Enzyme-Linked Immunosorbent Assays Based on *Neospora caninum* Dense Granule Protein 7 and Profilin for Estimating the Stage of Neosporosis

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*Neospora caninum* is an intracellular apicomplexan protozoan parasite that causes bovine and canine neosporosis, characterized by fetal abortion and neonatal mortality and by neuromuscular paralysis, respectively. Although many diagnostic methods to detect parasite-specific antibodies or parasite DNA have been reported, to date no effective serodiagnostic techniques for estimating pathological status have been described. Our study aimed to elucidate the relationship between the parasite-specific antibody response, parasite activation, and neurological symptoms caused by *N. caninum* infection by using a recombinant antigen-based enzyme-linked immunosorbent assay. Among experimentally infected mice, anti-*N. caninum* profilin (NcPF) antibody was only detected in neurologically symptomatic animals. Parasite numbers within the brains of the symptomatic mice were significantly higher than those in asymptomatic animals. In addition, anti-NcPF and anti-NcGRA7 antibodies were mainly detected at the acute stage in experimentally infected dogs, while anti-NcSAG1 antibody was produced during both acute and chronic stages. Furthermore, among anti-NcSAG1 antibody-positive clinical dogs, the positive rates of anti-NcGRA7 and anti-NcPF antibodies in the neurologically symptomatic dogs were significantly higher than those in the non-neurologically symptomatic animals. Our results suggested that the levels of anti-NcGRA7 and anti-NcPF antibodies reflect parasite activation and neurological symptoms in dogs. In conclusion, antibodies against NcGRA7 and NcPF may have potential as suitable indicators for estimating the pathological status of neosporosis.
MATERIALS AND METHODS

Parasite preparation. Tachyzoites of *N. caninum* strain Nc-1 (12) were propagated in monkey kidney adherent fibroblasts (Vero cells) cultured in Eagle’s minimum essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum. Purification of tachyzoites involved washing the parasites and host cell debris in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS before being passed through a 27-gauge needle and a 5.0-μm prefilter (Millipore, Bedford, MA).

Construction and expression of recombinant NcPF. CDNA was synthesized from RNA isolated with TRI reagent (Sigma-Aldrich), and we used a SuperScript first-strand synthesis system for reverse transcription-PCR (RT-PCR; Invitrogen, Carlsbad, CA). cDNA was used as a template to amplify the coding region of NcPF (accession number BK006901). Recombinant NcPF (rNcPF), which consisted of 163 amino acids (aa), was cloned using a designed set of oligonucleotide primers that included an EcoRI restriction enzyme site (shown in bold) in the forward primer (5’-ATTGACTACGGACTGGATTCG-3’) and an XhoI restriction enzyme site (5’-TACCGTCTAGTAACTGAGCGC-3’). PCR products were digested with EcoRI and XhoI before being ligated into a glutathione S-transferase (GST) fusion protein in the *Escherichia coli* expression vector pGEX-4T1 (GE Healthcare, Buckinghamshire, United Kingdom) that had been digested with the same set of restriction enzymes (pGEX-NcPF). Plasmid nucleotide sequences were determined using an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA). rNcPF was expressed as GST fusion proteins in the *E. coli* DH5α strain (Takara Bio Inc., Shiga, Japan). GST tags of the recombinant proteins were removed with thrombin proteases (GE Healthcare) according to the manufacturer’s instructions.

Expression of recombinant proteins of *NcSAG1* and *NcGRA7*. Recombinant *NcSAG1* (rNcSAG1) and *NcGRA7* (rNcGRA7) proteins were expressed in *E. coli* as GST fusion proteins and then purified using glutathione-Sepharose 4B as described previously (4, 16).

Mice and infection. Twenty-one (first trial) and 31 (second trial) 7-week-old female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). All mice used in the present study were treated under the guidance principles for the care and use of research animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine, Japan. They were intraperitoneally inoculated with 1 × 10⁷ tachyzoites of the *N. caninum* Nc-1 strain. Survival rates and clinical findings of the infected mice were monitored until 49 days and 44 days after infection in the first and second trials, respectively. Five and six mice from the first and second trials, respectively, exhibited clinical signs of neosporosis, including head tilt, limb paralysis, circling motion, and febrile response (starry stiff coat). Five and six asymptomatic mice were identified in the first and second trials, respectively, Eleven and 19 mice from the first and second trials, respectively, died before the end of monitoring.

Serum (20 μl) was obtained weekly from mice via the tail vein and used to measure levels of *N. caninum*-specific antibodies by ELISA, Blood was centrifuged at 1,000 × g for 10 min, and serum was collected and stored at −20°C until use. To confirm the lack of an antibody response in uninfected mice, control sera were taken from all animals 3 days before infection. Thereafter, all surviving mice were killed using a high level of anesthetic for postmortem examination at the end of monitoring. Tissue samples (liver, kidney, brain, spleen, lung, and heart) were obtained for DNA extraction and real-time PCR analysis. Tissues were stored at −20°C until use.

Dogs and infection. Four purebred female specific-pathogen-free (SPF) beagle dogs (14 to 15 months old) were used in this study. All dogs were purchased from Chugai Medical Animal Institute (Nagano, Japan) and were housed in separate rooms. Prior to experiments, dogs were proven to be free of *N. caninum*-specific antibody by ELISA based analysis of lysates of *N. caninum* (18) and rNcSAG1 as described below. They had never consumed uncooked meat or meat by-products and were fed dry dog food for the duration of the experiment. Dogs were released into a room for several hours each day to permit exercise. They were intravenously inoculated with 2 × 10⁷ tachyzoites of *N. caninum* strain Nc-1. Survival rates and clinical findings of the infected dogs were monitored until 24 weeks postinfection. Infected dogs showed no clinical symptoms or death until the end of the monitoring. Blood was collected from the saphenous vein and centrifuged at 1,000 × g for 10 min, and then the serum was collected and stored at −20°C until use. Dog experiments were conducted under the guiding principles for the care and use of research animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine, Japan.

Dog serum samples from animal hospitals. Clinical serum samples from dogs (n = 27) that exhibited neurological symptoms, such as disturbance of motility, were obtained from the Animal Medical Center of Yamaguchi University, Japan. Clinical serum samples from non-neurologically symptomatic dogs (n = 143) were collected from animal hospitals located in 35 prefectures of Japan. All serum samples were screened to detect *N. caninum* infection by rNSAG1-based ELISA as described below. Eighteen neurologically symptomatic and 45 non-neurologically symptomatic dog samples were considered *N. caninum* positive.

Measurement of *N. caninum*-specific antibodies by ELISA. Fifty microtiter plates were coated with purified rNcPF, rNcSAG1, rNcGRA7, and control GST, at a final concentration of 0.1 μg/ml, and used to coat ELISA plate. N. caninum* infection by rNSAG1-based ELISA as described below. Eighteen neurologically symptomatic and 45 non-neurologically symptomatic dog samples were considered *N. caninum* positive.

DNA extraction. Collected mouse tissues were thawed in a 10× volume of lysis buffer (0.1 mM Tris-HCl [pH 9.0], 0.1 M NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate) containing proteinase K (100 μg/ml; Sigma-Aldrich) and incubated for 4 days at 50°C. RNAase A (100 μg/ml; Sigma-Aldrich) was then added and incubated for 1 h at 37°C. Tissue DNA was extracted by phenol-chloroform-isooamyl alcohol followed by ethanol precipitation and resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The DNA concentration was adjusted to 50 ng/μl for each tissue sample, and the DNA was used as a template for real-time PCR analysis.

Real-time PCR. Oligonucleotide primers for the *N. caninum* Nc5 (GenBank accession number X84238) (20) were designed to amplify a 76-bp DNA fragment (5). The *N. caninum* Nc5 forward primer spanned nucleotides 248 to 257 (5’-ACTGGAGGACGCCTGAACAC-3’), and the *N. caninum* Nc5 reverse primer spanned nucleotides 303 to 323 (5’-AACAATGCTCAGCAAGGGA-3’). The PCR mixture was amplified in a 20-μl reaction volume containing 10 μl of 2× SYBR Green qPCR Mastermix (Bio-Rad Laboratories, Hercules, CA), 0.2 μl of 10 μM forward primer, 0.2 μl of 10 μM reverse primer, and 5 μl of DNA template in a real-time PCR system (Bio-Rad).
(25 μl, total volume) contained 1× SYBR green PCR buffer, 2 mM MgCl2, 200 μM concentrations of deoxynucleoside triphosphates dATP, dCTP, and dGTP, 400 μM dUTP, 0.625 U AmpliTaq Gold DNA polymerase, 0.25 U AmpErase UNG (uracil-N-glycosylase), all of which were included in the SYBR green PCR core kit (Applied Biosystems), 20 pmol of each primer, and 1 μl of DNA template (50 ng). Amplification was performed by a standard protocol recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min). Amplification, data acquisition, and data analysis were carried out using an ABI 7700 Prism sequence detector machine, and the calculated cycle threshold (C_T) values were exported to Microsoft Excel for analysis. Quantification was determined from the C_T values, based on the cycle at which the fluorescence exceeded the standard deviation of the mean baseline emission for the early cycles by 10-fold. The parasite number in the samples was calculated by interpolation of the standard curve, in which C_T values were plotted against the log of a known concentration of parasites. After Neospora Nc5 sequence amplification, the melting curves of PCR products were acquired by stepwise increases of the temperature from 55°C to 95°C for 20 min. Data analyses were performed using the Dissociation Curves software (version 1.0 f; Applied Biosystems).

**Statistical analysis.** Significant differences (P < 0.05) were calculated by using the chi-square test and Student’s t test.

**RESULTS**

*N. caninum*-specific antibodies in experimentally infected mice. To examine the relationship between neurological symptoms and *N. caninum*-specific antibody production, the IgG1 and IgG2a antibody responses against rNcSAG1, rNcGRA7, and rNcPF in experimentally infected mice were measured by ELISA. Initially, *N. caninum*-infected mice were grouped into dead, neurologically symptomatic, and asymptomatic animals. Furthermore, parasite numbers in the brains of neurological symptomatic mice were significantly higher than those of asymptomatic mice (Fig. 1A). This result revealed the association of parasites within brains of animals with neurological symptoms. In addition, there were no significant differences in the number of parasites in the spleen, heart, and lungs of neurologically symptomatic and asymptomatic mice (Fig. 1B to D).

Levels of IgG1 and IgG2a antibodies against rNcSAG1 increased from 7 days postinfection; thereafter, these antibodies remained at high levels until the end of the experiment (Fig. 2A and B). The detection of anti-rNcGRA7 antibody production was delayed by 1 week compared with that of the anti-rNcSAG1 antibody (Fig. 2C and D). Furthermore, we tested the sera at higher dilutions to confirm the differences in the serum antibody levels between the assessed mouse groups (Fig. 3). Antibody levels of anti-rNcSAG1 were higher than those of anti-rNcGRA7 at 7 and 14 days after infection. However, there were no marked differences in antibody responses against rNcGRA7 and rNcSAG1 among dead, neurologically symptomatic, and asymptomatic mice (Fig. 2A to D and 3). Interestingly, antibodies against rNcPF showed unique production dynamics. The anti-NcPF IgG1 antibody was detected in 3/5 neurologically symptomatic mice and in 2/11 dead mice, while the levels of this antibody were low in the asymptomatic mice (Fig. 2E). Moreover, high anti-NcPF IgG2a antibody production was observed in 4/5 neurologically symptomatic mice and 7/11 dead mice, while only 1 mouse from the asymptomatic group produced this antibody (Fig. 2F).

**Anti-*N. caninum*-specific antibodies in experimentally infected dogs.** ELISAs were used to measure IgG antibody responses against rNcGRA7, rNcSAG1, and rNcPF in experimentally infected dogs (Fig. 4) to investigate changes in antibody responses. All dogs infected with *N. caninum* produced IgG antibodies

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**FIG 1** Real-time PCR for determining *N. caninum* numbers in various organs of mice at 49 days after infection. (A) Brain; (B) spleen; (C) heart; (D) lungs. Group 1 contained mice exhibiting neurological symptoms (n = 5); group 2 comprised asymptomatic mice (n = 5). *, significant difference (P < 0.05), calculated with Student’s t test. The parasite DNA was not detected in kidney or liver. Reproducibility of the data was confirmed in two independent experiments, which both gave similar results.
against the lysates of this protozoan (data not shown). Serum antibody levels against rNcSAG1 peaked at 21 days after infection. Thereafter, the antibody level gradually decreased (Fig. 3A). The antibody levels against rNcGRA7 peaked between 14 and 21 days after infection, which were similar to the dynamics for anti-rNcSAG1 antibody production (Fig. 3B). However, antibody levels as a response against rNcGRA7 decreased quicker than those against rNcSAG1 (Fig. 3A and B). Furthermore, anti-rNcPF antibodies were detected at 14 to 21 days after infection and rapidly decreased (Fig. 3C). At 112 days after infection, serum antibodies against rNcPF were not detectable, although production of antibodies against rNcSAG1 and rNcGRA7 was still observed (Fig. 3). We further observed that anti-rNcPF antibody levels varied among animals.

Evaluation of anti-N. caninum-specific antibody levels in dogs exhibiting neurological symptoms. Our results suggested that anti-rNcPF antibody might be associated with the progression of neurological symptom. To investigate the relationship between the antibody response and neurological symptoms caused by N. caninum infection, dog serum samples from animal hospitals were examined (Table 1). To determine N. caninum infection status, serum samples were screened using an rNcSAG1-based ELISA, because anti-NcSAG1 antibody was detected at both the acute and chronic stages of the infection, as shown in Fig. 4A. Serum samples were then grouped into neurologically symptomatic and non-neurologically symptomatic dogs. Positive rates of anti-rNcGRA7 and anti-rNcPF antibodies in the neurologically symptomatic dogs were significantly higher than those in the non-neurologically symptomatic animals (P < 0.05) (Table 1). Four anti-rNcGRA7-positive and four anti-rNcPF-positive dogs were found in nine anti-NcSAG1-negative and neurologically symptomatic dogs (data not shown).

FIG 2 Production of IgG1 (A, C, and E) and IgG2a (B, D, and F) antibodies against rNcSAG1 (A and B), rNcGRA7 (C and D), and rNcPF (E and F) in mice infected with N. caninum tachyzoites. Serum samples diluted 1:250 were tested by ELISA. Blue lines indicate asymptomatic mice. Red lines indicate neurological symptomatic mice. Black lines correspond to mice that died during the experimental time course. Reproducibility of the data was confirmed by two independent experiments, which both gave similar results.
the levels of IgG antibody against rNcGRA7 in experimentally infected dogs decreased from 30 days after infection. These dynamics of anti-rNcGRA7 antibody production may relate to parasite activation. In clinical samples of anti-rNcSAG1 antibody-positive dogs, positive rates of IgG antibody against rNcGRA7 in neurologically symptomatic dogs were significantly higher than those in the non-neurologically symptomatic animals. This result suggested that neurological signs caused by \textit{N. caninum} infection might coincide with parasite activation.

The dynamics of anti-rNcPF antibody production were different between mice and dogs following \textit{N. caninum} infection. In mice, anti-NcPF antibody was detected in neurologically symptomatic animals but not in asymptomatic ones. In contrast, experimentally infected dogs produced anti-NcPF antibody only at the acute stage. The kinetics of antibody production, as assessed by determination of serum levels in intravenously infected dogs or intraperitoneally infected mice may not be the same as in naturally infected animals, in which the parasite disseminates from the gastrointestinal tract. Although such a difference in anti-NcPF antibody production may be due to differences between the host species or inoculation route of the parasite, we currently have no exact explanation for this.

Given that NcPF lacks a signal peptide and is a cytosolic protein (19), we therefore speculated that large amounts of NcPF are required to stimulate specific antibody production. During the acute stage of \textit{N. caninum} infection, host immune cells would control the parasite burden. For instance, T cells, such as CD8$^+$ T cells, kill the \textit{N. caninum}-infected host cells, and macrophage killing of the parasites supposedly occurs intracellularly, resulting in the release of NcPF from the dead parasites. Otherwise, NcPF may be released from the large number of free parasites during the activation stage or during host cell invasion. This released NcPF might be the antigen that stimulates antibody production. In mice, high levels of anti-NcPF antibody production may be due to the reactivation stage of \textit{N. caninum} in neurologically symptomatic animals. In the experimentally \textit{N. caninum}-infected dogs, high levels of anti-NcPF antibody production at the acute stage may reflect host immunity associated with infection control. In addition, NcPF itself may stimulate immune responses, because gamma interferon production was induced in mice after NcPF inoculation (19).

The number of parasites in brains of mice that exhibited neurological symptoms was higher than that in asymptomatic animals. This result indicated that neurological symptoms might be caused by \textit{N. caninum} infection of the central neuron system (CNS). Furthermore, anti-rNcPF antibody levels may correlate with parasite numbers in the CNS. Brain lesions examined by magnetic resonance imaging in neurologically symptomatic dogs were mainly found in both the cerebrum and cerebellum (data not shown). However, there were no statistically significant differences for the region of the brain lesions between neurologically symptomatic anti-rNcSAG1-negative versus -positive dogs. Furthermore, we analyzed the correlation of brain lesions with anti-NcGRA7 and anti-NcPF serum positivity. However, there was no significant correlation (data not shown). This result was supported by a previous study that suggested neosporosis is an important cause of progressive cerebellar ataxia and cerebellar atrophy in adult dogs (14). Thus, \textit{N. caninum} infection of the CNS, especially the cerebrum, is likely to cause neurological symptoms in infected animals.
In summary, we have described the possible use of NcGRA7 and NcPF recombinant proteins as useful diagnostic tools for dogs exhibiting neurological signs. To date, definitive diagnosis of neosporosis has been conducted based on immunohistochemical staining of *N. caninum* in neural tissue. Reliable ante-mortem diagnosis of neosporosis is necessary before an effective therapeutic strategy can be initiated. Our recombinant antigen-based ELISA may replace soluble extract-based ELISAs or tachyzoite-based IFAT in routine serodiagnosis and PCR assays to detect parasite DNA from CSF, and it may have the additional advantage of being capable of estimating pathological status. Increased levels of antibodies against NcSAG1, NcGRA7, and NcPF in dogs exhibiting neurological symptoms are most likely indicative of current *N. caninum* infection. In addition, by measuring parasite activation using rNcGRA7-based and rNcPF-based ELISAs, we might detect neosporosis earlier than via the direct observation of symptoms. Treatment of canine neosporosis is difficult and shows only partial effects (17). Therefore, to obtain effective therapeutic effects, treatment should be started before muscular contracture has occurred (8). The relevance of our findings for the clinical field must take into account that dogs likely will be checked for neosporosis once symptoms are noticed. In addition, the use-

### TABLE 1

| Groups                        | Anti-rNcGRA7 | Anti-rNcPF |
|-------------------------------|--------------|------------|
| Neurologically symptomatic    | 66.7 (12/18) | 66.7 (12/18) |
| Non-neurologically symptomatic| 35.6 (16/45) | 26.7 (12/45) |

*Serum samples screened by rNcSAG1-based ELISA were used. Serum samples were grouped into neurologically symptomatic (n = 18) and non-neurologically symptomatic (n = 45) dogs. Percentages of positive samples (number of positive/number of total rNcSAG1 positive) are shown, and the significant difference was calculated by chi-square test (P < 0.05).*

*Significant difference for the percentage positive between neurologically symptomatic and non-neurologically symptomatic dogs for the same antigen.*
fulness of this method for the economic relevant bovine host should be evaluated in future studies.

ACKNOWLEDGMENTS

We thank J. P. Dubey (U.S. Department of Agriculture, Agriculture Research Service, Livestock and Poultry Sciences Institute, Parasite Biology and Epidemiology Laboratory) for the gift of the N. caninum Nc-1 isolate.

This research was supported by the Japan Society for the Promotion of Science through the funding program for Next Generation World-Leading Researchers (NEXT Program), initiated by the Council for Science and Technology Policy (2011/LS003).

We declare no conflicts of interest.

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