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Usefulness of *Neospora caninum* dense granule 1 based-iELISA for the detection of canine neosporosis

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ABSTRACT

Neospora caninum is an apicomplexan parasite that causes abortion in cattle and sheep. Dog is the main host of *N. caninum* and infected one shows neurologic disorders. Accurate diagnosis is important particularly under lacking of treatment and vaccination measures. Herein, we targeted the evaluation of diagnostic ability of recombinant protein NcGRA1(rNcGRA1) for detection of specific antibody (IgG) against *N. caninum* in mice and dogs. The rNcGRA1 was expressed using the *E. coli* expression system. Diagnostic ability of rNcGRA1 was tested against experimental mouse and dog sera, as well as field dog sera. Simultaneously, same serum samples were tested against rNcSAG1 and *Neospora* lysate antigen (NLA)-based iELISA. In the *N. caninum* experimentally infected mice, specific IgG antibodies to NcGRA1 were detectable from 14 days post infection (dpi), and increased at 28 dpi with no detectable antibody levels in the negative control or *Toxoplasma gondii* infected-mouse sera. In experimentally infected dog sera, the performance of rNcGRA1 was proficient in demonstrating IgG antibody at 28 dpi. In case of field dog sera (N=18), seropositive rate was 44.4% and 22.2% for rNcGRA1 and rNcSAG1-based iELISAs, respectively. These results show that the rNcGRA1 is a novel useful diagnostic antigen for serological detection of *N. caninum* in dogs.

Keywords: *Neospora caninum*; Neosporosis; NcGRA1; ELISA; Dog

INTRODUCTION

Neospora caninum is a coccidian parasite with a wide range of intermediate hosts. Family Canidae is the final host that harbors the sexual stage of the parasite, sheds the oocyst in its feces, which is the only source of horizontal infection for the intermediate host. Ruminants and equids have been reported with some other wild and domestic animal species as intermediate hosts. The parasite transmitted horizontally in cattle and dogs via ingestion of infective oocyst or tissue cyst, respectively, while vertical transmission from the dam to fetus plays a key role in the epidemiology of bovine neosporosis (Dubey and Schares, 2011). Abortion and fetal abnormalities are the main signs of the disease in cattle. In congenitally infected pups, the parasite destroying the neural cells, causing neuromuscular paralysis. Sulfonamides and/or pyrimethamine, and clindamycin can be used successfully in the treatment of canine neosporosis, depending on the stage of infection (Dubey, 2003).

Various diagnostic methods have been developed to evaluate the infection status in bovine or canine neosporosis. The indirect fluorescent antibody test (IFAT), Indirect enzyme-linked immunosorbent assay (iELISA), immunoblotting and various agglutination tests were employed to detect anti-*N. caninum* antibodies in sera of infected animals. However, iELISA has many advantages including the time saving and capability of using for high number of samples. iELISA using single antigen provides an excellent opportunity to avoid cross-reactivity with closely related parasites (Dubey et al., 1996). Surface antigens (NcSAG1, NcSRS2) and numerous of dense granule antigens (NcGRA2, NcGRA6, NcGRA7) have been successfully used for detection of specific antibodies in infected cattle or dog mostly using iELISA (Sinnott et al., 2017).

Neospora caninum dense granule 1 (NcGRA1) was identified from tachyzoites by proteomics analysis (Lee et al., 2003). NcGRA1 secreted and accumulated in the cyst wall, it could be involved in formation of the tissue cyst wall during tachyzoite to bradyzoite conversion stage (Vonlaufen et al., 2004). The polypeptides encoded by NcGRA1 target for the host immune system during chronic infections of *N. caninum* in mouse and cattle (Atkinson et al., 2001). The antigen was evaluated as a vaccine candidate using pregnant mice model with low efficacy (Ellis et al., 2008). The diagnostic performance of rNcGRA1 was reported against sheep and donkey sera using DOT- ELISA test (Joaquin Patarroyo et al., 2013; Blanco et al., 2014). However, there is no report regarding its use as a diagnostic antigen for canine neosporosis or as a based antigen for the development of iELISA test.

Several studies have been conducted to identify and characterize the antigens of *N. caninum*, in order to improve the performance of serological diagnostic tests, and finally to participate in the control measures against *Neospora* infection (Bjerkas et al., 1994; Sinnott et al., 2017). However, further studies are required to identify more diagnostic antigens for detection of neosporosis, particularly in dog, for its epidemiological importance of disease transmission. In the current study, we have expressed the recombinant NcGRA1 (rNcGRA1) using glutathione S-transferase (GST) fusion protein in the *E. coli* expression system and evaluated its diagnostic ability against a number of experimental and field dog serum samples using the iELISA. The obtained results provide a promising novel serodiagnostic tool for specific antibody detection of *N. caninum* in dog.

MATERIALS AND METHODS

Ethics statement

All animal experiments strictly followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The study protocol was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers: 24-17, 25-66, 18-15). For mice, general anesthesia with isoflurane was conducted before painful experiments.

Experimental animals

Female BALB/c mice aged 6–7 weeks were obtained from Clea Japan (Tokyo, Japan). The mice were housed in cages in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan under specific-pathogen-free conditions. Experimentally infected dog sera, we evaluated the same samples used in a previous study (Hiasa et al., 2012). Briefly, four specific-pathogen-free (SPF) female beagle dogs (14 to 15 months old) were purchased from Chugai Medical Animal Institute (Nagano, Japan) and were housed in separate rooms. All dogs were tested by iELISA based analysis of NLA and rNcSAG1 to prove that they are free from *N. caninum*-specific antibodies.

Parasites and host cell cultures

The *N. caninum* (Nc-1 strain) was maintained in African green monkey kidney epithelial cells (Vero cells) cultured in Eagle's minimum essential medium (Sigma, St Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan) and 1% streptomycin–penicillin (Sigma). For purification of tachyzoites, parasites were washed in sterile phosphate-buffered saline (PBS), and then a cell scraper (BD Bioscience, San Jose, CA, USA) used to separate the infected cell monolayer. Finally, the cell pellet was resuspended in RPMI 1640 medium (Sigma) using a 27-gauge needle and passed through a 5 μm filter (Millipore, Bedford, MA, USA).

Cloning of the NcGRA1 gene into pGEX-4T-1 vector

To clone the *NcGRA1* gene (ToxoDB: NCLIV_036400), a pair of oligonucleotide primers with restriction enzyme sites was designed and used for the amplification of the *NcGRA1* gene from the *N. caninum* (Nc-1 strain) cDNA (forward primer: 5′- AA GGA TCC ATG GTT GCA GCC TCG GTG GTT -3′; reverse primer: 5′- GC GAA TTC TTA ATG TTG CCC TTG AAG -3′). The PCR product was ligated to the *BamH*I (underlined sequence in forward primer) and *EcoR*I (underlined sequence in reverse primer) restriction sites of the pGEX-4T-1 expression vector (GE Healthcare Life Sciences, UK). The resulting plasmid construct was checked for accurate insertion by digestion with restriction enzyme*s* according to the manufacturer instructions (ROCHE Diagnostics, Germany), and by sequencing using a Big Dye Terminator Cycle Sequencing Kit, and an ABI PRISM3100 genetic analyzer (AB Applied Biosystems, Carlsbad, CA, USA).

Expression and purification of rNcGRA1 in *E. coli*

An open reading frame (ORF) of the *NcGRA1* gene was expressed as GST fusion protein in the *E. coli* BL21 (DE3) strain according to the manufacturer's instructions (New England BioLabs Inc., Ipswich, MA, USA). Expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan) for 6 h at 37°C. Bacterial cells were harvested and the pellets were suspended in sonication buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol) then centrifuged at 7,180 × *g* at 4°C for 10–15 min. Lysozyme (final concentration of 500 μg/mL) and Triton X-100 (10%) in PBS were added followed by incubation on ice for 1 h. The lysate

was applied to Glutathione Sepharose 4B beads (GE Healthcare Life Sciences, Buckinghamshire, England) according to the manufacturer's instructions. Briefly, the supernatant was incubated with washed beads overnight at 4°C with gentle rotation. GST fusion proteins were eluted with elution buffer (100 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 5 mM EDTA, and 25 mM reduced glutathione powder; Wako Inc). The quantity and purity of each protein were determined by Sodium dodecylsulfate polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue R250 (MP Biomedicals Inc., Illkirch-Graffenstaden, France). The protein concentrations were assayed with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The recombinant proteins, NcGRA1-GST, and GST, were expressed abundantly with apparent molecular weights, 45 and 26 kDa, respectively, consistent with the expected molecular weights (Fig. 1).

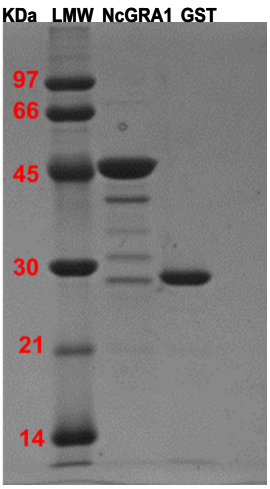

Fig. 1. SDS-PAGE of purified recombinant antigens. All recombinant proteins were expressed as GST fused protein and obtained with apparent molecular weights consistent with the expected molecular sizes of each protein. KDa; kilodalton, LMW; low molecular weight marker.

Preparation of lysate antigen

Lysate antigen from purified tachyzoites of *N. caninum* was prepared as described previously (Liao et al., 2005). The obtained extract was filtered through a 0.45 μm low-protein binding Supor® membrane (Pall Life Sciences, Ann Arbor, MI, USA), and the concentration was measured using a BCA protein assay kit.

Sera from experimentally-infected mice

A total 18 mice were divided into three groups representing non-infected (n = 6), acute (n = 6), and sub-acute infection (n = 6) groups. For infected sera, samples were collected from mice at 14 dpi, and 28 dpi. Blood samples were collected via cardiac puncture and added in tube without anticoagulants. The blood was incubated for 30 min at room temperature, and then centrifuged at 5,000 × *g*, 20 min, 20°C. After separation of serum from coagulated blood, it was centrifuged again at higher speed to harvest high purity serum and kept at -30°C until use.

Experimental and field dog serum samples

The samples evaluated in our study had been prepared and used in our previous study (Hiasa et al., 2012). In brief, four females of SPF beagle dogs (14–15 months) were intravenously inoculated with *N. caninum* Nc-1 strain tachyzoites (2×10^6). Clinical serum samples from dogs (n=18) were collected from animal hospitals located in Japan. The reactivity of the sera from experimentally infected animals was confirmed by a commercial immunofluorescent antibody test slide (VMRD, Pullman, WA, USA) and iELISA based on

rNcSAG1 (Hiasa et al., 2012; Abdelbaky et al., 2018). Sera collected at 0- and 28-days post infection were used in this study.

iELISA

iELISAs were performed as reported previously (Abdelbaky et al., 2018) with slight modifications. An amount 50 μL of the recombinant proteins (0.1 μM) and GST (0.1 μM) and NLA (5 μg/mL) were added to each well of a 96-well microtiter plate (Nunc, Roskilde, Denmark). All used antigens were prepared in coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4°C in the plate. On the next day, the plates were washed once with PBST (0.05% Tween-20 in PBS) and blocked with 100 μL of 3% skim milk prepared in PBS (PBS-SM) for 1 h at 37 °C. The plates were washed once, then 50 μL of the test sera diluted with PBS-SM at 1:600 for mouse sera and 1:200 for dog sera was added to the wells. The plates were incubated for 1 h at 37°C. After washing with PBST six times, 50 μL of horseradish peroxidase-conjugated goat anti-mouse and goat anti-dog IgG antibodies (Bethyl Laboratories, Montgomery, TX, USA) diluted with PBS-SM at 1:15,000 and 1:4,000 in PBS-SM, respectively, was added to plates at 37°C for 1 h. An amount 100 μL of substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/mL 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); Sigma–Aldrich, St. Louis, MO, USA] were added to each well after further six times of washing. The plates were incubated at room temperature for 1 h. Then, absorbance at 415 nm was measured using a microplate reader (MTP-120; Corona, Tokyo, Japan). Absorbance values for rNcSAG1 and rNcGRA1 antigens were determined after subtraction of the optical density for GST at 415 nm. Cutoff values for iELISA were estimated using negative control *N. caninum* dog sera (n = 4), using formula of cutoff value = mean + 3 × standard deviation.

Statistical analysis

Data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). For statistical analysis, one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test, or Student's *t*-test were performed. A P value < 0.05 was considered statistically significant. Degrees of statistical significance are shown as asterisks (*) defined in each figure legend.

RESULTS

Assessment of recombinant antigens using sera from experimentally infected mice

First, the diagnostic performance of rNcGRA1 as a test antigen, in addition to rNcSAG1 and NLA as control antigens, was evaluated using control mouse sera using iELISAs. In case of mice, antigens were assayed against sera on non-infected, 14 dpi (acute infection), and 28 dpi (sub-acute infection). The highest IgG level was observed against rNcSAG1 followed by rNcGRA1 and NLA in *N. caninum*-infected mouse sera at 14 dpi. While in case of sub-acute infection, the antibody level of NcGRA1 was comparable to NcSAG1 and to lower extent to NLA. Noteworthy, no reactivity for the recombinant antigens was observed against sera from non-infected or *T. gondii*-infected animals, unlike NLA that

showed slight cross- reactivity against related parasite *T. gondii* (Fig. 2). This result indicates the ability of rNcGRA1 for detection of anti-*Neospora* antibodies either in acute or sub-acute infections with no cross-reactivity against *T. gondii*-infected sera in mice.

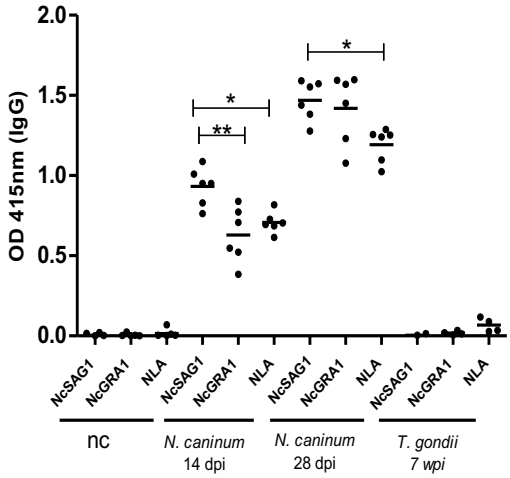

Fig. 2. Production of IgG antibodies in mouse sera against rNcGRA1, rNcSAG1 and *Neospora* lysate antigen (NLA). The mean optical density (OD) was determined at a wavelength of 415 nm. Each bar represents the mean of the tested sera (nc; sera from uninfected mice, n = 6, *N. caninum* 14 dpi; infected mice at 14 days post infection, n = 6, *N. caninum* 28 dpi; infected mice at 28 days post infection, n = 6, *T. gondii* 7 wpi; infected mice at 7 weeks post infection, n = 4). Statistically significant differences among the tested groups are shown as asterisks (*$P < 0.05$, one-way ANOVA plus Tukey–Kramer post hoc analysis).

Assessment of recombinant antigens using sera from experimentally and naturally infected dogs

Using sera from experimentally infected dog at 28 dpi, the three antigens, rNcSAG1, rNcGRA1 and NLA, were recognized by the specific IgG antibody (Fig. 3A). Although the levels of anti-NcSAG1 antibodies were higher than those of anti-NcGRA1 antibodies ($P < 0.05$), only antibody against NcGRA1 was not detectable in sera of non-infected control dogs (Fig. 3A). Regarding field dog sera (n = 18), these samples were tested using rNcGRA1 and rNcSAG1 as a control antigen. The prevalence rate based on rNcGRA1-iELISA (8/18; 44.4%) was higher compared with rNcSAG1-iELISA (4/18; 22.2%) (Fig. 3B). The two aforementioned antigens showed differences in the seropositive rates and antibody titers against IgG antibodies.

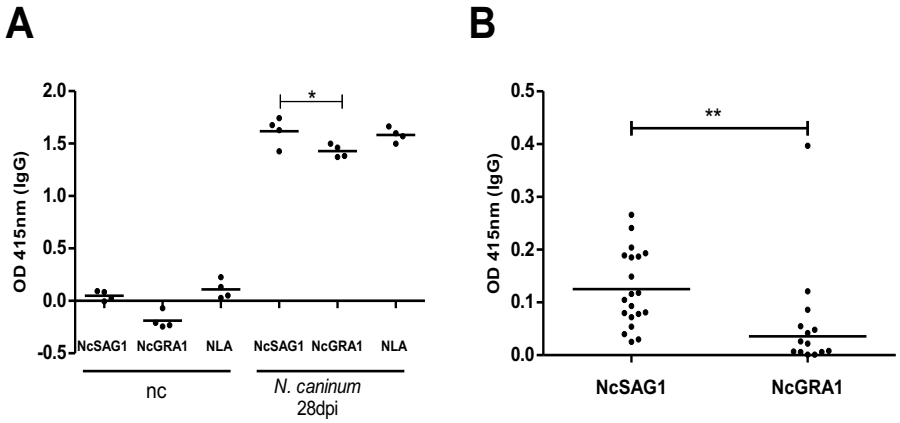

Fig. 3. The reactivity of rNcGRA1 and rNcSAG1 antigens against dog sera. (A) Performance of antigens against experimentally infected dog. Each bar represents the mean of tested sera (nc; uninfected dog sera, n = 4, *N. caninum* 28 dpi; infected dog sera collected at 28 days post infection, n = 6). Statistically significant differences among the tested groups are shown as asterisks (*$P < 0.05$, one-way ANOVA plus Tukey–Kramer post hoc analysis). (B) Performance of antigens against naturally infected dog sera. Each bar represents the mean of tested sera. Statistically significant differences among the tested groups are shown as asterisks (*$P < 0.05$, Student's *t*-test). The mean optical density (OD) was determined at a wavelength of 415 nm.

DISCUSSION

Regarding the definitive host and susceptibility to neosporosis, dog is epidemiologically important for transmission of *N. caninum*. Thus, the optimization and standardization of the diagnostic assays for detection of *N. caninum* infection are significant importance. To develop reliable diagnostic tool against *N. caninum* infection, diagnostic approaches using many antigenic proteins have been studied. Although there are numerous recombinant antigens can be used for iELISA to detect *N. caninum* antibodies in dogs, more serological assays are still needed for accurate diagnosis and further epidemiological investigations of risk factors and disease transmission.

Most of evaluated antigens have been used for testing cattle sera, while those used for dog are limited (Dubey et al., 1996; Sinnott et al., 2017). Moreover, some of previously tested antigens in dog exhibited a difficulty of protein expression because of variable degrees of solubility as reported for NcGRA7 (Lally et al., 1996), and NcSAG1 (Dong et al., 2012). For example, NcGRA7, profilin (Hiasa et al., 2012) and NcGRA2 (Jin et al., 2015), as well as NcGRA6 (Ghalmi et al., 2014), mostly detect their specific antibodies in case of acute infection. On the contrary, anti-NcSAG1 antibody is detectable both acute and chronic stages of the infection in dogs (Hiasa et al., 2012).

In the current study, rNcGRA1 was expressed by *E. coli* expression system and purified as GST-fusion protein. This can be extrapolated from efficient diagnostic ability of such antigen through the testing against *N. caninum* experimentally-infected mouse and dog sera. For either mouse or dog control samples, the performance of rNcGRA1-based iELISA was proficient and comparable to the reference antigens NcSAG1 and NLA. In case of mice, high antibody level of NcGRA1 was observed in the sub-acute status in compare to the acute one, suggesting its predominant role in late stage of infection. This effect can be deduced from its high antibody level against chronically infected dog. This result also coincides with those reported before by Atkinson et al. (2001), where the authors identified NcGRA1 using chronically infected mouse and cattle sera. Together, NcGRA1 may play a role in the chronic stage of infection in mice, dogs, and cattle.

In case of sera from naturally infected dog, rNcGRA1-based iELISA detected a higher number of seropositive samples compared to rNcSAG1-based iELISA. This might be attributed to the lower cut-off value of rNcGRA1 (0.008) compared to the cut-off value of NcSAG1 (0.189). Noteworthy, cut-off value was calculated from negative control samples kept in our laboratory and tested via both antigens as experimental samples (Fig. 3A). Nevertheless, the high performance of rNcGRA1 in detecting *N. caninum* in experimentally infected mice and dog, with no reactivity against non-infected sera in such animals and also no cross-reactivity against *T. gondii*-infected mice is supposedly regarded as a promising

result for considering rNcGRA1 as a useful antigen for detecting anti-*Neospora* antibody in dog. However, formulation of rNcGRA1 antigen with some other specific antigens as rNcGRA7, rNcGRA6 and rNcGRA2 (Hiasa et al., 2012; Jin et al., 2015; Ghalmi et al., 2014), can be employed to obtain wide-spectrum diagnostic antigen preparation. Further studies are needed to confirm the diagnostic ability of rNcGRA1 using higher number of control and field sera from dogs and also other species.

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CONFLICT OF INTEREST

All authors declare no conflict of interest in the study.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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