dog IgG peroxidase conjugate anti-dog IgG [H + L] (Jackson Immuno Research Laboratories) was applied in a dilution of 1:500.

To confirm positive findings for *N. caninum,* selected IFAT-positive sera were examined in immunoblot using nonreduced total lysate antigen as previously described.[2,34] Reactions against immunodominant antigens of 17- to 19-, 29-, 30-, 33-, and 37-kDa Mr were recorded.[31,34] As positive and negative controls, we used the same sera as described above for the *N. caninum* p38 IB in a dilution 1:500.

IFAT for *N. caninum* was performed as described by Paré et al in 1995,[26] using *N. caninum* (Nc-1) tachyzoites as the antigens for both tests. Fluorescein isothiocyanate (FITC) conjugate anti-swine IgG (Jackson ImmunoResearch Laboratories) and anti–*P. tajacu* IgG, produced in sheep by CCZ-SP, were used in a dilution of 1:250 after standardization. The dilution of 1:50 was used as the cutoff,[2] and positive samples were two fold serially diluted until the maximum positive dilution titer was reached. Positive and negative pig sera were added in each slide.[2] Only peripheral, but not apical fluorescence, was considered specific.

All secondary conjugated antibodies used in this study were nonreactive against antigen of protozoan parasites in IB and IFAT, i.e., antigen of *N. caninum, T. gondii*, and *Besnoitia besnoiti*, when applied in the dilutions mentioned above.

Specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were calculated and compared for both serologic tests (IFAT and p38 IB) using IB as the gold standard.[37] Level of agreement between the serologic tests was evaluated using κ value with 95% confidence interval (CI).[37]

The study was carried out under research permits, Animal Care and Use Committee approval from Intendencia Forestal y de Fauna Silvestre, Ministerio de Agricultura, Lima, Peru, following protocol 68-2008- INRENA-IFFS-DCB and R.D. 251-2009-AG-DGFFS-DGEFFS.

RESULTS AND DISCUSSION

From the 101 WLPs samples tested for anti–*T. gondii* antibodies, 85 (84%) were positive by IB, using the peroxidase anti-swine IgG conjugate. These results matched with those found by Solorio et al in 2010[36] that tested the same samples and found 90 (89%) were MAT positive, confirming the feasibility of a heterologous conjugate (anti-swine IgG conjugate for WLP IgG) for serologic diagnosis of coccidian infections.

From the total of 101 samples of WLPs examined for anti–*N. caninum* detection, 6 (5.9%) were IFAT positive for both conjugates (anti–*P. tajacu* IgG and anti-swine IgG FITC) and 5 (4.9%) of the 6 IFAT-positive samples were also positive in p38 IB (with a peroxidase anti-swine IgG conjugate). All serologically *N. caninum*–positive animals were also *T. gondii* positive. Antibody titers found in animals varied from 50 to 200 in the *N. caninum* IFATs independent of the conjugate (anti–*P. tajacu* IgG and anti-swine IgG FITC) (Table 1).

Although peccaries and pigs show similarities, their phylogenetic classification and original worldwide distribution are different. Peccaries belong to the Family Tayassuidae and are native to the New World, whereas pigs belong to the Family Suidae and are native to the Old World. Both families belong to the suborder Suina. In the present study, it was showed that antisera or conjugates directed to swine IgG represent effec-

Table 1. Comparison of the *N. caninum* positive results from IB and IFAT from *T. pecari* serum samples using heterologous conjugates and results on IB of the same animals using *T. gondii* antigens.

	N. caninum antigen			*T. gondii antigen*
Positive samples Identification	P38 IB (anti-swine IgG conjugate[a])	IFAT (anti-swine IgG conjugate[b])	IFAT (anti–*P. tajacu* IgG conjugate[b])	P30 IB (anti-swine IgG conjugate[a,c])
TP31	2	100	100	2
TP03	1	50	50	2
TP12	2	50	100	2
ST03	1	50	50	1
ST21	3	200	200	2
ST30	0	50	100	1
Total	5	6	6	6

[a] Intensity of the color of the bands in IB (1, faint, 2, clear, 3, strong).
[b] Reciprocal antibody titers.
[c] Samples were also positive for *T. gondii* as determined by MAT.

tive heterologous secondary antibodies or tools applicable in serodiagnostic techniques.

Comparing both IFAT tests and considering p38 IB as the gold standard, the relative sensitivity of IFATs was 100%, the specificity was 98.9%, the PPV was 83.3%, and the NPV was 100% (Table 2). Agreement was characterized by a κ value of 0.904 (95% CI: 0.717 to 1.0) and an SE of 0.095, so that the agreement is considered almost perfect.[23] Only one sample was positive in both IFATs and negative using IB. Five samples positive by IFAT and p38 IB were also tested positive by IB using total lysate antigen (Fig. 1). Despite confirmatory testing, one sample testing positive by IFAT but negative by immunoblot remains unexplained. Although an IFAT titer of 1:50 is usually regarded as specific,[14] cross-reactions after infections with other related parasites (e.g., *Sarcocystis* or *Besnoitia* spp.) cannot be completely ruled out, especially at less stringent cutoff titers.[14]

Antibodies against *T. gondii* (p30) antigens were detected in 84% (85 of 101) of the WLP samples using IB (anti-swine IgG peroxidase conjugate), a result that is very close to the 89% (90 of 101) found by MAT with the same samples.[36] These results also confirm the efficacy of the heterologous anti-swine IgG conjugate used to detect specific *N. caninum* IgG antibodies. The MAT was developed for the serologic diagnosis of *T. gondii* antibodies in humans and animals.[7] Unlike most

Table 2. Quantitative comparison of IB and IFAT for detection of antibody against *N. caninum* in *T. pecari*[a]

	IB positive	IB negative	Total
IFAT positive	5	1	6
IFAT negative	0	95	95
Total	5	96	101

[a] Sensitivity: 100%; specificity: 98.9%; PPV: 83.3%; NPV: 100%.

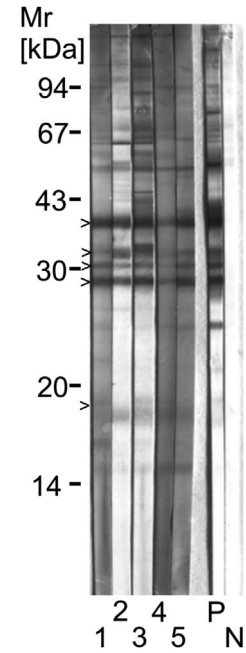

Figure 1. IB reactions of five white-lipped peccaries (*T. pecari*) positive in IFAT and p38 IB resemble those of a European fox (*V. vulpes*), experimentally infected with *N. caninum*. P, positive control; N, negative control; 1-5, sera of animals positive by p38 IB and IFAT: 1, TP31; 2, TP03; 3, TP12; 4, ST03; 5, ST21 (serum IDs in Table 1). Locations of the reactions against immunodominant antigens of 17- to 19-, 29-, 30-, 33-, and 37-kDa Mr are marked by arrowheads.

serologic tests, the MAT does not require species-specific conjugates, and so any animal species could be tested. This type of test is very useful for serologic studies in wild animal species. Tests for the detection of *N. caninum* antibodies that are based on agglutination are described (NAT,[28] N-MAT[25]); however, they are not commercially available.

Several possible routes of infection for WLPs with *N. caninum* could be considered: (1) the proximity of WLPs with domestic animals and human settlements; (2) the WLP foraging behavior; (3) the hunting methods of WLPs involving domestic dogs; and (4) evisceration of felled animals and the use of these viscera to feeding dogs, might perpetuate the cycle.

This is the first report of *N. caninum* antibodies in free-ranging *T. pecari*. In addition, the study showed that commercially available anti-swine IgG and anti–*P. tajacu* conjugates can be used as effective secondary antibodies for the detection of specific *T. gondii* and *N. caninum* antibodies in *T. pecari* and provides a useful framework for diagnostic test standardization for other related animal species.

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