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 NcGRA7m 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.

Keywords: *Neospora caninum*; antigenicity; dense granule protein 7

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. 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') (Fig. S1A). *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-4T3 plasmid vector treated with the same restriction enzymes. The recombinant fragments were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* BL21(DE3), as described previously [6] with slight modifications. The purities and quantities of the NcGRA7m-GST, NcGRA7m3-GST, and NcGRA7m4-GST recombinant proteins were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue R250 staining (MP Biomedicals Inc., Illkirch-Graffenstaden, France). The three recombinant GST tagged proteins and GST itself were obtained with apparent molecular weights of 54 kDa, 38 kDa, 40.5 kDa and 27 kDa, respectively (Fig. S1B). Slight variations were observed in the apparent sizes of NcGRA7m3 and NcGRA7m4 than those expected of 40.7 kDa and 38 kDa, respectively.

Because we confirmed that the NcGRA7m3 and NcGRA7m4 DNA sequences in the plasmids were consistent with those of the target regions, the composition ratio of hydrophilic to hydrophobic amino acids may have caused the unexpected gel shift. The protein concentrations were measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

ELISAs were performed as reported previously [19] with slight modifications. Purified recombinant antigens or synthetic peptides (50 µl aliquots), each at a final concentration of 0.1 µM in carbonate-bicarbonate buffer (pH 9.6), were coated onto the ELISA plates (Nunc, Roskilde, Denmark) at 4°C overnight. The plates were washed once with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 3% skimmed milk (PBS-SM) for 1 h at 37°C. The plates were washed once with PBS-T, and 50 µl aliquots of the serum samples, diluted 1:600 for mouse and dog sera or 1:300 for cattle sera in PBS-SM, were added to the wells. The plates were incubated at 37°C for 1 h. After washing six times with PBS-T, the plates were incubated with horseradish-peroxidase (HRP)-conjugated IgG (Bethyl Laboratories, Montgomery, TX, USA) at 37°C for 1 h. HRP-conjugated goat anti-mouse IgG1 and IgG2a diluted 1:15,000 with PBS-SM, sheep anti-bovine IgG1 and IgG2 diluted 1:10,000 with PBS-SM, and goat-anti dog IgG1 and IgG2 diluted 1:15,000 with PBS-SM were used as the secondary mouse, cattle and dog sera, respectively. The plates were washed an additional six times before the 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-sulfonic acid) (Sigma-Aldrich) was added to each well in 100 µl aliquots. The absorbance at 415 nm after 1 h of incubation at room temperature was measured using an ELISA reader. The readings for the recombinant antigens were subtracted from those of the GST protein.

Using an IgG1 and IgG2a-based ELISA with the mouse sera, the three NcGRA7m, NcGRA7m3 and NcGRA7m4 fragments could differentiate *N. caninum*-infected mouse sera

from uninfected mouse sera, and no cross-reactivity against the *T. gondii*-infected mouse sera was observed (Fig. 1A). The reactivity of the three fragments against the *N. caninum*-specific mouse IgG1 and IgG2a did not differ. Furthermore, for the experimentally infected dog sera, compared with the negative control sera from them, the three fragments each reacted strongly against IgG2 in particular, and a statistically significant difference was observed between NcGRA7m and NcGRA7m3 (Fig. 1B). The IgG1 in the sera from the *N. caninum*-infected dogs reacted against the three fragments also, but with lower reactivity than of IgG2. The IgG1 and IgG2 antibody responses indicate the presence of Th2 and Th1-immune responses in the dogs, respectively [20]. Accordingly, our results suggest that anti-NcGRA7 IgG2 may be an indicator of the cellular immune response against *N. caninum* infection in dogs. In dog field sera, the three fragments (NcGRA7m, NcGRA7m3 and NcGRA7m4) showed low reactivity against the IgG1 antibodies compared with the reactivity of the IgG2 antibodies, indicating the similar results of the experimentally infected sera. In comparison with the reaction of IgG2 antibodies in the experimentally infected dog sera, the levels of IgG2 antibodies against NcGRA7m3 were higher than those against NcGRA7m and NcGRA7m4. These results suggest that the antigen structure of NcGRA7m3 is critical for the recognition of *N. caninum*-specific IgG2 antibodies (Fig. 2A). Additionally, these results revealed the high diagnostic capabilities of the three fragments (NcGRA7m, NcGRA7m3 and NcGRA7m4) for *N. caninum* infections in mice and dogs, and only the C-terminal regions of the synthetic peptides (NcGRA7m5 and NcGRA7m6) were unreactive (Fig. 1). The NcGRA7 fragment (amino acids 27 to 160) has potential, therefore, as a diagnostic tool for *N. caninum* infection in dogs.

Conversely, when the different fragments were tested against the cattle sera, high IgG1 levels were observed for the NcGRA7m fragment compared with NcGRA7m3, NcGRA7m4, NcGRA7m5 and NcGRA7m6 (Fig. 1C). No IgG2 reactivity against any fragment was detected, which is consistent with the Hiasa et al. study results (2012) [9]. In cattle field sera, IgG1

antibodies against NcGRA7 were predominant compared with the IgG2 antibodies. The levels of IgG1 antibodies against NcGRA7m were significantly higher than those of IgG1 antibodies against NcGRA7m3 or NcGRA7m4. These results were consisted with the results of the experimentally infected sera (Fig. 2B). Thus, when NcGRA7 undergoes fragmentation in cattle this can negatively affect its antigenic potential. This result suggests that the structure of the mature NcGRA7 without the signal peptide may be essential for IgG1-specific antibody detection, and the result is consistent with the observations of numerous reports that have indicated that NcGRA7 predominantly enhances humoral immune responses as indicated by high IgG1 production [9,21]. Interestingly, our results revealed antibody production differences against NcGRA7 among mouse, dog and cattle, suggesting a species-specific immune response against *N. caninum* infection in the different host animals. Distinct variation in the humoral mechanisms against *N. caninum* infection has also been reported for different cattle breeds [22]. This variation in antibody production may be attributed to functional differences in the immunoglobulin subclasses among the host species [23]. Furthermore, genetic diversity in the IgG and cell-mediated effector molecule repertoires can determine the types and levels of the immune responses triggered, including antibody production [24]. In particular, the enormous variation in the genetic makeup of the major histocompatibility complex not only among different animal species but also within a species, are considered to underlie the species-specific immune response [25]. In conclusion, the current study presents valuable information about the antigenic regions of NcGRA7. The varied performance of NcGRA7 with cattle and dog sera highlights the need to consider the species factor when developing a diagnostic tool based on this antigen.

Acknowledgments

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Conflict of interest

None.

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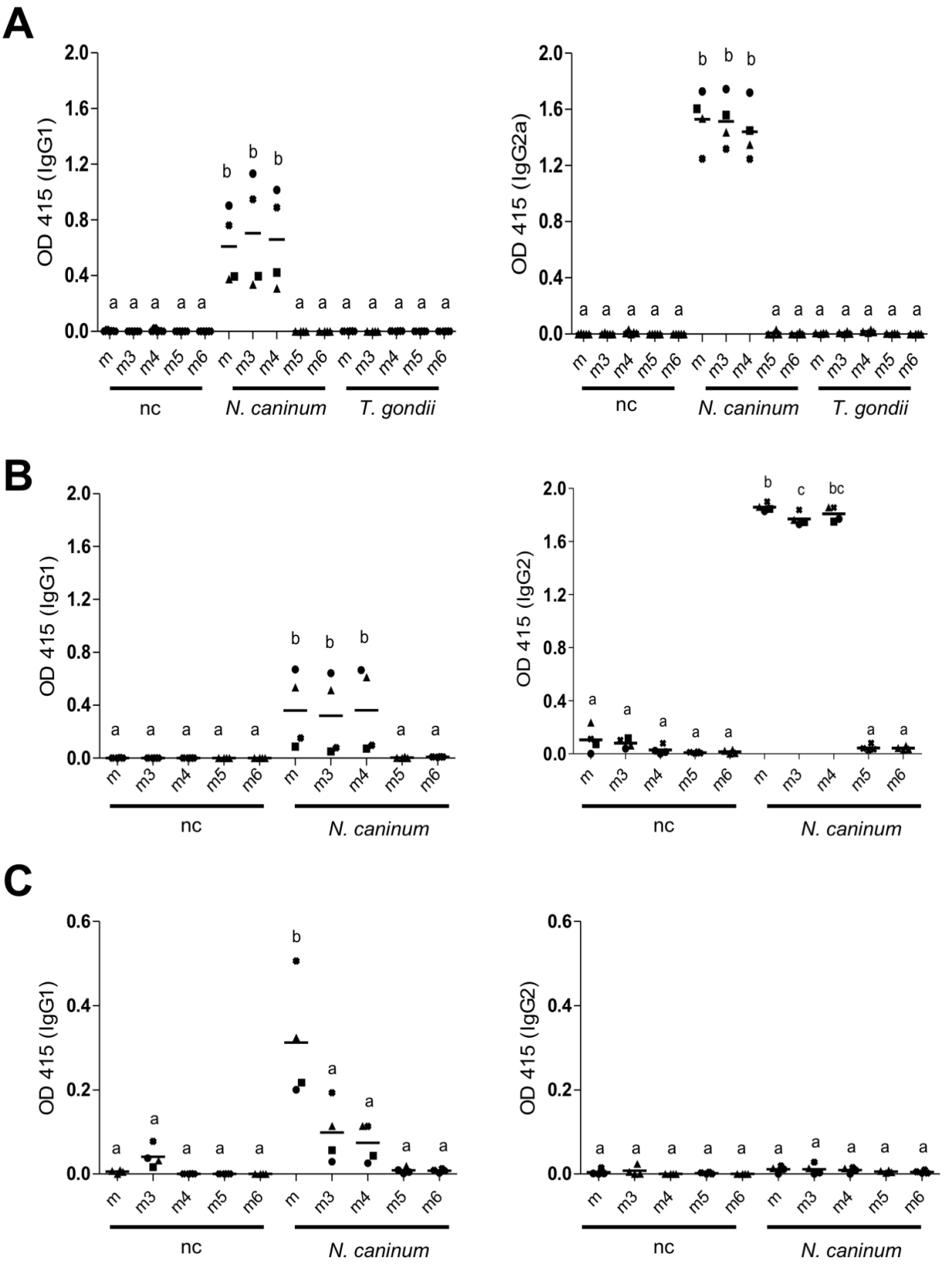
Figure legends

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.

Fig. 2. Reaction of field sera. (A) Reaction of dog IgG1 and IgG2 antibodies against the NcGRA7 fragments. (B) 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.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B
OD 415 (IgG1)
OD 415 (IgG2)
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OD 415 (IgG1)
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m
m3
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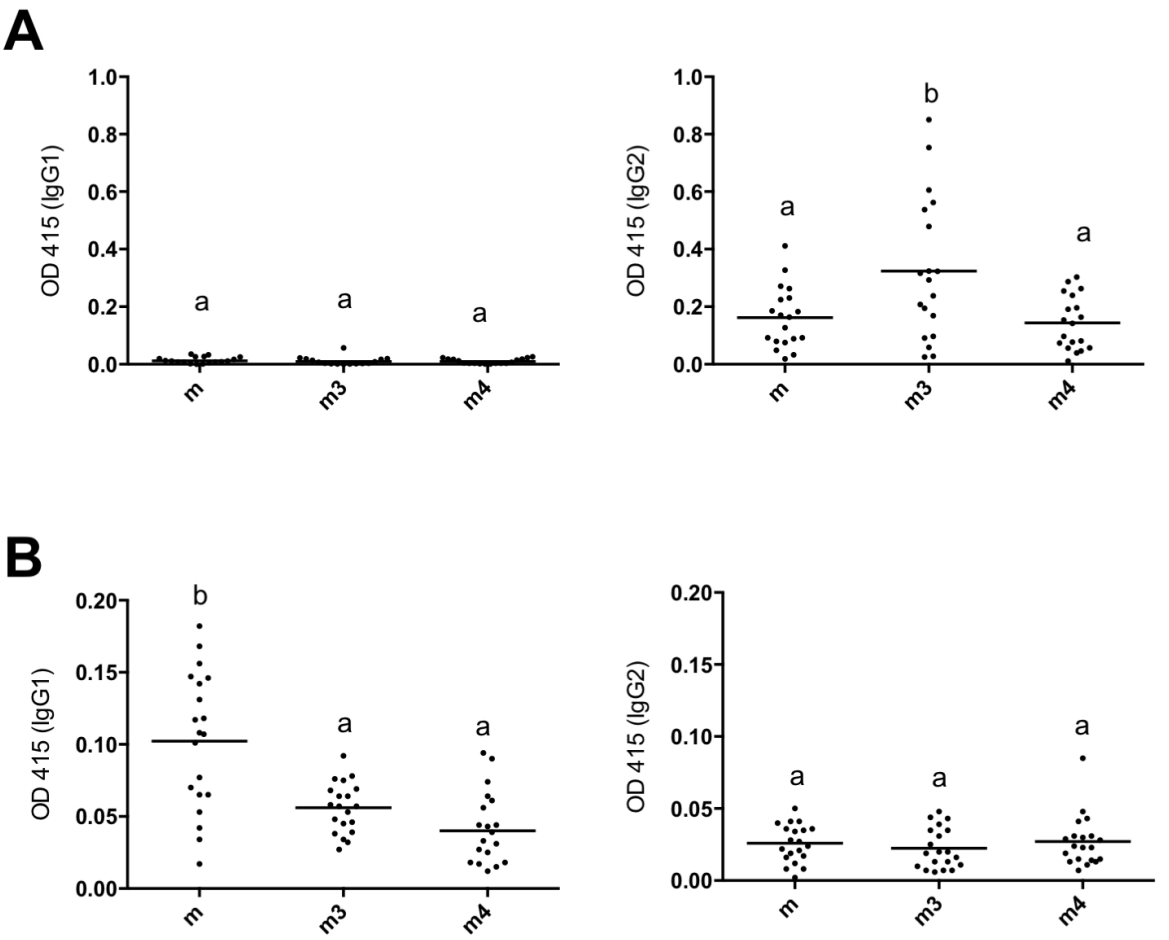
N. caninum
T. gondii

Fig. 1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OD 415 (IgG2)
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a
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OD 415 (IgG1)
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OD 415 (IgG2)
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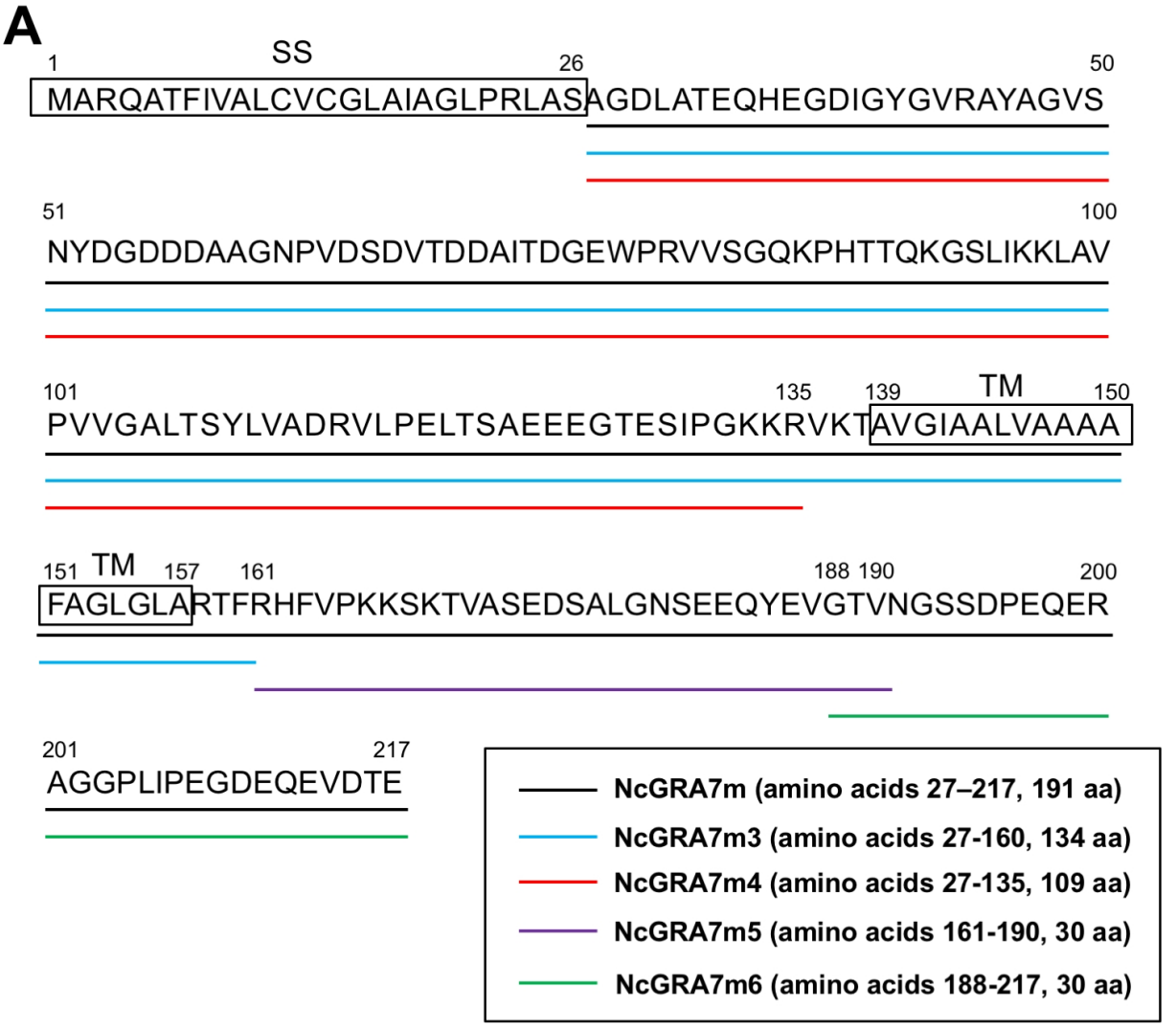
Fig. 2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101
135
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TM
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PVVGALTSYLVADRVLPELTSAEEEGTESIPGKKRVKTAVGIAALVAAAA
151
TM
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188
190
200
FAGLGLARTFRHFVPKKSKTVASEDSALGNSEEQYEVGTVNGSSDPEQER
201
217
AGGPLIPEGDEQEVDTE
NcGRA7m (amino acids 27–217, 191 aa)
NcGRA7m3 (amino acids 27-160, 134 aa)
NcGRA7m4 (amino acids 27-135, 109 aa)
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NcGRA7m6 (amino acids 188-217, 30 aa)

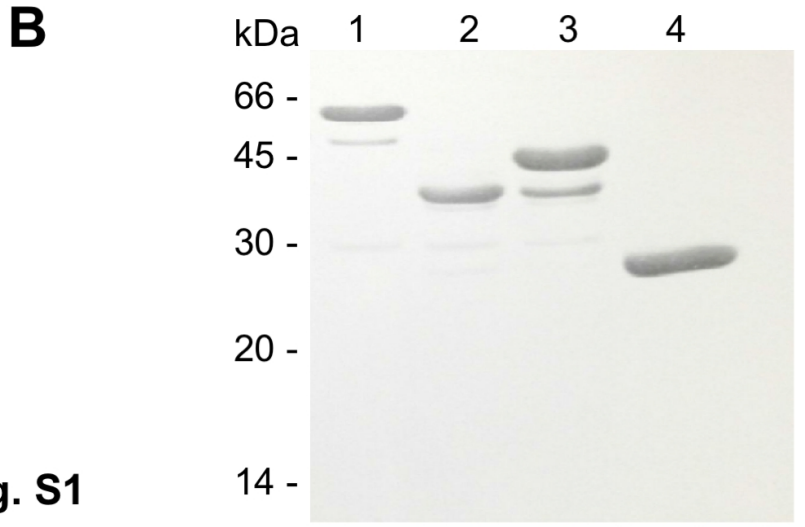
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Fig. S1

Supplemental information

Fig. S1. (A) Schematic depiction of the NcGRA7 fragments used in this study. The amino acid lengths of the fragment were 191, 134, 109, 30 and 30 for NcGRA7m, NcGRA7m3, NcGRA7m4, NcGRA7m5 and NcGRA7m6, respectively. SS, signal sequence; TM, transmembrane domain. In NcGRA7m fragment (amino acids 27–217), the first 26 amino acids (amino acids 1–26) which represent the signal peptides were removed. In NcGRA7m4 fragment (amino acids 27–135), the transmembrane region (amino acids 139–157) was removed. The C-terminal region (amino acids 161–217) was split in NcGRA7m3 (amino acids 27–160). The C-terminal region was dissected into two different regions; NcGRA7m5 (amino acids 161–190) and NcGRA7M6 (amino acids 188–217). (B) SDS-PAGE analysis of purified recombinant NcGRA7m-GST (lane 1), NcGRA7m3-GST (lane 2), NcGRA7m4-GST (lane 3), and GST (lane 4). All the recombinant proteins were GST fusions. M, molecular mass markers.