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Short Communication

Identification of the antigenic region of *Neospora caninum* dense granule protein 7 using ELISA

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ABSTRACT

Dense granule protein 7 (NcGRA7) is a potent diagnostic antigen of *Neospora caninum.* Some studies have reported on the difficult expression, low yield, and variable degree of solubility of recombinant NcGRA7. We aimed to unravel the possible causes for these issues and tested NcGRA7 antigenicity in enzyme linked immunosorbent assays (ELISAs). The NcGRA7 coding sequence (217 amino acids) was split into five amino acid regions: NcGRA7m (27–217), NcGRA7m3 (27–160), NcGRA7m4 (27–135), NcGRA7m5 (161–190), and NcGRA7m6 (188–217). Three fragments, NcGRA7m, NcGRA7m3 and NcGRA7m4, exhibited high antigenic properties when tested against experimentally-infected mice and dog sera by ELISA. High levels of IgG2 antibodies against NcGRA7m3 were observed in field dog sera. In experimentally and naturally-infected cattle, the *N. caninum*-specific sera only reacted with NcGRA7m, indicating that this antigenic region differs among the three animal species. This study presents valuable information about the antigenic properties and topology of NcGRA7, and highlights its suitability for diagnostic purposes.

Neospora caninum, an obligate intracellular protozoan parasite, can infect a wide range of domestic and wild animals, and also inflicts economic losses on the cattle industry [1]. *N. caninum* is widely distributed globally, and the infection rate is generally 10 to 40%, occasionally reaching 80% [2]. The parasite is transmitted *via* the oral route and from the dam to the fetus in cattle and dogs [3].

Without any potent therapy or vaccine for both cattle and dogs, early and efficient diagnosis of *N. caninum* infection will assist the establishment of effective control policies against neosporosis [3]. High cost and cross reactions with *Toxoplasma gondii*, a related parasite, are the main disadvantages of most serodiagnostic tools (*e.g.*, *N. caninum* commercial kits in dogs and equids) [4, 5]. A recombinant protein-based, enzyme-linked immunosorbent assay (ELISA) could overcome these drawbacks, and *N. caninum* surface antigens and dense granules (NcGRAs) have been reported to be promising tools because both are highly specific, sensitive and practical to use [6, 7].

With its specificity of antibody detection in infected animals and lack of cross reactivity against closely related *T. gondii*, NcGRA7 has strong potential for use as a diagnostic tool [8–15]. Two hydrophobic regions have been identified in NcGRA7 (amino acids 1–26, and 139–157). The first hydrophobic region appears to represent the signal sequence, while the other contains a putative transmembrane region. Consequently, NcGRA7 protein is highly hydrophobic and completely insoluble [16, 17], and removing the signal peptide from the full-length gene greatly improved its expression in *Escherichia coli* [18].

In this study, we mapped the antigenic regions of NcGRA7 by splitting the coding sequence into five fragments and examined the reactivity of each fragment against sera from *N. caninum* experimentally-infected mice, dogs and cattle. In addition, field sera of dog and cattle were tested. Identifying the relationship between antigenicity and animal species could potentially enable this antigen to be used diagnostically.

Nc-1 strain *N. caninum* tachyzoites and PLK strain *T. gondii* tachyzoites were propagated using monolayers of African green monkey kidney (Vero) cells in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum. Tachyzoite purification was performed by washing the parasites and host-cell debris in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS before passage through a 27-gauge needle and a 5.0-μm-pre filter (Millipore, Bedford, MA, USA).

We tested each NcGRA7 fragment against the serum samples obtained from the mice, cattle and dogs. For the mouse sera, female BALB/c mice (8 weeks old) were purchased from Clea Japan. *N.*

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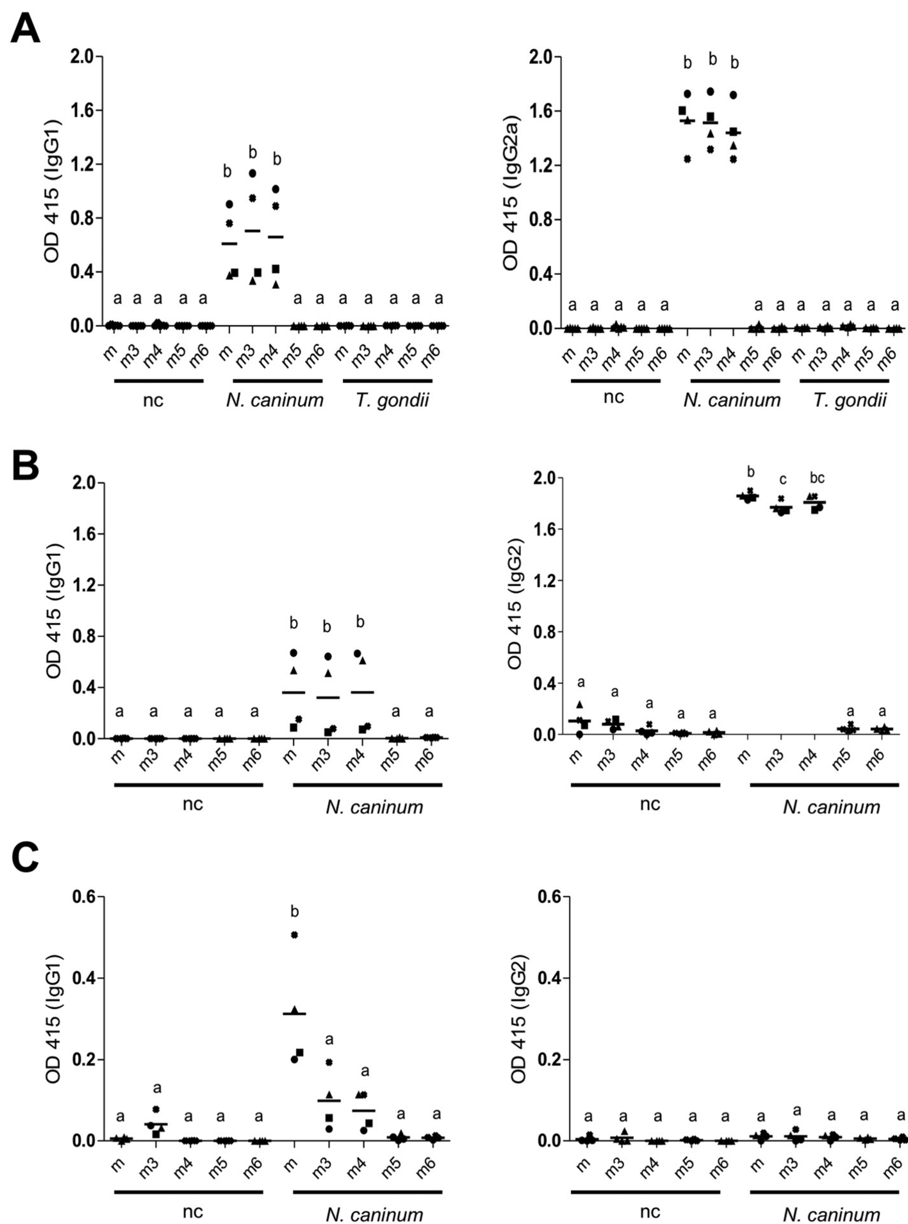

Fig. 1. Reaction of sera from experimentally infected animals. (A) Reaction of mouse IgG1 and IgG2a antibodies against the NcGRA7 fragments. (B) Reaction of dog IgG1 and IgG2 antibodies against the NcGRA7 fragments. (C) Reaction of cattle IgG1 and IgG2 antibodies against the NcGRA7 fragments. The data represent two independent experiments with similar results. The different letters above the bars in the graphs indicate statistically significant differences among the groups tested (one-way ANOVA plus Tukey–Kramer *post hoc* analysis, $P < 0.05$). m, NcGRA7m-GST. m3, NcGRA7m3-GST. m4, NcGRA7m4-GST. m5, NcGRA7m5. m6, NcGRA7m6. nc, sera from uninfected animals. Triangle, sample no. 1; circle, sample no. 2; ×, sample no. 3; square, sample no. 4.

caninum or *T. gondii* tachyzoites were intraperitoneally inoculated (1×10^5 and 1×10^3 tachyzoites respectively) into female BALB/c mice (9 weeks old, $N = 4$). Serum (200 μl) was obtained from mice at 0- and 7-weeks post-infection. The dog serum samples were the same as those used in a previous study [10]. Briefly, four purebred female specific pathogen-free (SPF) beagle dogs (14–15 months of age) were intravenously inoculated with 2×10^6 *N. caninum* Nc-1 strain tachyzoites. Sera collected at 0 and 28 days post-infection were used in this study. With the cattle, we evaluated the same samples used in a previous study [9]. Briefly, four serum samples collected from male Holstein calves aged 2–4 months at −13 and 29 days after intravenous infection with 1×10^7 tachyzoites of *N. caninum* Nc-1 strain were used. The reactivity of the sera from experimentally infected animals was confirmed by a commercial immunofluorescent antibody test slide (VMRD, Pullman, WA, USA) and ELISA based on recombinant NcSAG1 [9, 10]. Clinical serum samples from dogs ($N = 19$) were collected from animal hospitals located in Japan [10]. Cow serum samples ($N = 20$) were obtained from one Holstein dairy herd with a history of *Neospora*-associated abortions [9]. All serum samples were screened to detect *N. caninum* infection by NcSAG1-based ELISA [9, 10]. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 24-17, 25-66, 18-15).

The whole gene sequence of NcGRA7 (GenBank accession number, U82229.1) was sectioned into five fragments (Fig. S1A). Three of them (NcGRA7m, NcGRA7m3 and NcGRA7m4) were processed as recombinant proteins and two of them were synthetic peptides of NcGRA7m5 and NcGRA7m6 from Sigma Aldrich. The target sequences were PCR-amplified from *N. caninum* Nc-1 cDNA using oligonucleotide primers that included a restriction enzyme sequence, a sense primer (5′-AC **GAA TTC** CGC TGG AGA CTT GGC A-3′) for the three fragments, and the following anti-sense primers: NcGRA7m, 5′-GT **GAA TTC** CTA TTC GGT GTC TAC TTC CTG-3′; NcGRA7m3, 5′-GT **CTC GAG** TTA GAA TGT TCT CGC GAG ACC-3′; and NcGRA7m4, 5′-GT **CTC GAG** TTA ACG TTT TTT ACC GGG GAT-3′). *Eco*RI restriction enzyme sites were included in all the primers except the NcGRA7m3 and NcGRA7m4 anti-sense primers, which included *Xho*I sites. The digested PCR products were inserted into the pGEX-4 T3 plasmid vector treated with the same restriction enzymes. The recombinant fragments were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*

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