NOTE *Parasitology*

Studies on Serological Cross-Reaction of *Neospora caninum* with *Toxoplasma gondii* and *Hammondia heydorni*

Yoshifumi NISHIKAWA[1)], Florencia G. CLAVERIA[1)], Kozo FUJISAKI[1)] and Hideyuki NAGASAWA[1)]*

[1)]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inadacho, Obihiro, Hokkaido 080–8555, Japan*

(Received 21 August 2001/Accepted 11 October 2001)

ABSTRACT. The enzyme-linked immunosorbent assay (ELISA) was used to examine cross-reactivity of *Neospora caninum* with *Toxoplasma gondii* and *Hammondia heydorni*. Anti-*T. gondii* mouse and cat sera cross-reacted with *N. caninum* soluble antigen (NLA), but not with the recombinant surface antigen (NcSRS2). Anti-*H. heydorni* dog sera showed no cross-reactivity with either the NLA antigen or the NcSRS2. Lack of cross-reactivity between anti-*H. heydorni* sera and *N. caninum* antigens, and the cross-reactivity of anti-*T. gondii* sera with the NLA suggest that *N. caninum* has common antigens to *T. gondii* except for NcSRS2 based on serology. In light of several studies suggesting a closer relationship between *N. caninum* and *H. heydorni* than with *T. gondii*, examination of serological cross-reactivity with *N. caninum* may be necessary to further classify the parasites in addition to molecular and morphological studies and clarification of the life cycle.

KEY WORDS: ELISA, *Neospora caninum*.

J. Vet. Med. Sci. 64(2): 161–164, 2002

Neospora caninum is a protozoan parasite of livestock and companion animals that causes abortion, neuromuscular paralysis and death [1, 2]. Several studies have reported similarities and differences in the cyst wall morphology, tachyzoites ultrastructure, oocysts features, and life cycle of *N. caninum*, *Toxoplasma gondii*, *Hammondia heydorni* and *Hammondia hammondi*. The cyst wall of *N. caninum* bears resemblance to that of *T. gondii* and *H. hammondi* [12, 13, 17, 18]. *N. caninum* oocysts exhibit features similar to *H. heydorni* oocysts from dog's faeces, and to the oocysts of both *T. gondii* and *H. hammondi* from cat faeces [8]. Similarities between the intermediate and definitive (i.e. dogs) hosts of *N. caninum* and *H. heydorni* suggest a closer relationship between them [13] than with either *T. gondii* or *H. hammondi*, which utilize cats as definitive hosts [4]. Thus, it is difficult to distinguish *N. caninum* from *H. heydorni* based on their life cycle and morphology alone. While the coccidian nature of *N. caninum* has been recently confirmed through the demonstration of its oocysts in dog's faeces [11], its classification has remained controversial [10, 13]. In this paper, we investigate whether cross-reactivity occurs between *N. caninum*, *T. gondii* and *H. heydorni* using the surface protein NcSRS2 [5, 7] and the soluble antigen (NLA) of *N. caninum* by using enzyme-linked immunosorbent assay (ELISA).

Full-length NcSRS2 genes were cloned into baculovirus using the primer sets: 5'-ACG AAT TCA TGG CGA CGC ATG CTT-3', and 5'-GCG TCG ACT CAG TAC GCA AAG ATT-3'. The cDNA of *N. caninum* tachyzoites was used as the template in the polymerase chain reaction (PCR). PCR products were blunted by Klenow fragments, and ligated to a previously-digested *Sma* I baculovirus transfer vector pBacPAK8 (Clontech, Palo Alto, CA). Sf9 cells were co-transfected with a transfer vector and BacuoGold(r) Baculovirus DNA (PharMingen, San Diego, CA) using lipofectin^{TM} reagent (Gibco BRL, Grand Island, NY). After 4 days of incubation at 27°C, the culture supernatant containing recombinant viruses was harvested and subjected to plaque purification. After three cycles of purification, recombinant viruses-expressing NcSRS2 were obtained. *Autographa californica* nuclear polyhedrosis virus and its recombinant products were grown in *Spodoptera frugiperda* (Sf9) cells in a TC-100 insect medium (Gibco BRL) supplemented with 10% FBS and 0.26% bacto tryptose broth (Difco, Detroit, MI).

ELISA plates coated with the NLA antigen were prepared as described previously by Nishikawa *et al.* [15]. Monolayers of Sf9 cells that were grown in a 75 cm^2 flask and infected with 10 m*l* of recombinant baculovirus at 5 PFU/cell in Sf-900II SFM medium (Gibco BRL). After 4 days, infected cells were harvested and sonicated. The cells were treated with Triton-X100 at a final concentration of 1%. The suspension was left at room temperature for 2 hr, and then centrifuged at 10,000 × g for 10 min. The supernatant was dialysed against PBS and the protein concentration was determined using the biothinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Plates were coated with the antigen with carbonate-bicarbonate buffer (pH 9.6) for overnight at 4°C. After blocking with PBS containing 3% skim milk (PBS-SM) for 1 hr at 37°C, the plates were washed twice with PBS containing 0.05% Tween20 (PBS-T) and 100 μl of serum diluted with PBS-SM was added to each of the duplicate wells. Plates were incubated at 37°C for 1 hr, then washed 5 times with PBS-T. Plates were incubated with horseradish peroxidase-conjugated mouse (Amersham Pharmacia Biotech, Piscataway, NJ), dog

* CORRESPONDENCE TO: NAGASAWA, H., National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inadacho, Obihiro, Hokkaido 080–8555, Japan.

(Bethyl, Montgomery, TX) or cat IgG Ab (Bethyl) at 37°C for 1 hr. Colorimetric reactions were performed by adding substrate (0.1 M citric acid, 0.2 M $NaHPO_4$, 0.003% H_2O_2, 0.3 mg/m*l* of 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate] after 5 washes. Absorbance at 415 nm in each well was measured using an MTP-120 micro plate reader (Corona Electric, Ibaraki, Japan).

To produce mAbs against NcSRS2 or NcSAG1, immunization of BALB/c mice (Clea Japan, Tokyo, Japan), cell fusion, and selection of fused cells were performed according to the methods described previously [16]. Antibodies against NcSRS2 or NcSAG1 were produced by immunization of recombinant proteins expressed in *E. coli* into BALB/c mice as described previously [16]. To produce antisera, three 10-week old female BALB/c mice per group were intraperitoneally injected with 1×10^6 *N. caninum* tachyzoites (Nc-1 isolate) or 5×10^4 *T. gondii* tachyzoites (PLK isolate). Similarly, two 12-week old beagle dogs which were confirmed free of *N. caninum* and/or *T. gondii* antibodies by indirect fluorescence antibody test (IFAT) prior to the experiment were intravenously injected with 2×10^6 *N. caninum* tachyzoites (Nc-1 isolate). Anti-*Hammondia heydorni* dog sera were provided by Dr. Matsui of the Department of Parasitology, Kyorin University School of Medicine [9]. Two specific pathogen-free cats were orally inoculated with 1×10^4 *T. gondii* bradyzoites (Beverly strain). Sera from mice, dogs and cats were collected one month post-infection.

IFAT analysis showed that three mAbs (1B8, 2C8 and 2G2) and polyclonal antibody against NcSRS2 recognized the recombinant proteins expressed by recombinant baculovirus (data not shown), suggesting that the antigenic structure of the recombinant proteins was similar to the native parasite protein.

The results of the ELISA assay are shown in Figs. 1 and 2. Both the recombinant NcSRS2 and NLA showed strong reactivity with anti-*N. caninum* mouse and dog sera. With anti-*T. gondii* mouse sera, cross reaction was noted only with NLA, but not with the recombinant NcSRS2. In the case of anti-*T. gondii* cat sera, cross reaction was observed only with NLA due to antigen concentration while high concentration of sera showed non-specific reaction with NLA and the recombinant NcSRS2. Earlier findings in our laboratory have likewise demonstrated the inability of mAbs specific to *N. caninum* (NcSRS2 or NcSAG1) to recognize *T. gondii* [16]. These results demonstrate that both species are serologically cross-reactive while NcSRS2 and NcSAG1 may be absent in *T. gondii* surface protein. This finding is supported by earlier reports of a strong cross-reactivity of *T. gondii* polyclonal antibodies with *N. caninum* [19].

Available information on the molecular biological aspect of these parasites that may help clarify questions about their phylogenetic relationship are somehow confusing. While the sequence analyses of the large subunit of ribosomal RNA and the internal transcribed spacer-1 revealed a closer relationship between *N. caninum* and *H. heydorni* than with either *H. hammondi* or *T. gondii* [3, 14], *H. heydorni* genomic DNA could not be amplified in the PCR assay targeting the *N. caninum*-specific Nc5 genomic sequence [6]. Several reports on similarities in the life cycle and morphology of *N. caninum* and *H. heydorni* suggest a closer relationship between them than with *T. gondii*. In the present study, the absence of cross-reactivity of anti-*H. heydorni* dog sera with either the NLA or the recombinant NcSRS2, and the cross-reactivity of anti-*T. gondii* sera with NLA suggest that *N. caninum* is more closely related with *T. gondii* than with *H. heydorni* based on serology. The absence of cross-reaction between anti-*T. gondii* sera and the *N. caninum* specific surface antigen may be interpreted to further support the contention that *N. caninum* is a different species altogether. However, the examination of more anti-*H. heydorni* sera may be necessary to further clarify serological cross-reactivity between *N. caninum* and *H. heydorni*.

ACKNOWLEDGMENTS. We thank Dr. J. P. Dubey of the United States Department of Agriculture, Agriculture Research Service, Livestock and Poultry Sciences Institute, and Parasite Biology and Epidemiology Laboratory, and Dr. Matsui of the Department of Parasitology, Kyorin University School of Medicine for providing the *N. caninum* (NC-1 isolate), and the anti-*H. heydorni* dog sera, respectively. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The first author is a grantee of a Research Fellowship by the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

1. Dubey, J. P., Carpenter, J. L., Speer, A., Topper, M. J. and Uggla, A. 1988. *J. Am. Vet. Med. Assoc.* **192**: 1269–1285.
2. Dubey, J. P. and Lindsay, D.S. 1996. *Vet. Parasitol.* **67**: 1–59.
3. Ellis, J. T., Morrison, D. A., Liddell, S., Jenkins, M. C., Mohammed, O. B., Ryce, C. and Dubey, J. P. 1999. *Parasitology* **118**: 357–362.
4. Frenkel, J. K. and Dubey, J. P. 1975. *Science* **189**: 222–224.
5. Hemphill, A., Felleisen, R., Connolly, B., Gottstein, B., Hentrich, B. and Muller, N. 1997. *Parasitology* **115**: 581–590.
6. Hill, D. E., Liddell, S., Jenkins, M. C. and Dubey, J. P. 2001. *J. Parasitol.* **87**: 395–398.
7. Howe, D. K., Crawford, A. C., Lindsay, D. and Sibley, L. D. 1998. *Infect. Immun.* **66**: 5322–5328.
8. Lindsay, D. S., Upton, S. J. and Dubey, J. P. 1999. *Int. J. Parasitol.* **29**: 1521–1523.
9. Matsui. T., Morii, T., Iijima, T., Ito, S., Tsunoda, K., Kobayashi, F. and Fujino, T. 1986. *Jpn. J. Parasitol.* **35**: 215–222.
10. McAllister, M. M. 2000. *Parasitol. Res.* **86**: 860.
11. McAllister, M. M., Dubey, J. P., Lindsay, D. S., Jolley, W. R., Wills, R. A. and McGuire, A.M. 1998. *Int. J. Parasitol.* **28**: 1473–1478.
12. Mehlhorn, H. and Frenkel, J. K. 1980. *J. Parasitol.* **66**: 59–67.
13. Mehlhorn, H. and Heydorn, A.O. 2000. *Parasitol. Res.* **86**: 169–178.
14. Mugridge, N. B., Morrison, D. A., Heckeroth, A. R., Johnson, A. M. and Tenter, A. M. 1999. *Int. J. Parasitol.* **29**: 1545–1556.

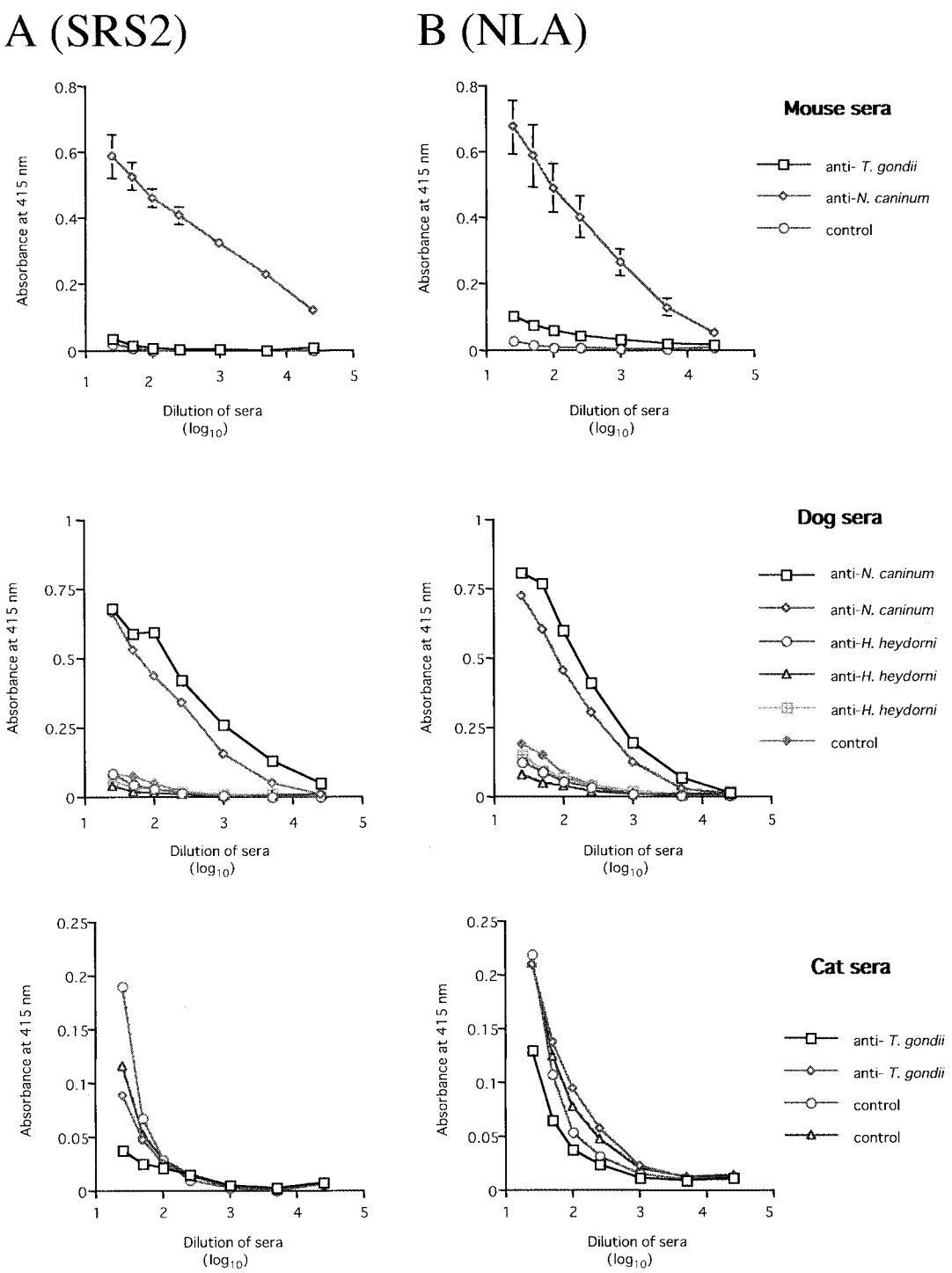

Fig. 1. ELISA using the recombinant surface antigen (A, NcSRS2) and the *N. caninum* soluble antigen (B, NLA), and reacted with anti-*N. caninum*, anti-*T. gondii* or anti-*H. heydorni* sera which were serially diluted. Antigen used was 60 *n*g/well. In mouse sera, the values are means ± standard deviation of three mice per group. Control: normal animal sera.

15. Nishikawa, Y., Kousaka, Y., Fukumoto, S., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H. and Mikami, T. 2000. *Parasitol. Res.* **86**: 934–939.

16. Nishikawa, Y., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H. and Mikami, T. 2000. *Int. J. Parasitol.* **30**: 51–58.

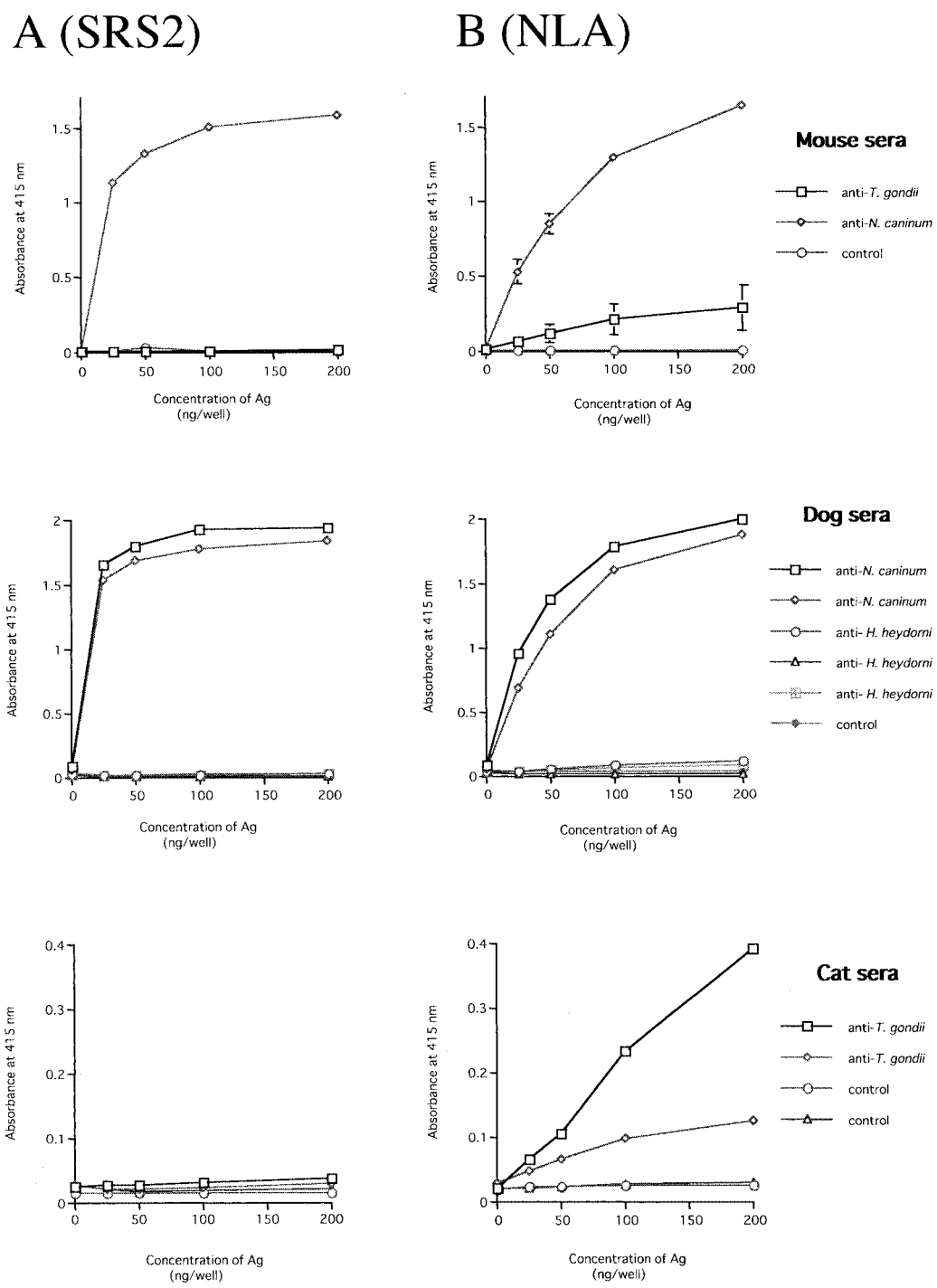

Fig. 2. ELISA using the recombinant surface antigen (A, NcSRS2) and the *N. caninum* soluble antigen (B, NLA), which were serially diluted, and reacted with anti-*N. caninum*, anti-*T. gondii* or anti-*H. heydorni* sera diluted at 1:250. In mouse sera, the values are means ± standard deviation of three mice per group. Control: normal animal sera.

17. Speer, C. A. and Dubey, J. P. 1989. *J. Protozool.* **36**: 488–493.
18. Speer, C. A. and Dubey, J. P. 1989. *J. Protozool.* **36**: 458–463.
19. Sundermann, C. A., Estridge, B. H., Branton, M. S., Bridgman, C. R. and Lindsay, D. S. 1997. *J. Parasitol.* **83**: 440–443.