**Neospora caninum Antigens Recognized by Mouse IgG at Different Stages of Infection Including Recrudescence**

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**ABSTRACT.** Western blotting was performed to analyze *Neospora caninum* tachyzoite antigens recognized by mouse IgG at different stages of infection including recrudescence. At the early stage of infection, a 36–38 kDa antigen was clearly recognized by the mouse antisera. After day 48 postinoculation, the signal of the 36–38 kDa antigen gradually weakened. Meanwhile, a 43 kDa antigen was intensively and continuously recognized from 48 to 125 days postinoculation. This 43 kDa antigen was clearly detectable with the antisera from the mice under immunosuppression. Sera from naturally infected cattle strongly reacted with the 43 kDa antigen. Therefore, the 43 kDa antigen may be useful for immunological reactions to detect infected animals except in the early stage of the infection.

**Key words:** immunosuppression, infection stage, *Neospora caninum*.

*Neospora caninum* (*N. caninum*) is a recently identified apicomplexan parasite and a major cause of abortion in dairy cattle worldwide [1, 8]. At present, immunohistochemistry and the indirect fluorescent antibody test (IFAT) are mainly used for diagnosing neosporosis [8, 29]. Although, various ELISA have been developed and tested [5–7, 14, 16, 17, 20, 21], it needs to be clarified the process of *N. caninum* antigens being recognized in infected animals at different stages of infection and which antigen is the most suitable for diagnostic systems. Therefore, it is necessary to identify potential candidate of recognizable protein throughout all infection stages. A number of *N. caninum* antigens have been identified by using polyclonal antibodies [2, 4, 10, 12], monoclonal antibodies [3, 23, 25], and molecular biological techniques [9, 13]. In the case of *Toxoplasma gondii*, the tachyzoite and bradyzoite antigens recognized by the host vary depending on the stage of infection, and the different reacting patterns is used for differentiating infection stages in clinical diagnosis [19, 26–28]. However, the *N. caninum* antigens predominantly detected by the hosts at various infection stages are remaining to be specified. In order to select the most suitable Neospora antigen for the serological diagnosis, we examined the antigens recognized in mice at different stages of infection including the recrudescent stage following immunosuppressive treatment. The Neospora antigens recognized in the serum of naturally infected cattle were also investigated for the purpose of confirming whether the reacting pattern of mice could be adapted to that of cattle.

Eight, female, 6-week-old ICR mice (CLEA Japan, Inc., Tokyo) were used for preparation of serum samples. The three out of eight mice were immunosuppressed with 4 mg of prednisolone (prednisolone INJ.NZ, Nippon Zenyaku Kogyo, Inc., Fukushima, Japan) 7 days prior to and at the time of subcutaneous inoculation with 2,000,000 *N. caninum* tachyzoites of the BT3 strain [22]. Mouse antisera were obtained on post inoculation day(s) (PID) 15, 20, 25, 48, 77 and 125. They included sera collected from eight mice on PID 15, 20, and 25, seven mice on PID 48 and 77, three mice on PID 125 (Table 1). Antisera sampled on PID 125 were obtained from three mice in which immunosuppression was induced by continuous medication with prednisolone every fifth day from PID 75. Tachyzoites of strain BT3 were purified by the Percoll gradient method [29]. The purified tachyzoites were solubilized in 0.1% non-ionic detergent Triton X-100. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Tris-glycin buffer, pH 8.3, containing 0.1% SDS, on 12% polyacrylamide gels, under reducing conditions, according to the method of Laemmli [15]. The tachyzoite antigens separated by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride microporous membranes (Immobilon-P Transfer-Membranes, Millipore, Bedford, MA). Membranes were blocked for 5 min in phosphate-buffered saline (PBS) containing 2% skim milk, and then exposed to the test serum diluted 1:50 (mouse sera) or 1:200 (cattle sera) with PBS containing 2% skim milk for 1 hr. After a wash, the membranes were incubated for 1 hr with horseradish peroxidase-conjugated goat anti-mouse and anti-bovine immunoglobulin G (ICN Pharmaceuticals, Inc., Aurora, OH) diluted 1:500 with the same buffer as above. After another wash, immunoreactive proteins were detected with horseradish peroxidase substrate, 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO), according to the method of the manufacturer.

The *N. caninum* antibody titers of all mouse sera were strongly positive as determined by ELISA using solubilized tachyzoite antigen [21]. The antibody titers of eight naturally infected cows were above 1:1,600, according to the previously described IFAT [29]. The results of the Western blotting are shown in Fig. 1 and Table 1. Neospora antigens commonly recognized with mouse sera were 36–38, 43, and...
65 kDa in size. On PID 15–25, the 36–38 kDa antigen was clearly recognizable. On PID 48 and 77, the expression of the 36–38 kDa antigen weakened, whereas the 43 kDa antigen appeared and was detectable until PID 125. Furthermore, the 43 kDa antigen was clearly recognized by the antisera from recrudescent mice. Tachyzoite antigen of 65 kDa was detected from an early stage of infection and present until the chronic stage, however the results were inconsistent among individuals. All the sera from the cattle naturally infected with *N. caninum* invariably recognized the 43 kDa antigen (Data not shown).

The *N. caninum* antigens recognized by the serum of infected mice changed depending on the stage of infection. Tachyzoite antigen of 36–38 kDa was recognized until PID 25, but not easily detected after PID 48. On the other hand, all the serum samples collected after PID 48 reacted with the 43 kDa antigen. The 36–38 kDa antigen is a surface protein of tachyzoites and has not been detected in bradyzoites [9, 23]. Therefore, this molecule is considered useful for differentiating tachyzoites from bradyzoites [23]. In the present study, the antibody titer to 38 kDa antigen was elevated at the early stage of infection and disappeared until the activation of tachyzoites by immunosuppression. The results indicated that the active proliferation of tachyzoites in mice occurred at the early stage of infection and recrudescence.

Nc-p43 is a recently identified major surface protein which is functionally involved in adhesion to and invasion of host cells by this parasite [11]. The protein has been reported to be expressed in both tachyzoites and bradyzoites [9]. In our previous study, *N. caninum* was isolated from the brain of a naturally infected cow which was clinically normal except for repeated abortion [22]. This finding suggested that *N. caninum* infected latently in the brain was reactivated and infected the fetus transplacentally via the bloodstream. A serum antibody profile showing consistent increasing pattern during pregnancy also indicated a reactivation rather than a reinfection of the parasite at mid-gestation [24]. Therefore, *N. caninum* may be reactivated by factors such as pregnancy and immunosuppression in cows of latent infections. In the present study, we treated latently infected mice with an immunosuppressive drug and confirmed that the serum antibody of the recrudescent mice strongly reacted with various antigens, particularly the 43 kDa protein. Therefore, the 43 kDa antigen may be useful in immunological reactions to detect animals infected with *N. caninum* except in the early stage of the infection. Currently, ELISA using recombinant Nc-p43 has been proposed as highly specific and sensitive method for serodiagnosis of *N. caninum* infection [20]. Baszler, *et al.* [3] reported that 65 kDa antigen was recognized consistently and specifically in cattle infected with *N. caninum*. Nc-p65 of 65 kDa was found to be a proteolytic enzyme in *N. caninum* [18]. In the present study, the antibody titer against the 65 kDa antigen

### Table 1. Molecular weight of solubilized *N. caninum* tachyzoite antigens recognized by mouse sera at different time points post-infection with *N. caninum*

<table>
<thead>
<tr>
<th>Molecular weight of antigen (kDa)</th>
<th>Post infection (days)</th>
<th>No. (%)</th>
<th>No. (%)</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15–25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38</td>
<td>38</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>48–77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8 (100)</td>
<td>2 (25)</td>
<td>6 (75)</td>
</tr>
<tr>
<td></td>
<td>125&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3 (100)</td>
<td>7 (100)</td>
<td>5 (71)</td>
</tr>
<tr>
<td></td>
<td>(immunosuppressed)</td>
<td></td>
<td>3 (100)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>total examined</td>
<td></td>
<td>8</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

a) Post inoculation days 15, 20, or 25.

b) Post inoculation days 48 or 77.

c) Immunosuppression was induced by continuous medication with prednisolone every fifth day from post inoculation days 75.

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**Fig. 1.** Representative immunoblot of *N. caninum* antigens separated by SDS-PAGE under reducing conditions with sera from 15 (lane 1), 48 (lane 2), 125 (lane 3) days postinoculation, immunosuppressed in the chronic stage (lane 4), and uninfected (lane 5). Numbers on the left indicate molecular mass in kilodaltons.
was variable in individual mice and the antigen might not be suitable for the diagnostic system.

REFERENCES