

Serological Survey of *Toxoplasma gondii* Infection Among Slaughtered Pigs in Northwestern Taiwan

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ABSTRACT: A serological survey of *Toxoplasma gondii* infection among slaughtered pigs in the largest slaughterhouse located in Taoyuan County of northwestern Taiwan was conducted using the latex agglutination (LA) test during 1998. The overall seroprevalence of *T. gondii* infection was 28.8% (32/111) with LA titers of 1:32 (6, 18.8%), 1:64 (10, 31.2%), 1:128 (9, 28.1%), 1:256 (6, 18.8%), and 1:512 (1, 3.1%). No significant difference ($P > 0.05$) in seroprevalence between male (28.6%, 20/70) and female (29.7%, 12/41) slaughtered pigs was observed. A decreasing trend in the seroprevalence among slaughtered pigs examined in the same slaughterhouse was observed because of a lower seroprevalence ($P < 0.05$) than that (44.4%, 128/288) previously reported about 10 yr ago using the LA test. Nevertheless, it is important to avoid eating raw or undercooked pork in order to prevent the acquisition of *T. gondii* infection among people in Taiwan.

Infection by the protozoan parasite *Toxoplasma gondii* is prevalent in animals and humans worldwide. Humans become infected with *T. gondii* usually by ingesting oocysts in food and water contaminated by cat feces or by consuming tissue cysts in undercooked meat (Dubey and Beattie, 1988). Pork is considered to be the most important meat source of *T. gondii* infection in the United States (Dubey, 1986). Two outbreaks of acute toxoplasmosis involving 8 adult patients in Korea were linked to eating uncooked pork (Choi et al., 1997). Most infections are asymptomatic, but in some persons it eventually becomes symptomatic. Thus, for example, toxoplasmic encephalitis is a major disease in acquired immunodeficiency syndrome patients. Prenatal infection may also occur, resulting in newborns with congenital toxoplasmosis (Jones et al., 2001). In the United States, the *T. gondii*-associated annual economic public health burden purportedly exceeds \$400 million (Roberts et al., 1994).

In our previous studies, the seroprevalence of latent *T. gondii* infection among Taiwanese, especially of mountain aboriginal populations, was not low, with a range of 2.7–26.7% using the latex agglutination (LA) test (Fan et al., 1998, 2001, 2002). In addition, data from questionnaires concerning risk factors of acquiring *T. gondii* infection showed that the consumption habits of frequently eating raw or undercooked pork were considered the chief factor contributing to *T. gondii* infection among these people (Fan et al., 2001, 2002). Infective tissue cysts have been found repeatedly in commercial cuts of pork of both experimentally and naturally infected pigs (Dubey, 1988). In Taiwan,

studies concerning *T. gondii* infection in pigs are rather rare. Kundin et al. (1972) were the first to investigate the *T. gondii* infection in slaughtered pigs and indicated the low prevalence (1%, 10/999) in Taiwan. Lee et al. (1975) reported, in contrast, that the prevalence in slaughtered pigs was high (52%, 26/50) in southern Taiwan. In the 1980s, a large-scale survey of pig toxoplasmosis was conducted in 8 counties of Taiwan using the LA test, and it was found that the overall seroprevalence of latent *T. gondii* infection in slaughtered pigs was 27.7% (1,073/3,880), of which the highest seroprevalence (44.4%, 128/288) was recorded in the largest slaughterhouse located in Taoyuan County of northwestern Taiwan (Chang et al., 1991). Since then, information concerning the status of pig toxoplasmosis in Taiwan is not available. The purpose of this study was to reexamine the prevalence of antibodies to *T. gondii* in pigs in the largest slaughterhouse located in Taoyuan County of northwestern Taiwan.

Between January and October 1998, 111 blood samples were randomly collected from pigs having a slaughter weight of 110 kg in a slaughterhouse located in Taoyuan County. Sera were separated by centrifugation and kept at -70°C until analysis. A commercial LA test kit (Eiken Chemical Co., Tokyo, Japan) was used to test serum anti-*T. gondii* antibodies (Chang et al., 1991; Gajadhar et al., 1998; Kim et al., 2002). The reactions were performed using a 96-well U-bottom polystyrene microplate at 2 dilutions, i.e., 1:16 to 1:1,024. To each well was then added 25 μl of *T. gondii* antigen-coated latex particles suspension, which was incubated overnight at room temperature. An agglutination titer $\geq 1:32$, i.e., 1:32 to 1:1,024, was considered positive. For calculation of the significant differences in seroprevalence between male and female slaughtered pigs, a chi-square test was used and a P value < 0.05 was considered significant. Although several studies have indicated that the LA test is not the best serological tool to detect latent *T. gondii* infection in pigs (Dubey et al., 1995), we still used the LA test because of its moderate agreements with the dye test and modified agglutination test (Dubey et al., 1995).

Of the 111 serum samples tested, 32 (28.8%) were found to be positive with LA titers of 1:32 (6, 18.8%), 1:64 (10, 31.2%), 1:128 (9, 28.1%), 1:256 (6, 18.8%), and 1:512 (1, 3.1%) (Table I). No significant difference ($P > 0.05$) was observed in the seroprevalence between male (28.6%, 20/70) and female (29.7%, 12/41) pigs in this study (Table I). The results of this study were higher than those of pigs reported in China (10.4%) (Lin et al., 1990), Indonesia (6.3%) (Inoue et al., 2001), Japan (9.1%) (Horio et al., 2001), the Netherlands (1.8%) (van Knapen et al., 1995), and the United States (2.2%) (Dubey et al., 1995). Nevertheless, a decreasing trend in the seroprevalence among pigs examined in the same slaughterhouse was observed because of the lower seroprevalence ($P < 0.05$) than that (44.4%, 128/288) previously reported about 10-yr ago using the LA test (Chang et al., 1991). The probable reasons for the decreasing seroprevalence are believed to be an improved zoohygienic situation on farms, good sanitation, and the exclusive use of commercially marketed feeds. However, it is important for people in Taiwan to avoid eating raw or undercooked pork in order to prevent the acquisition of *T. gondii* infection.

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TABLE I. Seroprevalence of *Toxoplasma* antibody among pigs slaughtered in northwestern Taiwan using the latex agglutination (LA) test during 1998.

Sex/LA titer	Male*		Female*		Total	
	Tested	Positive (%)	Tested	Positive (%)	Tested	Positive (%)
	70	20 (28.6)	41	12 (29.7)	111	32 (28.8)
1:32	—	4 (20.0)	—	2 (16.7)	—	6 (18.8)
1:64	—	7 (35.0)	—	3 (25.0)	—	10 (31.2)
1:128	—	6 (30.0)	—	3 (25.0)	—	9 (28.1)
1:256	—	2 (10.0)	—	4 (33.3)	—	6 (18.8)
1:512	—	1 (5.0)	—	0 (0.0)	—	1 (3.1)
1:1,024	—	0 (0.0)	—	0 (0.0)	—	0 (0.0)

* No significant difference was observed ($P > 0.05$).

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J. Parasitol., 90(3), 2004, pp. 654–657
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Infectivity of Microsporidia Spores Stored in Seawater at Environmental Temperatures

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ABSTRACT: To determine how long spores of *Encephalitozoon cuniculi*, *E. hellem*, and *E. intestinalis* remain viable in seawater at environmental temperatures, culture-derived spores were stored in 10, 20, and 30 ppt artificial seawater at 10 and 20 C. At intervals of 1, 2, 4, 8, and 12 wk, spores were tested for infectivity in monolayer cultures of Madin Darby bovine kidney cells. Spores of *E. hellem* appeared the most robust, some remaining infectious in 30 ppt seawater at 10 C for 12 wk and in 30 ppt seawater at 20 C for 2 wk. Those of *E. intestinalis* were slightly less robust, remaining infectious in 30 ppt seawater at 10 and 20 C for 1 and 2 wk, respectively. Spores of *E. cuniculi* remained infectious in 10 ppt seawater at 10 and 20 C for 2 wk but not at higher salinities. These findings indicate that the spores of the 3 species of *Encephalitozoon* vary in their ability to remain viable when exposed to a conservative range of salinities and temperatures found in nature but, based strictly on salinity and temperature, can potentially remain infectious long enough to become widely dispersed in estuarine and coastal waters.

Fourteen species of microsporidia have been reported to infect humans (Kotler and Orenstein, 1999; Cali and Takvorian, 2003). Of these, *Encephalitozoon cuniculi*, *E. hellem*, *E. intestinalis*, and *Enterocytozoon bieneusi* are zoonotic, infecting domesticated animals (Deplazes et al., 1996; Mansfield et al., 1997; Breitenmoser et al., 1999; Mathis et al., 1999; Rinder et al., 2000; Buckholt et al., 2002; Fayer, Santin, and Trout, 2003) and wildlife (Hersteinsson et al., 1993; Mathis et al., 1996; Thomas et al., 1997; Sulaiman et al., 2003). *Encephalitozoon cuniculi* has been identified in wild and pet rabbits, wild rats and mice, dogs, cats, foxes, mink, and a variety of monkeys (Bryan and Schwartz, 1999; Deplazes et al., 2000). *Encephalitozoon hellem* and *E. hellem*-like mi-

crosporidia have been found in psittacine birds, budgerigar chicks, a wild yellow-streaked lory (Bryan and Schwartz, 1999; Deplazes et al., 2000), and have experimentally infected domesticated chickens (Fayer, Santin, Palmer et al., 2003). *Encephalitozoon intestinalis* spores have been reported from feces of farm animals in Mexico (dog, pig, cow, goat) (Bornay-Llinares et al., 1998). Although the actual routes of transmission are not known, it is possible that the infectious spore stage in urine or feces can contaminate surface waters used for recreation or drinking water (Sparfel et al., 1997; Dowd et al., 1998; Cotte et al., 1999; Fournier et al., 2000). Microscopic and molecular detection of spores in surface waters and circumstantial evidence of waterborne transmission has been reviewed by Bryan and Schwartz (1999). Under experimental conditions, spores of *E. cuniculi*, *E. hellem*, and *E. intestinalis* stored in water at environmental temperatures ranging from 10 to 30 C remained infectious long enough to become widely dispersed if exposed to similar conditions in the environment (Li et al., 2003). For example, at 10 C, spores of *E. intestinalis* were still infectious after 12 mo, whereas those of *E. hellem* and *E. cuniculi* were infectious for 9 and 3 mo, respectively. At 30 C, the former 2 species were infectious for 3 wk and 1 mo, respectively, and the latter species for 1 wk. Little is known of how long microsporidians remain infectious in seawater. On the basis of artificially induced filament extrusion from spores of the microsporidian fish parasite *Loma salmonae*, a decrease was found after storage in seawater, suggesting that spores lost viability (Shaw et al., 2000). Using a similar experimental design as that of Li et al. (2003), the present study was conducted to determine the effect of salinity and temperature on longevity of spores of zoonotic species of *Encephalitozoon* that might be found in estuaries and coastal marine

TABLE I. Viability of *Encephalitozoon hellem* spores held in 0, 10, 20, and 30 ppt of seawater at 10 and 20 C for 1, 2, 4, 8, and 12 wk, as determined by observation of clusters of proliferating intracellular parasites in Madin Darby bovine kidney cells. (0, 0 clusters; 1, 1–10 clusters; 2, 11–100 clusters; 3, 101 + clusters.)

TP*	Positive control 5 C	0 ppt, 10 C	10 ppt, 10 C	20 ppt, 10 C	30 ppt, 10 C	0 ppt, 20 C	10 ppt, 20 C	20 ppt, 20 C	30 ppt, 20 C
1	3, 2, 3† (TP = 0)	2, 2, 2	2, 2, 2	2, 1, 1	2, 2, 2	2, 2, 1	2, 1, 1	2, 1, 1	1, 1, 1
2	3, 3, 2 (TP = 12)	2, 2, 2	2, 2, 2	2, 2, 2	2, 2, 2	2, 2, 1	1, 1, 1	1, 0, 0	1, 0, 0
4		2, 1, 1	2, 1, 1	1, 1, 1	2, 1, 1	1, 1, 1	1, 1, 0	1, 0, 0	0, 0, 0
8		1, 1, 1	1, 1, 0	1, 0, 0	1, 1, 1	1, 1, 0	1, 0, 0	0, 0, 0	0, 0, 0
12		1, 1, 1	2, 1, 1	1, 1, 0	1, 0, 0	1, 1, 0	0, 0, 0	0, 0, 0	0, 0, 0

* TP, time period (number of weeks spores were held before in vitro viability testing).

† Number of clusters of proliferating parasites within each of 3 wells of MDBK cells.

TABLE II. Viability of *Encephalitozoon intestinalis* spores held in 0, 10, 20, and 30 ppt of seawater at 10 and 20 C for 1, 2, 4, 8, and 12 weeks, as determined by observation of clusters of proliferating intracellular parasites in Madin Darby bovine kidney cells. (0, 0 clusters; 1, 1–10 clusters; 2, 11–100 clusters; 3, 101 + clusters.)

TP*	Positive control	0 ppt, 10 C	10 ppt, 10 C	20 ppt, 10 C	30 ppt, 10 C	0 ppt, 20 C	10 ppt, 20 C	20 ppt, 20 C	30 ppt, 20 C
1	3, 2, 2† (TP = 0)	2, 2, 2	2, 2, 1	2, 1, 1	1, 1, 1	2, 1, 1	2, 2, 1	1, 0, 0	1, 0, 0
2	2, 2, 2 (TP = 12)	2, 2, 1	2, 1, 1	1, 1, 1	0, 0, 0	2, 1, 1	1, 1, 0	1, 0, 0	1, 0, 0
4		1, 1, 1	1, 1, 1	1, 0, 0	0, 0, 0	1, 1, 1	1, 0, 0	1, 1, 0	0, 0, 0
8		1, 1, 1	2, 1, 0	1, 1, 0	0, 0, 0	1, 1, 1	1, 0, 0	0, 0, 0	0, 0, 0
12		1, 1, 1	1, 0, 0	1, 0, 0	0, 0, 0	1, 1, 1	1, 1, 0	0, 0, 0	0, 0, 0

* TP, time period (number of weeks spores were held before in vitro viability testing).

† Number of clusters of proliferating parasites within each of 3 wells of MDBK cells.

waters used for recreation and shellfish harvesting. Knowledge of the effect of salinity and temperature on infectivity of microsporidia in seawater is necessary for evaluating the risk of waterborne contamination.

Many isolates of *E. cuniculi*, *E. hellem*, and *E. intestinalis* have been propagated in vitro in many types of cells (Visvesvara, 2002). In the present study, spores were obtained and propagated as described previously (Li et al., 2003). Briefly, *E. cuniculi* and *E. intestinalis* were propagated in monolayer cultures of Madin Darby bovine kidney (MDBK) cells, and *E. hellem* was propagated in human lung fibroblasts (WI-38). MDBK cells were cultured in Dulbecco modified Eagle medium supplemented with 1% nonessential amino acids, 2% *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid, 5% fetal calf serum (FCS), and 1% penicillin–streptomycin in a 5% CO₂ atmosphere at 35 C. WI-38 cells were similarly cultured, but in minimum essential medium with 10% FCS, as well as 1% L-glutamine, and 1% sodium pyruvate. Spores harvested from culture supernatant by centrifuging at 1,500 *g* for 15 min were resuspended in deionized water, stained with calcofluor white (Becton Dickinson Microbiology Systems, Sparks, Maryland), pipetted into a well of a Teflon-coated 3-well glass microscope slide (Cel-Line, Erie Scientific, Portsmouth, New Hampshire), and counted with the aid of an epifluorescence microscope. For each species, morphologic features and staining intensity appeared uniform,

indicating that the forms examined were spores. Spores of each species were pipetted at 1.5×10^5 spores per tube into 40 microcentrifuge tubes, centrifuged, and the pellets resuspended in artificial seawater at concentrations of 10, 20, and 30 ppt salinity or in deionized water (Tables I–III). Seawater was constituted from Forty Fathoms Crystal Sea Marine Mix (Marine Enterprises International, Inc., Baltimore, Maryland) dissolved in deionized water. Spores suspended in deionized water served as controls. Tubes were capped and held in either of 2 circulating water baths at 10 and 20 C that were monitored for temperature twice daily, except on weekends. At 1, 2, 4, 8, and 12 wk, 1 tube for each species of microsporidia at each concentration of salinity was removed from each water bath, spores were aspirated from that tube, and 5×10^4 spores were inoculated into each of the 3 wells of an 8-well Lab-Tek chamber slide (Nalge Nunc Intl., Naperville, Illinois), each well containing a monolayer of MDBK cells. After 4 days incubation at 35 C in a 5% CO₂ atmosphere, the culture medium was decanted, wells were flooded with 100% methanol for 30 min, and slides were air dried. After removing the plastic frame and silicon gasket that formed the wells, each slide was stained by the quick-hot gram-chromotrope method (Moura et al., 1997), a coverslip was affixed, and the entire area of cells within each well was examined by brightfield microscopy. Spores were considered viable and infectious on finding intracellular clusters of pro-

TABLE III. Viability of *Encephalitozoon cuniculi* spores held in 0, 10, 20, and 30 ppt of seawater at 10 and 20 C for 1, 2, 4, 8, and 12 weeks, as determined by observation of clusters of proliferating intracellular parasites in Madin Darby bovine kidney cells. (0, 0 clusters; 1, 1–10 clusters; 2, 11–100 clusters; 3, 101 + clusters.)

TP*	Positive control	0 ppt, 10 C	10 ppt, 10 C	20 ppt, 10 C	30 ppt, 10 C	0 ppt, 20 C	10 ppt, 20 C	20 ppt, 20 C	30 ppt, 20 C
1	2, 2, 2† (TP = 0)	2, 2, 2	1, 1, 1	0, 0, 0	0, 0, 0	1, 1, 1	1, 1, 0	0, 0, 0	0, 0, 0
2	2, 2, 2 (TP = 12)	1, 1, 1	1, 1, 0	0, 0, 0	0, 0, 0	1, 1, 1	1, 0, 0	0, 0, 0	0, 0, 0
4		1, 1, 1	0, 0, 0	0, 0, 0	0, 0, 0	1, 1, 0	0, 0, 0	0, 0, 0	0, 0, 0
8		1, 1, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
12		1, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0

* TP, time period (number of weeks spores were held before in vitro viability testing).

† Number of clusters of proliferating parasites within each of 3 wells of MDBK cells.

liferating microsporidia. The number of clusters in each well was counted, and the numbers 0, 1, 2, and 3 were assigned to represent counts of 0, 1–10, 11–100, and 101 or more clusters per well, respectively (Tables I–III). At the onset (time 0) and termination (12 wk) of the study, 5×10^4 spores of each species held as positive controls in deionized water at 5 C were also pipetted into each of 3 wells containing MDBK cell monolayers, then processed and examined in the same manner as wells that received spores stored in artificial seawater.

For spores of all 3 species held in deionized water at 10 and 20 C compared with those held in deionized water at 5 C for 12 wk, infectivity decreased both with elevated temperatures and length of storage time (Tables I–III). At all time periods for all species at both 10 and 20 C (with 2 exceptions), spores held in seawater were less infectious than those held in deionized water (Tables I–III), indicating a negative effect on infectivity from elevated salinity alone. The degree to which spores were affected was species dependent. Some spores of *E. hellem* remained infectious at 30 ppt at 10 and 20 C for 12 and 2 wk, respectively, fewer spores of *E. intestinalis* remained infectious at 30 ppt at 10 and 20 C for 1 and 2 wk, and spores of *E. cuniculi* remained infectious at only 10 ppt at 10 and 20 C for 2 wk.

Spores of microsporidia have been detected in a variety of surface waters (Avery and Undeen, 1987; Dowd et al., 1998), and water as a source of human infections has been implied from epidemiological data (Cotte et al., 1999), but information is lacking on the presence of and survival in seawater of microsporidia infectious to humans and other mammals. General interest in survival of microsporidian spores dates back nearly 90 yr, with most efforts to determine the effects of time versus temperature on the viability of spores of *Nosema apis*, a microsporidian parasite of bees, held in water at various temperatures (White, 1919; Revell, 1960; Kramer, 1970; Bailey, 1972; Vavra and Maddox, 1976; Malone et al., 2001) or the mammalian microsporidia *E. hellem* and *E. intestinalis* (Kucerova-Pospisilova et al., 1999; Li et al., 2003) and *E. cuniculi* (Shaddock and Polley, 1978; Waller, 1979; Koudela et al., 1999; Kucerova-Pospisilova et al., 1999; Li et al., 2003) held in water or culture medium. The only study to determine the effect of seawater on spores of microsporidia was that of Shaw et al. (2000), who examined the microsporidian fish parasite *L. salmonae*.

Infection with all microsporidia begins when the polar filament extruded from the spore forms a tube through which the sporoplasm passes into a host cell (Vavra and Larsson, 1999). This process of germination can be artificially induced. However, it is technique dependent, and polar filaments can fail to extrude from spores that are potentially infectious or can extrude from spores that lack infectivity. Shaw et al. (2000) examined the germination rate of *L. salmonae* and found that it decreased from 51 to 0% after 100 days storage at 4 C, suggesting that spores lost viability, although after 95 days, infectivity for fish appeared not to be diminished. Germination was induced in spores of *E. intestinalis*, *E. hellem*, and *E. cuniculi* stored in culture medium at 4 C for 48 mo, and the microscopic appearance of intact versus recently germinated spores versus those that had lost extruded polar filaments was reported (Kucerova-Pospisilova et al., 1999). When spores of *E. cuniculi*, *E. hellem*, and *E. intestinalis* stored in deionized water at elevated temperatures for 2, 8, and 10 mo were examined by DIC microscopy and chromotrope-stained spores were examined by brightfield microscopy, no extruded filament was detected despite the fact that other spores stored under the same conditions were infectious to cultured mammalian cells (Li et al., 2003). These findings suggested that factors other than extrusion of the filament were involved in the loss of infectivity (Li et al., 2003). On the basis of those findings, spores in the present study were not examined for polar filament extrusion but were considered infectious based solely on their ability to actually invade and multiply within cultured mammalian cells.

The present study has demonstrated that as the temperature of storage in deionized water increased from 5 to 20 C, infectivity of microsporidian spores decreased and as salinity increased from 0 to 30 ppt, infectivity of microsporidian spores decreased. At the highest level of salinity (30 ppt) at both 10 and 20 C, spores of *E. hellem* were more robust, i.e., remained infectious longer or more were infectious for longer periods, than those of *E. intestinalis*, which were more robust than those of *E. cuniculi*, indicating species differences with respect to the effects of salinity. These findings suggest that spores of *E. hellem* and *E. intestinalis* could potentially remain infectious in estuarine and ocean waters for weeks and those of *E. cuniculi* could remain infectious in

low-salinity estuarine waters for weeks, which is sufficient to infect humans and marine mammals or to contaminate shellfish.

The technical assistance of Robert Palmer is gratefully acknowledged.

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J. Parasitol., 90(3), 2004, pp. 657–659
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Ticks (Acari: Ixodidae) Parasitizing Wild Carnivores in Phu Khieo Wildlife Sanctuary, Thailand

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ABSTRACT: Ixodid ticks were collected and identified from 8 wild carnivore species in Phu Khieo Wildlife Sanctuary, northeastern Thailand. Six tick species belonging to 4 genera were recovered and identified from 132 individuals. These included *Amblyomma testudinarium* (n = 36), *Haemaphysalis asiatica* (n = 58), *H. hystrix* (n = 31), *H. semeris* (n = 3), *Rhipicephalus haemaphysaloides* (n = 3), and *Ixodes granulatus* (n = 1). Leopard cats (*Prionailurus bengalensis*) (n = 19) were infested with 4 tick species, whereas yellow-throated marten (*Martes flavigula*) (n = 4), clouded leopard (*Neofelis nebulosa*) (n = 2), and dhole (*Cuon alpinus*) (n = 1) were infested with 3 tick species, Asiatic golden cat (*Catopuma temminckii*) (n = 2) with 2 species, and marbled cat (*Pardofelis marmorata*), binturong (*Arctictis binturong*), and large Indian civet (*Viverra zibetha*) each infested with 1 species. This information contributes to the knowledge available on the ectoparasites of wild carnivores in Southeast Asia.

The collection and identification of ectoparasites from wild carnivores in North America and Europe is well documented in the literature, including host species such as raccoon (*Procyon lotor*) (Sonenshine and Stout, 1971; Rhodes and Norment, 1979; Whitaker and Goff, 1979;

Brillhart et al., 1994; Pung et al., 1994), striped skunk (*Mephitis mephitis*) (Durden and Richardson, 2003), coyote (*Canis latrans*) (Eads, 1948; Pence et al., 1981), red fox (*Vulpes vulpes*) (Aubert, 1975; Tou-toungi et al., 1991), and river otter (*Lutra canadensis*) (Eley, 1977). Tick research in Southeast Asia has mainly covered tick identification, distribution, and disease transmission (Toumanoff, 1944; Petney and Keirans, 1994, 1995, 1996; Voltzit and Keirans, 2002), a checklist of Thai ticks (Tanskul et al., 1983), and a tick survey of Malaysian carnivores and other mammals (Hoogstraal and Wassef, 1984). Except for research on the endoparasites of Thai wild cats (Patton and Rabinowitz, 1994), parasitological research on wild carnivores in Thailand remains largely unstudied. Our objective was to add information about the tick–host relationships of carnivores from Southeast Asia.

Ticks were collected as part of an ecological study of carnivores in northeastern Thailand (Grassman, 2004). Situated in Chaiyaphum Province (16°5′–16°35′N, 101°20′–101°55′E), Phu Khieo Wildlife Sanctuary (PKWS) is a large, 1,560-km² evergreen forest dominated by an approximately 1,000-m elevation plateau (Anonymous, 2000). Carnivores were livetrapped from October 1998 to October 2002. Captured carnivores were anesthetized for physical examination and to attach a radio

TABLE I. Wild carnivore hosts and ticks from Phu Khieo Wildlife Sanctuary, Thailand, October 1998 to October 2002.

Host	No. of carni- vores	No. of tick species	Tick species* and stage†
<i>Prionailurus bengalensis</i>	19	4	1 ^{LN} , 2 ^{NA} , 5 ^A , 6 ^A
<i>Martes flavigula</i>	4	3	1 ^N , 3 ^{LNA} , 4 ^A
<i>Neofelis nebulosa</i>	2	3	2 ^A , 3 ^A , 4 ^A
<i>Cuon alpinus</i>	1	3	1 ^{NA} , 2 ^N , 4 ^A
<i>Catopuma temminckii</i>	2	2	1 ^N , 2 ^A
<i>Pardofelis marmorata</i>	1	1	3 ^N
<i>Arctictis binturong</i>	1	1	4 ^A
<i>Viverra zibetha</i>	1	1	1 ^{LN}

* 1, *Amblyomma testudinarium*; 2, *Haemaphysalis asiatica*; 3, *H. hystricis*; 4, *Rhipicephalus haemaphysaloides*; 5, *H. semermis*; and 6, *Ixodes granulatus*.

† L, larvae; N, nymphs; A, adults.

collar. During examination, attached and unattached ticks were removed by forceps and placed in plastic vials containing 70% ethanol. Tick specimens were stored at room temperature ≤ 4 yr before identification. Parasites were identified by the Parasitology Section of the National Institute of Animal Health, Bangkok, Thailand. Ticks were examined by microscope and identified according to the methods of Yamaguti et al. (1971a, 1971b), Tanskul and Inlao (1989), and Walker et al. (2000).

Eight carnivore species (31 individuals) were captured and examined for ticks. Hosts examined included clouded leopard (*Neofelis nebulosa*) (n = 2), Asiatic golden cat (*Catopuma temminckii*) (n = 2), marbled cat (*Pardofelis marmorata*) (n = 1), leopard cat (*Prionailurus bengalensis*) (n = 19), dhole (*Cuon alpinus*) (n = 1), yellow-throated marten (*Martes flavigula*) (n = 4), binturong (*Arctictis binturong*) (n = 1), and large Indian civet (*Viverra zibetha*) (n = 1). All ticks (n = 132) were identified and classified into 6 species: *Amblyomma testudinarium* (n = 36), *Haemaphysalis asiatica* (n = 58), *H. hystricis* (n = 31), *H. semermis* (n = 3), *Rhipicephalus haemaphysaloides* (n = 3), and *Ixodes granulatus* (n = 1) (Table I). *Haemaphysalis asiatica* was identified the most frequently (43.6%), followed by *A. testudinarium* (27.1%); however, *A. testudinarium* was found on more hosts (5) than *H. asiatica* (4).

No record exists of ixodid ticks parasitizing clouded leopard and marbled cat. In this study, we found *H. asiatica*, *H. hystricis*, and *R. haemaphysaloides* infesting clouded leopards and *H. hystricis* from a marbled cat. Leopard cats were parasitized by *A. testudinarium*, *H. asiatica*, *H. semermis*, and *I. granulatus*. Similarly, ticks parasitizing leopard cats from Thailand recorded by Tanskul et al. (1983) included *H. asiatica*, *I. granulatus*, and *I. ovatus*. Other parasite records of leopard cats from Nepal included *I. ovatus* (Hoogstraal et al., 1973) and *I. acutitarsus* (Clifford et al., 1975). The occurrence of *H. asiatica* and *A. testudinarium* identified from the Asiatic golden cat is similar to ticks found on a golden cat in Laos. Robbins et al. (1997) identified *H. asiatica*, *R. haemaphysaloides*, and *Amblyomma* sp. from a single Asiatic golden cat.

Tanskul et al. (1983) recorded *H. bispinosa* and *H. koningsbergeri* from a Thai binturong and *H. asiatica* and *I. ovatus* from a large Indian civet. We identified *R. haemaphysaloides* from a binturong and *A. testudinarium* from a large Indian civet. One record of tick parasitism on dhole from Nepal was identified as *I. ovatus* (Hoogstraal et al., 1973). Three tick species were identified from a dhole in PKWS, i.e., *A. testudinarium*, *H. asiatica*, and *R. haemaphysaloides*. Two records exist for the yellow-throated marten. Clifford et al. (1975) identified *I. tanuki*, and Mitchell (1979) identified *R. haemaphysaloides* and *I. tanuki* from this mustelid in Nepal. We identified 3 species of ticks from yellow-throated martens, i.e., *A. testudinarium*, *H. hystricis*, and *R. haemaphysaloides*.

This study contributed new information on the host-tick fauna of Southeast Asia. Additional research is needed on host-tick identification, distribution, and disease agent transmission.

Voucher specimens of ticks were deposited at the Ohio State Museum of Biological Diversity (Columbus, Ohio). Accession numbers: (*Haemaphysalis asiatica*) OSAL 003360 (4 specimens), (*H. hystricis*) OSAL

003361 (4 specimens), (*H. semermis*) OSAL 003362 (2 specimens), (*Rhipicephalus haemaphysaloides*) OSAL 003363 (2 specimens), (*Ixodes granulatus*) OSAL 003364 (1 specimen), and (*Amblyomma testudinarium*) OSAL 003365 (4 specimens).

This study was supported by the Bosack and Kruger Foundation through the Cat Action Treasury. Support also was provided by the Caesar Kleberg Wildlife Research Institute at Texas A&M University-Kingsville, Sierra Endangered Cat Haven, Hexagon Farm, Parco Faunistica La Torbiera, Columbus Zoo, Point Defiance Zoo, and Mountain View Farms Conservation Breeding Centre. Research permission was granted by the National Research Council of Thailand (#0004.3/0301) and Royal Forest Department of Thailand. This project was part of the Joint Ph.D. Program between Texas A&M University-Kingsville and Texas A&M University, College Station. Research was approved by the TAMUK Institutional Animal Care and Use Committee (#2003-8-12). This is publication #04-101 of the Caesar Kleberg Wildlife Research Institute. We thank Dr. Hans Klompen of the Museum of Biological Diversity, Ohio State University, for cataloging our tick specimens.

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J. Parasitol., 90(3), 2004, pp. 659–660
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First Report of the Giant Kidney Worm (*Diectophyme renale*) in a Harbor Seal (*Phoca vitulina*)

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ABSTRACT: A male harbor seal (*Phoca vitulina*) was found moribund on the coast of New Jersey in January of 2003 and died a few hours later in the Marine Mammal Stranding Center. On necropsy, a single female *Diectophyme renale* was recovered from the peritoneal cavity, and a tissue mass was found adjacent to the pelvic urethra and urinary bladder. Within this tissue mass were found *D. renale* ova. This is the first report of this nematode in the harbor seal and in a North American marine mammal.

An 18.9-kg male harbor seal (*Phoca vitulina*) was found stranded at Barnegat Lighthouse State Park in New Jersey (latitude 39.76, longitude 74.10) in January 2003. At time of capture the animal had labored breathing and what appeared to be blood was present on the abdominal skin. The animal was treated at the Marine Mammal Stranding Center in Brigantine, New Jersey with 500 ml electrolyte solution, vitamins, and Baytril. The seal was dewormed with 92 mg of levamisole about 6.5 hr before death. The seal was inactive and shivering. The animal died approximately 7 hr after arriving at the stranding center.

At necropsy the urinary bladder was severely distended. It contained watery red fluid that had a strong odor of ammonia. The mucosa was also reddened with a coarsely roughened surface. The ureters were distended measuring 4 mm in diameter bilaterally, but both kidneys were grossly normal. A firm swelling, measuring 5.6 × 2.6 × 1.8 cm was present adjacent to the pelvic urethra, extending from the trigone of the urinary bladder to the caudal margin of the pelvic bone. The swelling contained a central cavity measuring 4.4 × 1.2 × 0.6 cm that contained thick opaque fluid. Aerobic cultures of this fluid were negative. The cavity did not communicate with the urethra. Microscopically the wall of the cavitated structure consisted of dense fibrous connective tissue with scattered small bundles of skeletal muscle near the outer surface. Numerous ova were present within multifocal to coalescing inflammatory foci. The ova had a double-contoured shell with a mammillated surface (Fig. 1). They contained central granular hypereosinophilic material and occasionally a single nucleus. Some ova were pale, staining with disrupted and collapsed shells (Fig. 1). Inflammatory cells consisted of large numbers of epithelioid macrophages and neutrophils with fewer multinucleated giant cells, lymphocytes, and plasma cells. The urinary bladder had severe necrotizing inflammation with fibrinoid necrosis of blood vessels and numerous surface bacteria. The kidneys had

mild multifocal nonsuppurative interstitial nephritis. A large nematode was found adjacent to the rectum in the retroperitoneal space. Parasite eggs were not present in the kidney or the urinary bladder.

Other necropsy findings included numerous lice on the skin of the dorsum (identified as *Echinophthirius horridus*), chronic ulcerative dermatitis, and cheilitis. Dilated lymphatic vessels were present on the pleural surfaces of the lung. The stomach contained a 2-cm-long white nematode identified as an anisakine. Additional histologic findings included mild multifocal acute encephalitis of the cerebellum and medulla, scattered pulmonary granulomatous and eosinophilic inflammation, and ulcerative gingivitis and dermatitis with intracytoplasmic amorphous inclusions in epithelial cells.

The nematode recovered from the peritoneal cavity was a female, brownish-red in color, and measured 22.3 cm long by 4.5 mm wide. The worm was alive when recovered (about 1 day after the death of the seal). The anus was terminal, and the mouth was surrounded by 2 circles of 6 papillae. This led to its identification as *Diectophyme renale*.

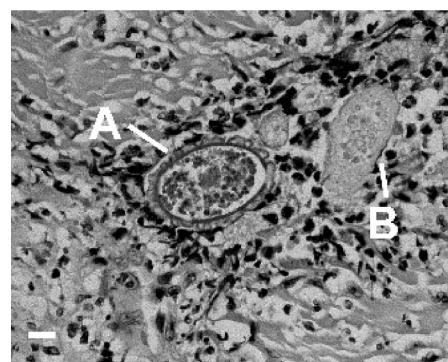


FIGURE 1. Histological section of the mass found in the peritoneal cavity of the harbor seal. Viable (A) and degenerate (B) *Diectophyme renale* ova are surrounded by neutrophils and macrophages. Hematoxylin and eosin. Bar = 20 µm.

Eggs recovered from the sediment of the vessel in which the worm was fixed and those seen in histological sections were consistent with this identification (Fig. 1). Ten eggs recovered from the formalin were measured with an ocular micrometer, and their mean size ($\pm$ SD) was $41.3 \pm 2.3 \mu\text{m}$ wide by $68.9 \pm 3.7 \mu\text{m}$ long.

At necropsy, dioctophymosis is frequently diagnosed by finding the adult worm, *D. renale*. In biopsy or cytology material, diagnosis can be definitively made by identifying the mammillated eggs in tissues or urine. Because ova in the seal were found in the wall of the periurethral abscess, it is probable that the worm was residing there after it had migrated into the peritoneal cavity. The intrapelvic lesion could have acted as a functional obstruction leading to the distended and inflamed urinary bladder and hydroureter. The resulting bloody urine lead to the initial observation of bloody fluid on the abdominal skin.

The usual definitive host for *D. renale* is the mink, although other mustelids and canids are commonly infected. Adult worms are normally found in the right kidney. They are blood-red, and females can measure up to 103 cm long (Measures, 2001). Eggs pass out in the urine, mature to the first stage (within the egg) in about 35 days, after which they are infectious to *Lumbriculus variegatus*, a freshwater oligochaete. Fish or frogs that ingest the infected oligochaete may act as paratenic hosts. Lesions usually associated with *D. renale* in mink include destruction of the right kidney. Renal lesions include dilated pelvises, fibrosis, tubular atrophy, and chronic inflammation. The worms can survive for years in the natural host without evidence of clinical signs. If the left kidney is also infected or if there is migration into the peritoneal cavity, the animal can die from renal failure or peritonitis, respectively (Mace, 1976; Dyer, 1998).

Immature *D. renale* have been reported in the body cavities of Caspian Seals (*Phoca caspica*) from the Caspian Sea. (Popov and Taikov, 1985), but this communication is the first report of this parasite in a harbor seal and the first report in a North American marine mammal. Because the life cycle of this parasite takes place in freshwater, the seal must have been infected while feeding in freshwater. Harbor seals are known to venture up freshwater rivers, and some populations stay in freshwater habitats (Baird, 2001).

Cases of *D. renale* in the peritoneal cavities of its host are not unusual

(Mace, 1976; de Souza Junior and de Padua, 1977; Celerin and McMullen, 1981), however, the eggs laid by such worms cannot pass out of the host to complete the life cycle. It is not known if the eggs recovered from this seal were fertile (unfertilized females will lay eggs). No male was found, and although the seal had been treated with levamisole 1 day before its death, any dead worms would still be intact. Fertile eggs of *D. renale* passing in the urine of naturally infected hosts measure 45–47 wide by 73–83 long (Measures, 2001), slightly larger than the formalin-fixed eggs recovered from this worm.

We wish to acknowledge the help of the staff of the Marine Mammal Stranding Center, the staff of the Pathology Laboratory at the University of Pennsylvania School of Veterinary Medicine, and Dr. Lena Measures for a translated copy of Popov and Taikov's report.

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J. Parasitol., 90(3), 2004, pp. 660–663
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Localization of a 56-kDa Antigen That is Present in Multiple Developmental Stages of *Neospora caninum*

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ABSTRACT: The purpose of the present study was to characterize the intracellular distribution of a native *Neospora caninum* 56-kDa protein that is recognized by sera from *N. caninum*-infected dairy cattle. The complementary DNA coding for this protein was expressed in *Escherichia coli* as a polyHis fusion protein to which antiserum was prepared and used to localize the antigen in *N. caninum* tachyzoites and bradyzoites. By sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunoblotting, antirecombinant Nc56 serum recognized a major 56-kDa protein and 2 minor (43 and 39 kDa) proteins of *N. caninum* tachyzoites. Antiserum to recombinant 56-kDa protein showed this antigen to be present in both *N. caninum* tachyzoites and bradyzoites/cysts as detected by immunofluorescence staining. Immunoelectron microscopy revealed the 56-kDa antigen to be present in the apical end of both tachyzoites and bradyzoites and possibly extracellularly secreted by tachyzoites.

Prevention of neosporosis in dairy cattle may rely on drug therapy or vaccination to inhibit reactivation of *Neospora caninum* during preg-

nancy (Dubey, 1999). Several research groups have identified gene sequences coding for parasite enzymes on the surface or associated with intracellular organelles. DNA sequences for nucleotide triphosphate hydrolase (Asai et al., 1998) and a subtilisinlike serine protease (Nc-p65; Louie and Conrad, 1999) have been reported. Gene sequences have also been described for surface antigens (NcSAG1 [Nc-p36], Hemphill, Fuchs et al., 1997; NcSRS2 [Nc-p43], Hemphill, Felleisen et al., 1997) and proteins associated with micronemes (NcMIC2, Lovett et al., 2000; NcMIC3, Naguleswaran et al., 2001; NcMIC1, Keller et al., 2002) and dense granules (NcGRA7, Lally et al., 1997; NcGRA6, Liddell et al., 1998; NcGRA2, Ellis et al., 2000). In the present study, a complementary DNA (cDNA) coding for a recombinant *N. caninum* antigen that is related to a native 56-kDa protein located in the apical end of *N. caninum* tachyzoites and bradyzoites is described.

A *N. caninum* tachyzoite cDNA library prepared in UNIZAP-XR vector (Stratagene, La Jolla, California) was immunoscreened with rabbit sera specific for a native 56-kDa *N. caninum* protein. Antinative Nc56 serum was prepared by excising a horizontal strip of nitrocellulose

membrane containing sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE)–fractionated whole *N. caninum* tachyzoite protein corresponding to a 56-kDa protein that was identified by sera from *N. caninum*-infected cattle. The nitrocellulose strip was minced into small pieces, mixed with ImmunoMax SR adjuvant (Zonagen Inc., The Woodlands, Texas), and injected into 2 female New Zealand white rabbits (Covance, Denver, Pennsylvania) using an 18-gauge needle and 1.0-ml syringe. The rabbits received a booster immunization with the native Nc56-impregnated nitrocellulose membrane at 1-mo postprimary immunization and were bled for serum 2 wk later.

Using an in vivo excision protocol, pBluescript plasmid harboring the Nc56 cDNA was prepared and subjected to dideoxy sequencing using BigDye terminators and analysis on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, California). The cDNA was excised from pBluescript by restriction enzyme digestion and inserted into the pBAD-His expression vector (Invitrogen, Carlsbad, California). DNA sequencing of the recombinant pBAD-His–Nc56 was conducted to ensure that Nc56 was cloned in-frame with the pBAD-His open-reading frame (ORF). Recombinant polyHis–Nc56 protein was expressed by 0.2% arabinose induction of pBAD-His–Nc56-transformed *Escherichia coli* DH5 at midlog growth at 37 C. Recombinant protein was extracted under denaturing conditions (3 M urea), and soluble Nc56 protein was purified by NiNTA affinity chromatography following manufacturer's instructions (Invitrogen). NiNTA-purified Nc56 was emulsified in ImmunoMax SR adjuvant (Zonagen) and used for intramuscular injection into 2 female New Zealand white rabbits (Covance, 1 µg/rabbit). The rabbits received a booster immunization with the same amount of purified antigen at 1-mo postprimary immunization and were bled for serum 2 wk later.

Northern blot hybridization was conducted to estimate the size of the Nc56 messenger RNA transcript. *Neospora caninum* tachyzoite RNA was prepared using Trizol reagent and procedures recommended by the manufacturer (Invitrogen). RNA was suspended in diethyl pyrocarbonate–treated H₂O. The RNA concentration was estimated by measuring absorbance at optical density 260/280. RNA (20 µg) was mixed with deionized formamide and formaldehyde, heat denatured for 5 min at 65 C, chilled on ice, mixed with RNA sample buffer, and electrophoresed in 1× TBE at 7.5 V/cm on 1.2% agarose containing 20 mM guanidine thiocyanate using described procedures (Goda and Minton, 1995). RNA molecular weight markers (New England Biolabs, Beverly, Massachusetts) were denatured in the same manner and electrophoresed in a separate well to allow for size estimation of the hybridizing RNA band. After electrophoresis, the RNA was transferred overnight to Nylon membrane (Roche, Indianapolis, Indiana) using a Turboblotter (Schleicher and Schuell, Keene, New Hampshire) and exposed to 1,200 µJ UV light in a Stratilinker (Stratagene). The Northern blot was treated with blocking reagent (Roche) for 1 hr at 50 C and then probed for 16 hr with digoxigenin (DIG)–labeled Nc56 insert DNA. DIG labeling was performed using Nc56 bacteriophage DNA and the DIG PCR-probe kit following manufacturer's instructions (Roche). After hybridization, the blots were washed 2 times at room temperature (RT) with 2.0× standard saline citrate (SSC) and 0.1% SDS and 2 times at 50 C with 0.2× SSC and 0.1% SDS. The blots were washed with maleic acid buffer, treated with blocking reagent, and then incubated overnight with a 1:25,000 dilution of alkaline phosphatase–labeled anti-DIG reagent (Roche). The blots were washed 3 times with maleic acid wash buffer, once with alkaline phosphatase buffer, treated with CDP-Star substrate (Roche), and analyzed using EpiChemII Darkroom and LabWorks Software (UVP Inc., Upland, California).

To estimate the size of Nc56-related *N. caninum* proteins, NC-1 tachyzoites were harvested and purified from cell culture using described procedures (Bjerkas et al., 1994). *Neospora caninum* tachyzoite total protein was extracted as described (Bjerkas et al., 1994), treated with sample buffer with or without 2-mercaptoethanol, size-fractionated by SDS-PAGE, and transferred to Immobilon (Millipore, Bedford, Massachusetts) on a semidry blotter (BioRad, Hercules, California). The blots were treated with phosphate-buffered saline (PBS) containing 2% nonfat dry milk (NFDm) for 1 hr at RT, followed by incubation for 2 hr at RT with a 1:500 dilution of rabbit anti-Nc56 or negative control serum. The blots were then incubated for 1 hr with 10 µg/ml biotinylated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Missouri), for 1 hr with 10 µg/ml avidin-peroxidase (Sigma), followed by perox-

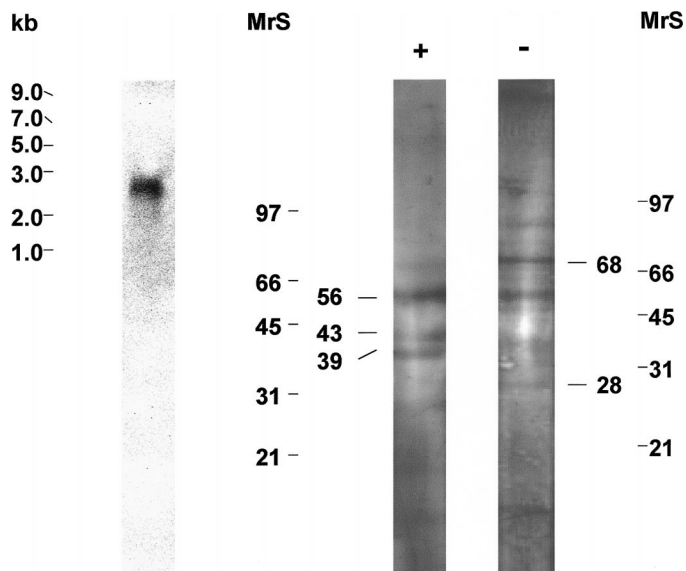


FIGURE 1. Characterization of Nc56 transcript and protein in *Neospora caninum* tachyzoites. Left panel, Northern blot hybridization of DIG-labeled Nc56 DNA to *N. caninum* tachyzoite RNA size-fractionated on a guanidine thiocyanate agarose gel; kb, RNA molecular size standards. Right panel, immunoblotting analysis of *N. caninum* tachyzoite protein probed with antisera to recombinant Nc56 protein. +, reducing (+2-mercaptoethanol) SDS-PAGE; –, nonreducing (–2-mercaptoethanol) SDS-PAGE; MrS, molecular weight markers.

idase substrate 4-chloro-1-naphthol (Sigma). The blots were washed 3 times for 5 min per wash between each incubation step.

For immunofluorescence antibody (IFA) staining, *N. caninum* tachyzoites were dried onto multiwell slides and then treated for 1 hr with PBS-NFDM to block nonspecific immunoglobulin binding in subsequent steps. The slides were washed once with PBS, air-dried, incubated for 2 hr with a 1:100 dilution of rabbit antiserum against recombinant Nc56 protein, then incubated with a 1:50 dilution of fluorescein-labeled goat anti-rabbit IgG (H+L chain specific, Sigma) for 1 hr. The slides were washed 3 times with PBS and air-dried between each incubation step. After the last step, the slides were air-dried, overlaid with anti-bleaching mounting medium (Vector Laboratories, Burlingame, California) and a coverslip, and then examined under epifluorescence microscopy at ×400 magnification.

To localize Nc56 antigen, in vitro-cultured *N. caninum* tachyzoites or bradyzoites (Vonlaufen et al., 2002) were suspended in 0.1 M cacodylate buffer containing 3% paraformaldehyde and 0.5% glutaraldehyde for 10 min at RT. The fixed tachyzoites were washed 2 times with 0.1 M cacodylate buffer and pelleted by centrifugation. The parasites were dehydrated in a graded ethanol series, infiltrated overnight in LR White hard-grade acrylic resin (London Resin Company, London, U.K.), and cured at 55 C for 24 hr. Thin sections (90 nm thick) were obtained using a Diatome diamond knife on a Reichert/AO Ultracut microtome and collected on 200-mesh nickel grids. The grids were floated on drops of PBS containing 0.1 M glycine and 1% bovine serum albumin for 10 min, washed with PBS, floated on drops of PBS-NFDM-Tw20, and then floated on drops containing a 1:100 dilution of rabbit antiserum in PBS-NFDM-Tw20. The grids were incubated for 2 hr at RT, washed 3 times with PBS-NFDM-Tw20, and floated for 1 hr at RT on drops of a 1:50 dilution of gold particle (10-nm diameter)–labeled goat anti-rabbit IgG (H+L chain specific, Sigma). The grids were washed 3 times with PBS-Tw20, twice with deionized H₂O, air-dried, stained with 5% uranyl acetate for 30 min, and examined under a Hitachi H500H transmission electron microscope at 75 kV.

Immunoscreening *N. caninum* tachyzoite cDNA libraries with antisera specific for a native 56-kDa *N. caninum* tachyzoite protein revealed a 2,607-nucleotide (nt) DNA insert (GenBank AY340638). An ORF beginning at nt 3 and terminating at nt 1,004 coding for a predicted ~41-kDa protein was present. This ORF was in-frame with the lacZ

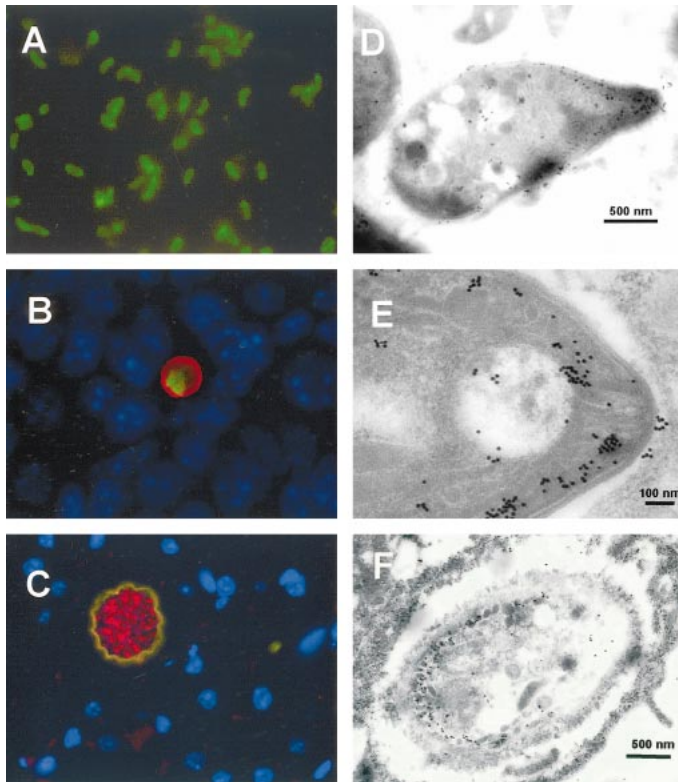


FIGURE 2. Immunofluorescence (A–C) or IEM (D–F) staining with antisera specific for recombinant Nc56 protein of in vitro-cultured *Neospora caninum* tachyzoites (A,D,E) or bradyzoites/cysts (B,F) or tissue cysts (C) from *N. caninum*-infected mice.

gene of UNIZAP-XR. BLAST-N searching of the DNA database revealed rNc56 to have 61.4% similarity to rat pyruvate carboxylase. BLAST-P searching of the protein database using the Nc56 ORF revealed a nearly 60% similarity to various eukaryotic pyruvate carboxylases. Northern blot hybridization of DIG-labeled Nc56 to *N. caninum* RNA revealed a single ~2.8-kb transcript (Fig. 1, left panel).

Expression of Nc56 cDNA from the pBAD-His expression vector revealed a 45-kDa protein, which is similar to the predicted size of rNc56 (41 + 4 kDa polyHis; M. Jenkins, unpubl. obs.). Antisera specific for rNc56 recognized a major 56-kDa and 2 minor 39- and 43-kDa *N. caninum* tachyzoite proteins by reducing SDS-PAGE and immunoblotting (Fig. 1, right panel). Anti-rNc56 sera recognized 2 proteins, 68 and 28 kDa, under nonreducing SDS-PAGE and immunoblotting. (Fig. 1, right panel). In nonreducing gels, a 50-kDa band was recognized by both pre- and postimmunization sera, indicating that this protein was probably not related to Nc56. The relatedness of recombinant Nc56 to the respective protein in *N. caninum* tachyzoites was supported by the reactivity of native Nc56 protein to purified polyHis-rNc56 (data not shown).

Anti-rNc56 sera recognized an antigen in the apical complex of in vitro-cultured *N. caninum* tachyzoites and bradyzoites (Fig. 2A, B) as well as tissue cysts from brains of *N. caninum*-infected mice (Fig. 2C). Similar to IFA results, immunoelectron microscopy (IEM) revealed the Nc56 antigen to be concentrated in the apical end of *N. caninum* tachyzoites and bradyzoites (Fig. 2D, F). The antigen was also observed extending toward the posterior end of the parasite. Some labeling was observed outside of tachyzoites, suggesting that Nc56 protein may be secreted during intracellular development (Fig. 2E).

In the present study, an *N. caninum* cDNA, designated as Nc56, with similarity to the gene for pyruvate carboxylase was cloned and expressed in *E. coli*. Antisera to purified recombinant Nc56 revealed that the respective native protein was present in both tachyzoite and bradyzoite/cyst stages of *N. caninum*. This antigen appears to be concentrated in the apical end of both tachyzoites and bradyzoites. Unlike other

apical complex antigens of *N. caninum* that have been described, Nc56 does not appear to be incorporated into the parasitophorous vacuole. IEM showed the Nc56 protein to be almost exclusively associated with an, as yet undefined, internal structure of the parasite. An occasional tachyzoite was observed to display extracellular Nc56, but it is not known whether this is active secretion by the parasite or an artifact of fixation and embedding associated with processing of in vitro-cultured *N. caninum* tachyzoites for IEM.

Whether Nc56 is a homologue of pyruvate carboxylase is unknown. Although the DNA sequence similarity between Nc56 and the gene coding for rat or mouse pyruvate carboxylase is somewhat high (61%), there is an appreciable discrepancy between reported sizes of eukaryotic pyruvate carboxylases and native Nc56 protein. Pyruvate carboxylase is a ~650-kDa protein consisting of 4 identical 147-kDa subunits. Under reducing SDS-PAGE, antisera to rNc56 identified a major 56-kDa protein and 2 minor 39- and 43-kDa proteins. Under nonreducing electrophoresis conditions, anti-rNc56 identified 2 proteins—68 and 28 kDa. Also, in preliminary SDS-PAGE and immunoblotting studies, antisera against the native Nc56 protein did not bind a high Mr protein (~147-kDa), which indicates that Nc56 is not a breakdown product of *N. caninum* pyruvate carboxylase. The lower apparent Mr of recombinant Nc56 (41 kDa) may indicate that rNc56 represents a partial sequence of the cDNA coding for native 56-kDa protein or that the native protein is glycosylated. Evidence supporting the latter is that there is 1 potential N-glycosylation site (AA residues 246–248) in the rNc56 sequence and that baculovirus-expressed rNc56 migrates at 67 kDa (M. Jenkins, unpubl. obs.). Our results suggest that Nc56 is not a homologue of pyruvate carboxylase for several reasons. Northern blot hybridization results showed that the Nc56 transcript is nearly full length (~2.8 kb) and appreciably less than the transcript size expected for pyruvate carboxylases (~3.5 kb). Also, avidin does not bind to native Nc56 or recombinant Nc56 (M. Jenkins, unpubl. obs.), as has been demonstrated for *Toxoplasma gondii* pyruvate carboxylase (Jelenska et al., 2001). Anti-rNc56 serum binds to an antigen in the apical complex of *N. caninum*, which also argues against Nc56 being pyruvate carboxylase because this enzyme is found to be exclusively associated with mitochondria in the closely related protozoan, *T. gondii*, (Jelenska et al., 2001). The function of native Nc56 is unknown, except that in earlier studies, serum from cows experiencing a recent *N. caninum* infection showed preferential recognition of a 56-kDa tachyzoite antigen (R. O'Handley, unpubl. obs.). Additional studies are required to test the potential of rNc56 antigen for detecting cows infected with *N. caninum*.

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J. Parasitol., 90(3), 2004, pp. 663–664
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Occurrence of Acanthocephalans in Largemouth Bass and Smallmouth Bass (Centrarchidae) From Gull Lake, Michigan

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ABSTRACT: A total of 65 largemouth bass, *Micropterus salmoides*, and 27 smallmouth bass, *M. dolomieu*, collected in April–September 2000 and April–July 2001 from Gull Lake, Michigan, were examined for acanthocephalans. *Leptorhynchoides thecatus* and *Neoechinorhynchus cylindratus* infected all the bass examined. *Leptorhynchoides thecatus* had the highest mean intensity (258.2 ± 185.4 in 2000 and 145.0 ± 61.0 in 2001) of the species infecting smallmouth bass. Although *N. cylindratus* had higher mean intensities (42.1 ± 37.9 in 2000 and 68.9 ± 70.5 in 2001) than did *L. thecatus* in largemouth bass, the values were not significantly different between bass species. The prevalence, mean intensity, and mean abundance of *Pomphorhynchus bulbocollis* in the bass species were below the values for the other acanthocephalan species. *Leptorhynchoides thecatus* and *N. cylindratus* are the most abundant intestinal helminths in bass from Gull Lake.

The parasite fauna of centrarchid fishes from Gull Lake, Michigan, has been studied by Esch (1971), Esch and Huffines (1973), Esch et al. (1975, 1976), and Muzzall et al. (1995). Although Esch (1971) and Esch et al. (1975) reported on the occurrence of acanthocephalans in bass from this lake, intensities and abundances of the species were not provided. During a survey of fishes for parasites from this lake, bass were found infected with *Leptorhynchoides thecatus* (Linton, 1891) Kostylew, 1924 (Rhadinorhynchidae), *Neoechinorhynchus cylindratus* (Van Cleave, 1913) Van Cleave 1919 (Neoechinorhynchidae), and *Pomphorhynchus bulbocollis* Van Cleave, 1919 (Pomphorhynchidae). The objectives of this study were to calculate infection values for each acanthocephalan species in largemouth bass and smallmouth bass from Gull Lake, compare infections between bass species, and compare the infections of these acanthocephalan species with the results of earlier studies in this lake.

Gull Lake, located in Barry and Kalamazoo counties in southwestern Michigan, is a mesotrophic lake with a total surface area of 822 ha. A total of 65 largemouth bass and 27 smallmouth bass were collected by angling, electrofishing, and fyke net from 4 locations in Gull Lake in April–September 2000 and April–July 2001 and examined for acanthocephalans. These locations have sand and cobble substrate, and vegetation is sparse. Some bass were examined within 2 hr of collection. Most bass were frozen immediately after capture, and the gastrointestinal tract, mesentery, liver, and spleen were examined later when the standard length (mm) of the fishes was measured and sex was determined. Fish length and the prevalence, intensity, and abundance of each parasite species were not statistically different among sites (Kruskal–

Wallis test, $P > 0.05$) so the data were pooled for each year. Use of prevalence, mean intensity, and mean abundance is consistent with that reported by Margolis et al. (1982). Voucher specimens have been deposited in the U.S. Parasite Collection, Beltsville, Maryland: *Leptorhynchoides thecatus* (94136), *Neoechinorhynchus cylindratus* (94137), and *Pomphorhynchus bulbocollis* (94138).

The number and mean standard lengths (mm) $\pm$ SD (95% CI) of largemouth bass and smallmouth bass examined by year were 2000, $n = 23$, $\bar{x} = 204 \pm 30$ (191–217); 2001, $n = 42$, $\bar{x} = 231 \pm 45$ (215–245); and 2000, $n = 24$, $\bar{x} = 225 \pm 45$ (205–247); 2001, $n = 3$, $\bar{x} = 250 \pm 18$ (205–295), respectively. There was no significant difference in the lengths among largemouth bass in 2000, largemouth bass in 2001, and smallmouth bass in 2000 (Kruskal–Wallis test, $P > 0.05$).

Leptorhynchoides thecatus and *N. cylindratus* infected all smallmouth bass and largemouth bass examined from Gull Lake (Table 1). *Leptorhynchoides thecatus* had the highest mean intensity of the species infecting smallmouth bass, and it had a significantly higher mean intensity in smallmouth bass in 2000 than in largemouth bass in 2000 and 2001 (Kruskal–Wallis test, $H = 31.9$, $P < 0.001$). Data from the 3 smallmouth bass examined in 2001 were not included in the analysis. Differences in bass species length cannot account for the significant differences in the intensities of *L. thecatus* among smallmouth bass in 2000 and largemouth bass in 2000 and 2001 because their lengths were not significantly different. Although *N. cylindratus* had higher mean intensities than did *L. thecatus* in largemouth bass in both years, the values were not significantly different (Mann–Whitney test, $P > 0.05$). There was no significant difference in the intensities of *N. cylindratus* among smallmouth bass in 2000 and largemouth bass in 2000 and 2001 (Kruskal–Wallis test, $P > 0.05$). The prevalence, mean intensity, and mean abundance of *P. bulbocollis* were well below the values for *L. thecatus* and *N. cylindratus* in these bass species. Although the number of smallmouth bass infected with *P. bulbocollis* was significantly higher than the number of largemouth bass infected in 2000 (chi-square test, $\chi^2 = 3.31$, $P < 0.05$), mean intensities and mean abundances were not significantly different (Kruskal–Wallis test, $P > 0.05$).

There was no significant difference in the number of infected and uninfected fish (chi-square, $P > 0.05$) and intensities or abundances (Mann–Whitney test, $P > 0.05$) for each *L. thecatus*, *N. cylindratus*, and *P. bulbocollis* between female and male bass of each species. The only significant Spearman's correlation coefficient involved largemouth bass length and intensity of *N. cylindratus* in 2001 ($r_s = -0.54$, $P < 0.001$). The correlation, however, in 2000 involving this pair was positive and nonsignificant. When the data for 2000 and 2001 were combined, 12

TABLE 1. Prevalence (P), mean intensity (MI), mean abundance (MA), and maximum of *Leptorhynchoides thecatus*, *Neoechinorhynchus cylindratius*, and *Pomphorhynchus bulbocolli* in *Micropterus dolomieu* and *M. salmoides* from Gull Lake in 2000 and 2001.

Acanthocephalan species	<i>M. dolomieu</i>					<i>M. salmoides</i>					
	Year	n*	P	MI $\pm$ SD (95% CI)	MA $\pm$ SD (95% CI)	Maximum	n*	P	MI $\pm$ SD (95% CI)	MA $\pm$ SD (95% CI)	Maximum
<i>L. thecatus</i>	2000	24	100	258.2 $\pm$ 185.4 (179.9 to 336.5)	—	741	23	100	40.0 $\pm$ 53.4 (35.4 to 81.5)	—	—
	2001	3	100	145.0 $\pm$ 61.0 (-6.7 to 296.7)	—	214	42	100	61.3 $\pm$ 100.2 (30.1 to 92.5)	—	486
<i>N. cylindratius</i>	2000	24	100	46.2 $\pm$ 82.0 (11.6 to 80.9)	—	367	23	100	42.1 $\pm$ 37.9 (25.7 to 58.5)	—	161
	2001	3	100	17.7 $\pm$ 1.5 (13.9 to 21.5)	—	19	42	100	68.9 $\pm$ 70.5 (46.9 to 90.8)	—	273
<i>P. bulbocolli</i>	2000	24	41	1.5 $\pm$ 0.85 (0.89 to 2.11)	0.63 $\pm$ 0.92 (0.24 to 1.01)	3	23	17	1.3 $\pm$ 0.5 (0.45 to 2.0)	0.22 $\pm$ 0.52 (0.01 to 0.44)	2
	2001	3	66	4.5 $\pm$ 0.71 (-1.85 to 2.04)	3.0 $\pm$ 2.64 (-3.57 to 9.57)	5	42	14	2.3 $\pm$ 1.9 (0.38 to 4.3)	0.33 $\pm$ 1.10 (0.01 to 0.66)	6

* Number of fish examined.

(44%) smallmouth bass and 10 (15%) largemouth bass were simultaneously infected with *L. thecatus*, *N. cylindratius*, and *P. bulbocolli*.

Gravid *L. thecatus* were found in the pyloric ceca and anterior intestine of largemouth bass and smallmouth bass in the 6 and 4 mo sampled in 2000 and 2001, respectively. In 3 smallmouth bass, unencysted *L. thecatus* (numbers not included in prevalence, intensity, and abundance) were found in the liver (1 gravid individual) and mesentery. It appears that this is the first report of gravid parenteral *L. thecatus* in bass. In 6 largemouth bass, encysted and unencysted *L. thecatus* occurred in the liver, mesentery, and spleen, and 1 gravid individual occurred half way out of a pyloric cecum with its anterior end protruding into the body cavity. Gravid *N. cylindratius* were found in the intestine of largemouth bass and smallmouth bass throughout the sampling periods. The livers and other extraintestinal viscera of all the bass examined were negative for *N. cylindratius*. Nongravid *P. bulbocolli* occurred in the mid- and posterior intestine of bass.

Esch (1971) did not find any of the 61 smallmouth bass and 34 largemouth bass from Gull Lake infected with *Pomphorhynchus* sp. These results are in contrast to the present study where *P. bulbocolli* infected 44% of the smallmouth bass and 15% of the largemouth bass in 2000 and 2001. Gravid *P. bulbocolli* were not found in bass from Gull Lake. Amin (1987) found gravid *P. bulbocolli* in smallmouth bass but not in largemouth bass.

Esch et al. (1975) reported that the most abundant intestinal helminths in bass from Gull Lake were *N. cylindratius* and *L. thecatus*. Largemouth bass and smallmouth bass are the preferred hosts for *L. thecatus* and *N. cylindratius* in Gull Lake based on the occurrence of gravid worms and their high prevalences and mean intensities. The mean intensities of *L. thecatus* in largemouth bass and smallmouth bass and the mean intensity of *N. cylindratius* in largemouth bass in the present study are the highest reported to date. Esch (1971) studied *Leptorhynchoides* sp. and *Neoechinorhynchus* sp. in smallmouth bass; assuming that these were *L. thecatus* and *N. cylindratius* in smallmouth bass from Gull Lake, the prevalence of *L. thecatus* in smallmouth bass was high in both studies (92% in the 1971 study and 100% in the present study). The prevalence of *N. cylindratius* in smallmouth bass from Gull Lake dramatically increased from 30% in the study by Esch (1971) to 100% in the present study. Also, 29 yr later, these acanthocephalan species are still the most abundant intestinal helminths in bass from Gull Lake. Acanthocephalan species have not been lost from these bass species nor have any new species colonized them in the lake since the study of Esch (1971).

We thank Jim Dexter, Michigan Department of Natural Resources, for his insight and help with finding collection locations; and Doyle Boss, Dan Anson (MDNR), Karl Strauss, Denise Kay, Matt Zwiernik, Carolyn Gilliland, Gale Gilliland, and Ryan Gilliland for assistance in collecting fish.

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