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Antibody reaction of human anti-*Toxoplasma gondii* positive and negative sera with *Neospora caninum* antigens

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Abstract: Anti-*Neospora caninum* antibody was detected in anti-*Toxoplasma gondii* positive and negative human sera by ELISA, western blot and immunofluorescence assay (IFA). Twelve cases out of 172 (6.7%) *Toxoplasma*-positive sera cross-reacted with both *T. gondii* and *N. caninum* antigens, and one out of 110 *Toxoplasma*-negative sera reacted with *N. caninum* antigen by ELISA. By western blot, all 12 sera reacted with *T. gondii* antigens with various banding patterns but specifically at 30 kDa (SAG1), and 22 kDa (SAG2) bands. With *N. caninum* antigen, the number of reactive bands was reduced, however a 43 kDa band reacted in three cases in *Toxoplasma*-positive sera in addition to one in *Toxoplasma*-negative control sera. All sera of the *Toxoplasma*-positive group labeled surface membrane of *T. gondii*, but reacted differently with *N. caninum*. Fluorescence was detected in surface membrane, subcellular organelles, or both in *N. caninum*. And one case in the *Toxoplasma*-negative group also reacted with *N. caninum* strongly in subcellular organelles. This suggested that the antibody against *N. caninum* may be present in human sera although the positive rate was very low in this study. The possibility of human infection with *N. caninum* remains to be evaluated further.

Key words: *Neospora caninm, Toxoplasma gondii*, antigenicity, cross reaction, ELISA, wester blot, IFA

INTRODUCTION

Neospora caninum is a protozoan (Apicomplexa) parasite that was first identified in 1988 during a retrospective study of dogs previously diagnosed with fatal toxoplasmosis (Dubey *et al.*, 1988). This parasite causes serious neurologic and muscular diseases in dogs, cattle, sheep, goats and horses (Dubey and Lindsay, 1993). However, this coccidian has not been found in humans until now, but it has been reported that it can infect monkeys experimentally (Barr *et al.*, 1994). *Neospora caninum* shares many features with, but is clearly distinct from *Toxoplasma gondii*. Before 1988, *N. caninum* was misdiagnosed as *T. gondii* because of the existence of morphologic and biologic similarities of tachyzoites between the two coccidia and their ubiquitous host ranges. In addition, there appears to be serologically cross-reactive due to shared antigens between *N. caninum* and *T. gondii* (Bjerkas *et al.*, 1994).

However, they are differentiated genetically (Marsh *et al.*, 1995) and evolve divergently (Guo and Johnson, 1995). More clearly, they are different from each other in antigenicity of

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major surface proteins; for example, SAG1 (p30) and SAG2 (p22) of *T. gondii* are absent in *N. caninum* (Brindley *et al.*, 1993), whereas a 43 kDa (Nc-p43) protein, a major surface protein of *N. caninum*, is absent in *T. gondii* (Hemphill and Gottstein, 1996).

We tried to detect antibody against *N. caninum* in anti-*Toxoplasma* positive and negative human sera to differentiate antigenic cross reactivity and to seek unique reactions with *N. caninum* which could suggest a possibility of zoonotic infection of this parasite.

MATERIALS AND METHODS

Antigen preparation

RH tachyzoites of *T. gondii* were maintained by a peritoneal passage in mice and purified by centrifugation over 40% Percoll (Pharmacia, Sweden). Tachyzoites of *N. caninum* were maintained in Vero cell monolayer in DMEM supplemented with 10% FBS (Gibco, USA), and they were purely obtained from the supernatant of 3-4 days culture.

Sera

A total of 172 anti-*Toxoplasma* positive sera was composed of 75 indirect latex agglutination (ILA) positive cases (> 1:32) (Choi *et al.*, 1989), 76 ILA and ELISA positive cases from Choi *et al.* (1992), 8 toxoplasmosis patients (Choi *et al.*, 1997), and 13 ELISA positive cases screened. Anti-*Toxoplasma* negative sera were collected from 110 healthy students previously screened by ELISA.

Analysis of antigenic cross reaction

ELISA was performed according to the method of Choi *et al.* (1992). Briefly, *T. gondii* and *N. caninum* extracts, 1 μg/well each, were used as antigens. Sera diluted at 1:100 with PBS/0.05% Tween-20 and HRP-conjugated goat anti-human IgG antibody (Cappel, USA) diluted at 1:1,000 were used as the primary and secondary antibodies, respectively. Substrate solution was composed of 1 ml 1% *o*-phenylene diamine, 50 μl of 30% H_2O_2 in 99 ml distilled water. After stopping the reaction with H_2SO_4, absorbances were read at 490 nm with an ELISA reader (Dynatech, USA).

Western blot was done by the method of Towbin *et al.* (1979). Nitrocellulose (NC) papers were incubated with sera with 1:500 dilution and then with 1:2,000 diluted HRP-conjugated goat anti-human IgG antibody. They were soaked in ECL solution (Amersham, USA) for 1 min and exposed to X-ray film (Fuji, Japan) for 5 to 10 sec.

For immunofluorescence assay (IFA), freshly prepared *T. gondii* and *N. caninum* tachyzoites suspended in PBS were cytospinned onto 18-mm coverslips. Tachyzoites were fixed with cold absolute methanol for 5 min. Sera were applied with 1:100 dilution in incubation solution (3% BSA in PBS) and coverslips were incubated with 1:500 diluted FITC-conjugated goat anti-human IgG antibody (Cappel). Fluorescence was observed under a fluorescence microscope (Axiophot, Zeiss Co.).

RESULTS

By ELISA, the cut-off value of anti-*Neospora* positive was determined as an absorbance of 0.24 (mean + 3SD) (Table 1). According to this cut-off value, 12 cases out of 172 (6.7%) *Toxoplasma*-positive sera showed cross reaction with both *T. gondii* and *N. caninum* antigens, and one case out of 110 in the *Toxoplasma*-negative sera reacted with *N.*

Table 1. Absorbance values of ELISA

Group	Absorbance at 490 nm	
	Anti-*Toxoplasma*	Anti-*Neospora*
Toxoplasma-positive	0.44 ± 0.18	0.14 ± 0.07
(Toxoplasmosis[a])	0.77 ± 0.13	0.10 ± 0.05
Toxoplasma-negative	0.11 ± 0.04	0.11 ± 0.04[b]

[a]Patients of toxoplasmosis outbreaks (Choi *et al.*, 1997).
[b]Cut-off value was determined as 0.24 by mean + 3 × SD.

caninum antigen. There were no significant correlations in absorbances between *T. gondii* and *N. caninum* as shown in Figure 1.

Cross-reacted sera were subjected to western blot with both *T. gondii* and *N. caninum* antigens. As shown in Figure 2, all 12 sera reacted with *T. gondii* antigens with various banding patterns, however specific reactions were observed at 30 kDa (SAG1) and 22 kDa (SAG2) bands (Fig. 2A). When the same sera were applied to *N. caninum* antigen, the number of reactive bands was reduced. But a 43 kDa band was observed in three sera in *Toxoplasma*-positive group (lanes 1, 7 and 10 in Fig. 2B) in addition to one in *Toxoplasma*-negative group.

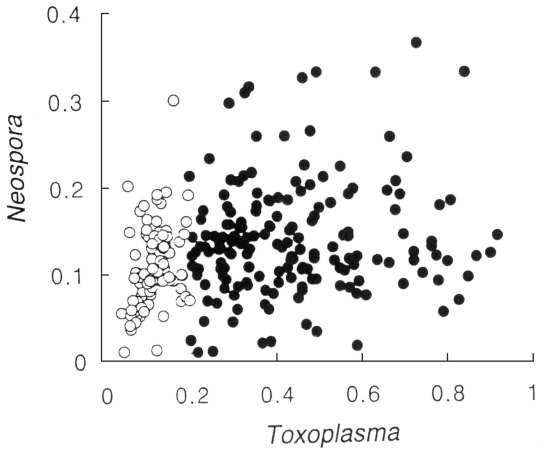

Fig. 1. Distribution of absorbance spots of *Toxoplasma* vs. *Neospora*. Closed circles indicate the *Toxoplasma*-positive group and open circles indicate the *Toxoplasma*-negative group.

These sera were tested further by IFA (Fig. 3). All sera of the *Toxoplasma*-positive group labeled surface membrane (subcellular organelles also in some cases) of *T. gondii*, but reacted differently with *N. caninum*. In the case of lane 1 in Figure 2, the surfaces of both *T. gondii* and *N. caninum* were fluorescent (Fig. 3B). With the serum of lane 4, fluorescence of *N. caninum* was much brighter than that of *T. gondii* (Fig. 3C). Especially with the serum of lane 7, surface of *N. caninum* was labeled distinctly (Fig. 3D). The serum of lane 10 reacted with subcellular organelles of *N. caninum* (Fig. 3E). And the serum of *Toxoplasma*-negative lane 2 also reacted with *N. caninum* strongly in subcellular organelles (Fig. 3F). Sera of the other lanes reacted with *T. gondii*, but not with *N. caninum* (data not shown).

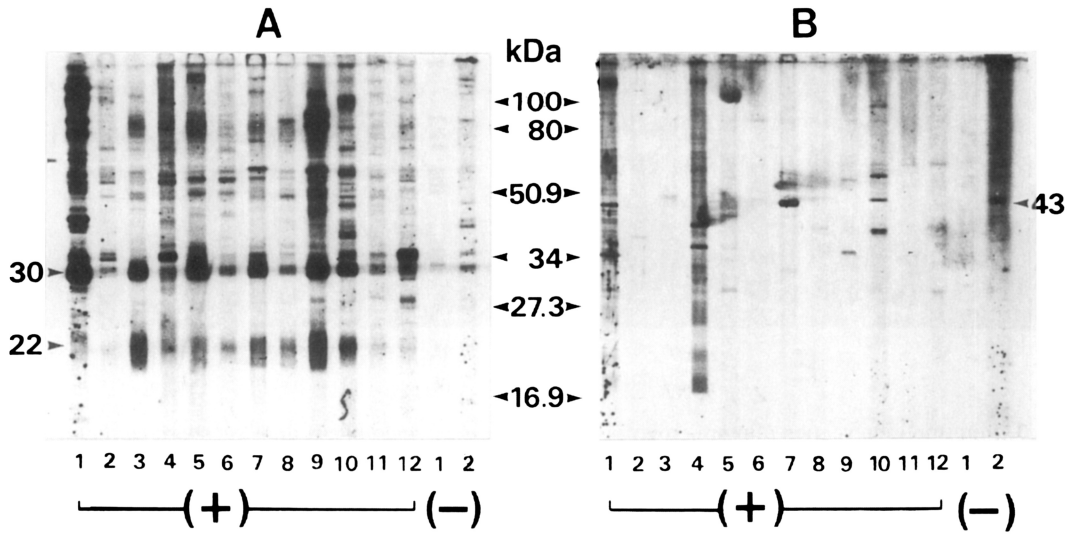

Fig. 2. Western blot pattern of human sera with *Toxoplasma gondii* (A) and *Neospora caninum* (B) antigens. Sera were applied to the same lanes to both antigens. (+) means 12 cases of cross reactions from the *Toxoplasma*-positive group by ELISA and (-) means 2 cases from the *Toxoplasma*-negative group.

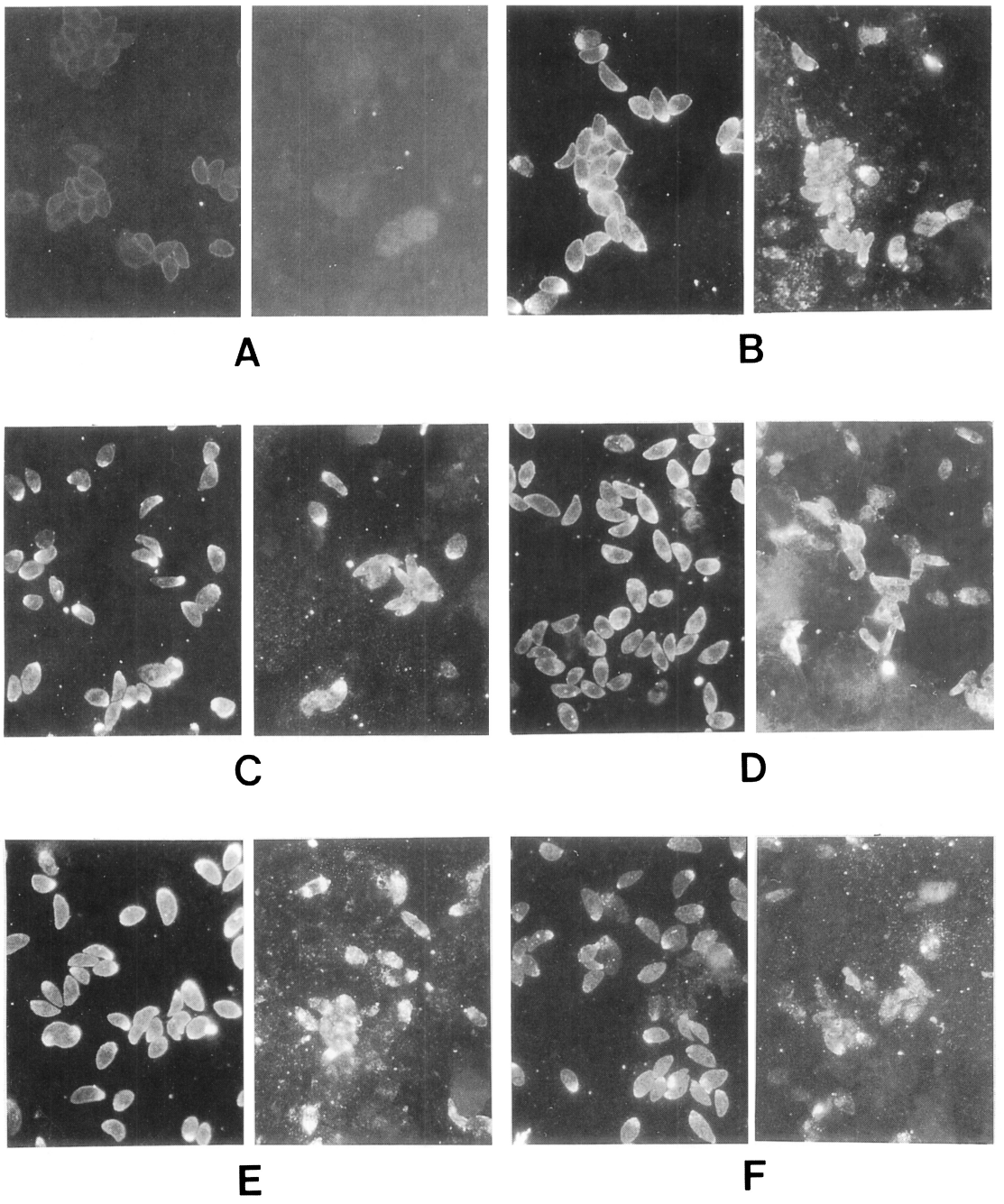

Fig. 3. Immunofluorescence photomicrographs of *Toxoplasma gondii* (left) and *Neospora caninum* (right). **A,** cotrol with serum of lane 1 of the *Toxoplasma*-negative group; **B,** serum of lane 1; **C,** lane 4; **D,** lane 7; **E,** lane 10 of the *Toxoplasma*-positive group; **F,** lane 2 of the *Toxoplasma*-negative group, respectively.

DISCUSSION

Twelve out of 172 (6.7%) *Toxoplasma*-positive sera cross-reacted with both *T. gondii* and *N. caninum* antigens, and one out of 110 *Toxoplasma*-negative sera reacted with the *N. caninum* antigen by ELISA. By western blot, all

of 12 sera showed various banding patterns, however they reacted specifically with 30 kDa (SAG1), and 22 kDa (SAG2) bands of *T. gondii*. With the *N. caninum* antigen, the number of reactive bands was reduced, but three sera in *Toxoplasma*-positive group and one in negative group reacted with a 43 kDa protein. The surface membrane of *T. gondii* was labeled with all *Toxoplasma*-positive sera. In case of *N. caninum*, fluorescence was detected in the surface membrane, subcellular organelles, or both. Interestingly, one *Toxo-plasma*-negative serum strongly reacted with the subcellular organelles of *N. caninum*.

We focused on the binding of 43 kDa protein in western blot using *N. caninum* antigen, which might be the most antigenic during infection because the Nc-p43 was identified as a major surface protein of *N. caninum* (Hemphill, 1996; Hemphill and Gottstein, 1996). In western blot of *N. caninum*, approximate molecular weights of 29, 30, 37, 51/52 kDa proteins were detected in addition to 43 kDa protein. These proteins were already characterized to be immunogenic, but they were not located in the surface membrane but in the rhoptries, dense granules, micronemes, tubular network, and membrane of the parasitophorous vacuole (Barta and Dubey, 1992; Bjerkas *et al.*, 1994). Whereas proteins of subcellular organelles or specific DNA sequences of *N. caninum* have been identified one by one (Cole *et al.*, 1994; Lally *et al.*, 1996 & 1997), organelles and their functions are seemingly conserved evolutionarily within the phylum Apicomplexa. So there are proteins which cross-react with subcellular organelles of both species as demonstrated in IFA. Bjoerkman *et al.* (1994) characterized antigens that are currently used in an ELISA for diagnosis of *N. caninum* infections. This group of antigens comprised four proteins of 52-61 kDa, and three proteins of 31-36 kDa, the location of which are suggested to be associated with membrane. Moreover, without Nc-p43 binding in western blot, the surface membrane was detected to be fluorescent. Further study is needed to clarify the surface proteins of *N. caninum* including Nc-p43.

As repeatedly proven in antibody surveys for toxoplasmosis, the positive rates were not high in Korea (Choi *et al.*, 1992) compared with those reported in European and American countries (Dubey and Beattie, 1988), which may be related with the differences in the food habit and/or the pet-loving behavior. Recently, dogs were identified as the definitive host of *N. caninum* (McAllister *et al.*, 1998) and Kim *et al.* (1998) isolated *N. caninum* from a calf in Korea. Thus human may have a chance to be exposed to *N. caninum* antigen as an infective form or an antigenic form merely. With this study, although specific binding to the surface membrane was not differentiated between *T. gondii* and *N. caninum*, the presence of antibody against *N. caninum* in human sera could not be overlooked.

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=초록=

톡소포자충 양성 및 음성인 사람 혈청에 대한 네오포자충 (*Neospora caninum*) 항원의 교차반응

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톡소포자충과 네오포자충 (*Neospora caninum*)의 충체 항원으로 톡소포자충 양성 혈청 및 톡소포자충증 환자의 혈청과 ELISA, western blot 및 면역형광법을 실시하였다. ELISA에서는 172 명의 톡소포자충 양성 혈청에서 12명 (6.7%)이 두 항원에 모두 반응하였으며, 톡소포자충 음성인 110명의 혈청에서 1명이 네오포자충과 반응하였다. 교차반응을 보인 12명의 혈청을 western blot으로 확인하였을 때, 톡소포자충 항원과는 다양한 양상으로 반응하였으나, 30 kDa (SAG1)과 22 kDa (SAG2) 항원과 강하게 반응하였다. 네오포자충과의 반응은 급격히 감소하였으나, 세 경우에서 43 kDa 단백질과 반응하였으며, 음성 혈청군의 1명도 43 kDa 단백질과 반응하였다. 면역형광법에서는 모든 양성 혈청이 톡소포자충의 세포막에 표지되었으나, 네오포자충과의 반응은 세포막, 세포 내 소기관, 혹은 둘 다를 표지하였다. 이로써, 톡소포자충과 네오포자충의 항원적 교차반응과 사람 혈청에서 네오포자충에 대한 항체의 존재를 확인하였으며, 네오포자충에 의한 인체감염 가능성에 대해서는 추후 연구가 필요할 것으로 판단된다. 또, *N. caninum*의 우리말 이름을 네오포자충으로 제안한다.

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