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Expression of recombinant dense granule protein 7 of *Neospora caninum* and evaluation of its diagnostic potential for canine neosporosis

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ABSTRACT

Neospora caninum infection is an important disease affecting bovine and canine populations worldwide. The dense granule antigen 7 of *N. caninum* (NcGRA7) is considered to be an immunodominant antigen. In the present study, the gene encoding truncated NcGRA7 (NcGRA7t) lacking an N-terminal signal peptide was expressed in *Escherichia coli*, and its diagnostic potential in an enzyme-linked immunosorbent assay (ELISA) was evaluated. The ELISA could clearly discriminate between known *N. caninum*-positive and -negative sera from dogs. Serum samples randomly collected from dogs in Japan and China were examined for the diagnosis of neosporosis using the ELISA. Twenty-three of 135 samples (17.03%) and 9 of 95 (9.47%) samples from Japan and China, respectively, were positive for antibodies to *N. caninum*. Of the 32 ELISA-positive samples, 29 (90.62%) were confirmed as positive by Western blot analysis with whole tachyzoite antigens. These results indicate that the recombinant NcGRA7t would be a reliable antigen in ELISA for the diagnosis of canine neosporosis.

Key Words: Canine, ELISA, *Neospora caninum*, NcGRA7

INTRODUCTION

Neosporosis is an important disease affecting bovine and canine populations worldwide (Dubey, 2003). The causative organism is an apicomplexan protozoan parasite, which was first recognized in 1984 from a dog in Norway (Bjerkas *et al.*, 1984) and eventually led to the description of a new genus and species, *Neospora caninum* (Dubey *et al*., 1988). This parasite induces abortion and stillbirths in both dairy and beef cattle, which caused an alarming economic concern in the cattle industry (Anderson *et al.*, 2000). In dogs, neosporosis is a neuromuscular disease usually characterized by ascending paralysis and death (Dubey *et al*., 1998). Studies revealed that *N. caninum* also infects a range of warm-blooded animals, such as sheep, goats, deer, and horses, but the disease is predominant in dogs and cattle (Dubey, 2003). Although found to be morphologically similar to certain related species, such as *N. hughesi*, *Toxoplasma gondii*, *Hammondia heydorni*, and *Sarcocystis* spp., *N. caninum* is distinguished by the antigenic and ultrastructural differences of its tissue cysts and tachyzoites (Bjerkas *et al*., 1994; Dubey *et al*., 2002; Hemphill *et al*., 1998; Howe and Sibley, 1999).

The dense granule antigens of *N. caninum* that have been identified so far include NcGRA1, NcGRA2, NcGRA6 (NCGRA6), NcGRA7 (NCDG1/Nc-p33), NcPI-S, a serine protease inhibitor, and NTPase, a nucleoside triphosphate hydrolase (Howe and Sibley, 1999; Morris *et al*., 2004). NcGRA6 and NcGRA7 were immunogenic and had been prepared in recombinant forms as antigens in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of neosporosis among cattle (Lally *et al*., 1996). The

recombinant NcGRA7 ELISA can clearly differentiate *N. caninum*-infected cattle sera from uninfected cattle sera (Howe and Sibley, 1999; Jenkins *et al*., 1997). Although this gene had 42% homology with *T. gondii* GRA7 (Howe and Sibley, 1999; Jacobs *et al*., 1998), no cross-reactivity with *T. gondii* was detected, as determined by Western blot analysis (Fuchs *et al*., 1998; Hemphill *et al*., 1998). The evaluation of the serodiagnostic potential of NcGRA7 was carried out exclusively for bovine assays (Jenkins *et al*., 1997; Lally *et al*., 1996), but the antigen was found to be highly hydrophobic and to yield a completely insoluble protein (Lally *et al*., 1996). Unlike those in cattle, studies concerning neosporosis in dogs are limited, and prevalence data are lacking. Serology remains the most useful diagnostic method for canine neosporosis. However, a practical and cost-effective method of antigen preparation is also an important consideration in establishing a diagnostic assay without compromising the specificity and sensitivity of the test. In this study, the approach is to use NcGRA7 with a truncated hydrophobic region in order to produce a soluble recombinant antigen for ELISA.

Therefore, the present study aims to evaluate the diagnostic potential of recombinant truncated NcGRA7 (NcGRA7t) for the detection of *N. caninum* infection in dogs through an ELISA. A survey on the prevalence of *N. caninum* using this diagnostic test in dogs from different areas in Japan and China is reported.

MATERIALS AND METHODS

Parasites

N. caninum tachyzoites of the NC-1 strain were cultured and purified as described previously (Chahan *et al*., 2003).

Cloning of NcGRA7 gene

Immunoscreening of the *N. caninum* tachyzoite cDNA expression library using an *N. caninum*-positive bovine serum was carried out as described previously (Liao *et al*., 2004), and several positive cDNA clones were obtained. The sequences of all clones were identical to the previously identified NcGRA7 (GenBank accession no. U82229). The entire NcGRA7 ORF without flanking UTR regions was PCR-amplified with a primer set, a sense primer (5’-AC GAATTC TAA AAT GGC CCG ACA AG-3’) and an antisense primer (5’-AC GAATTC CTA TTC GGT GTC TAC TT-3’). The truncated NcGRA7 (NcGRA7t) gene without a sequence encoding signal peptide was PCR-amplified with a primer set, a sense primer (5’-AC GAATTCC GCT GGA GAC TTG GCA) and an antisense primer (3’-AC GAATTCC TAT TCG GTG TCT ACT TCC). Both primer sets were flanked with the introduced *Eco*RI sites (underlined nucleotides) to facilitate cloning. The PCR products were digested with *Eco*RI and then cloned into the EcoRI site of the bacterial expression vector, pGEX-4T-3 (Amersham Biosciences, USA). The resulting plasmids were designated as pGEX/NcGRA7 and pGEX/NcGRA7t.

Expression and purification of recombinant NcGRA7 and NcGRA7t

Both NcGRA7 and NcGRA7t were expressed as glutathione S-transferase fusion proteins (GST-NcGRA7 and GST-NcGRA7t) in *E. coli* (DH5α strain) and purified using Glutathione Sepharose 4B as described previously (Chahan *et al*., 2003).

Production of anti-GST-NcGRA7t sera in mice

Six-week-old female BALB/c mice were injected intraperitoneally with 100 μg/head of recombinant GST-NcGRA7t and GST (control) proteins emulsified in complete Freund’s adjuvant (Difco, USA). Booster shots with incomplete Freund’s adjuvant (Difco) were performed at 2-week intervals until desired antibody titers were obtained. Sera were collected 10 days after the final immunization.

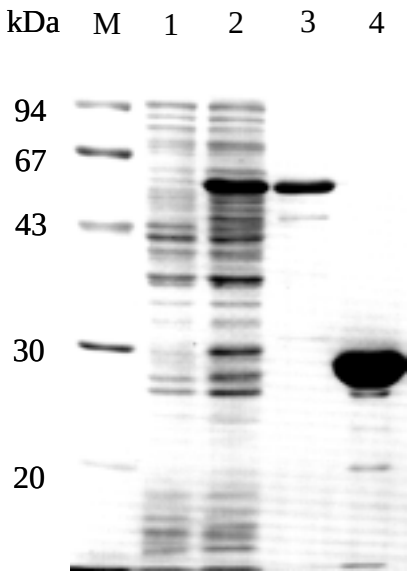

Figure 1. SDS-PAGE analysis of the recombinant NcGRA7 expressed in *E. coli.* Lane M, molecular mass standards; lane 1, lysates from uninduced *E. coli* transformed with pGEX/NcGRA7t; lane 2, lysates from IPTG-induced *E. coli* transformed with pGEX/NcGRA7t; lanes 3, purified GST-NcGRA7t; lane 4, purified GST.

ELISA

ELISA with GST-NcGRA7t was carried out as previously described (Chahan *et al*., 2003). Briefly, purified recombinant antigens (GST-NcGRA7t and GST, 2 μg /ml) in a coating buffer (a 50 mM carbonate-bicarbonate buffer, pH 9.6) were prepared to sensitize each flat-bottomed well (50 μl/well) of the ELISA plates (Nunc, Denmark). The antibody titer was computed as the difference in the OD_{415nm} values between the GST-NCGRA7t antigen and the control GST antigen. The cut-off point was established as the mean absorbance value plus 3 standard deviations of known negative control sera. Positive samples were subjected to Western blot analysis for further confirmation.

Western blot analysis

Western blot analysis was carried out as previously reported (Chahan *et al*., 2003).

Sera

The canine serum samples used in this study were as follows: sera from dogs experimentally pre- and post-infection with *N. caninum* (n=4); sera from specific pathogen-free dogs (n=30); sera randomly collected from dogs in Japan (n=135, the age and gender of the dogs were not specified); sera randomly collected from dogs in China (n=94, the age and gender of the dogs were not specified); sera from dogs experimentally infected with *B. gibsoni* (n=2); sera from dogs experimentally infected with *B. canis canis* (n=2); sera from dogs experimentally infected with *B. canis rossi* (n=2); sera from dogs experimentally infected with *B. canis vogeli* (n=2); and sera from dogs experimentally infected with *L. infantum* (n=2).

RESULTS

Cloning and expression of NcGRA7 gene

In order to identify an immunodominant antigen during a natural infection in cattle, the *N. caninum* tachizoite cDNA library was immunoscreened with known *N. caninum*-positive bovine serum samples collected from China (Liao *et al*., 2005). A total of 20 positive clones were obtained, and 17 of these were shown to correspond to a previously identified dense granule protein, NcGRA7 (Lally *et al*., 1997).

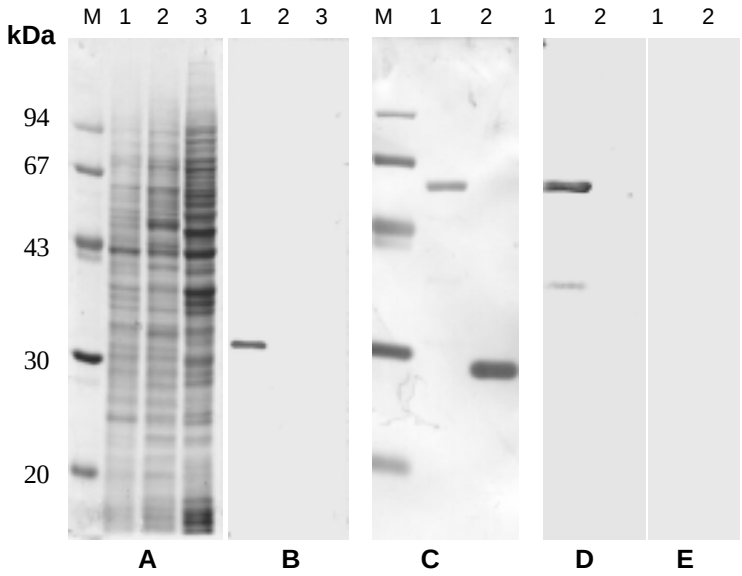

Figure 2. Western blot analyses of parasite NcGRA7 and recombinant NcGRA7t. Panel A shows *N. caninum* tachyzoite (lane 1), *T. gondii* tachyzoite (lane 2) and Vero cell (lane 3) lysates stained with amido black. Panel B represents the same antigens with probed with mouse anti-NcGRA7t serum. Panel C shows GST-NcGRA7t (lane 1) and control GST (lane 2) stained with amido black. Panels D and E represent the same antigens reacted with sera from a dog post- and pre-infected with *N. caninum*, respectively.

Both the full-length NcGRA7 gene and the NcGRA7t gene without a signal sequence were PCR-amplified and cloned into the bacterial expression vector pGEX-4T-3 and then expressed as a GST fusion protein in *E. coli*. As shown in Fig. 1, the molecular masses of GST and GST-NcGRA7t were estimated as 26 kDa and 57 kDa, respectively, as expected. When the amounts of soluble productions between GST-NcGRA7 and GST-NcGRA7t were compared, the latter was ten times greater (data not shown).

Evaluation of the antigenicity of recombinant NcGRA7

In order to determine whether the recombinant GST-NcGRA7t corresponds to the native protein, *N. caninum* tachizoite lysate blots were probed with the mouse anti-GST-NcGRA7t serum as the primary antibody. As shown in Fig. 2, the mouse antibody to recombinant GST-NcGRA7t bound to a single *N. caninum* tachyzoite antigen with a molecular mass of 33 kDa (Fig. 2B, lane 1). The *T. gondii* tachyzoite and host cell (Vero cell) lysates did not show the same reaction when probed with the same anti-serum (Fig. 2B, lanes 2 and 3). On the other hand, the recombinant GST-NcGRA7t was evaluated for its antigenicity in Western blot analysis with *N. caninum*-infected canine sera. The GST-NcGRA7t was specifically reacted with sera from dogs experimentally infected with *N. caninum* (Fig. 2D) but not with sera from uninfected dogs (Fig. 2E) and sera from dogs experimentally infected with *Babesia* spp. and *Leishmania* spp. (data not shown).

Localization of NcGRA7

Immunofluorescent analysis using mouse anti-GRA-NcGRA7t serum showed that the native NcGRA7 is specifically localized at the anterior and posterior portions with characteristic punctuated, granular staining within the *N. caninum* tachyzoites (data not shown). This observation agrees with previous reports on NcGRA7 concerning the antigen location (Hemphill *et al*., 1998; Lally *et al*., 1997).

Evaluation of the ELISA with recombinant NcGRA7: To evaluate whether the recombinant NcGRA7 expressed in *E. coli* can be a suitable antigen for the diagnosis of *N. caninum* infection, the purified

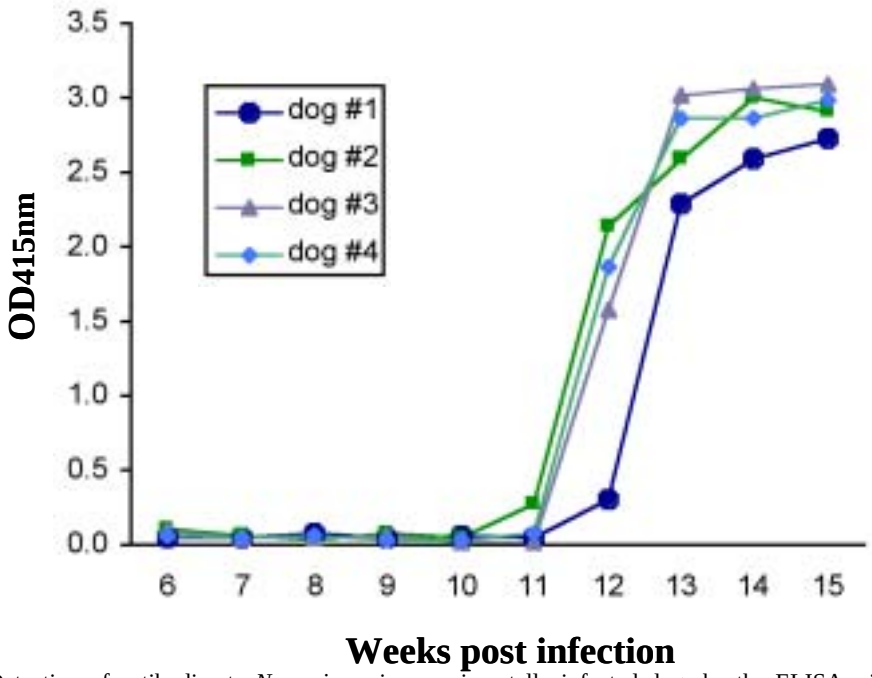

Figure 3. Detection of antibodies to *N. caninum* in experimentally infected dogs by the ELISA with recombinant NcGRA7t.

Table 1. Detection of antibodies to *N. caninum* in dog serum samples by the ELISA and Western blot analysis.

Country	No. ELISA[a)] positive/ no. examined (%)	No. Western blotting[b)] positive/ no. ELISA positive (%)
Japan	23/135 (17.03)	20/23 (86.95)
China	9/95 (9.47)	9/9 (100)
Total	32/230 (13.91)	29/32 (90.62)

[a)]Antibodies to *N. caninum* were detected by ELISA using recombinant GST-GRA7t expressed in *E. coli*. The ELISA was considered positive when an optical density at 415 nm equal to or greater than 0.238 was observed at serum dilution of 1:100.

[b)]Antibodies to *N. caninum* were detected by Western blot analysis using the parasite lysate. The assay was considered positive when the specific antigen bands were observed at serum dilution of 1:100.

GST-NcGRA7t was tested in an ELISA using sera from dogs pre- and post-experimental infection with *N. caninum*. As shown in Fig. 3, all sera from dogs post-infection with *N. caninum* were positive, whereas all sera from dogs pre-infection with *N. caninum* as well as SPF dogs were negative. In addition, the experimentally infected dogs exhibited an elevated antibody titer from 1-5 weeks post-infection, as determined by the ELISA (Fig. 3). To assess the specificity of the ELISA, serum samples from dogs experimentally infected with other protozoan parasites, such as *B. gibsoni*, *B. canis canis*, *B. canis rossi*, *B. canis vogeli*, and *L. infantum*, were tested. All sera exhibited OD415nm values lower than the cut-off point (0.238) except one serum from a *B. canis canis*-infected dog, which had an OD415nm of 0.4.

Serum samples randomly collected from dogs in Japan and China were tested for the detection of antibodies to *N. caninum* using the ELISA. As shown in Table 1, 23 of 135 samples (17.03%) and 9 of 95 (9.47%) samples from Japan and China, respectively, were positive for antibodies to *N. caninum*. Of the 32 ELISA-positive samples, 29 (90.62%) were confirmed as positive by Western blot analysis with whole tachyzoite antigens.

DISCUSSION

In the present study, the *N. caninum* tachizoite cDNA library was immunoscreened with known *N. caninum*-positive bovine serum samples in order to identify the immunodominant antigen(s) during natural infection in cattle. Most of the positive clones obtained were shown to correspond to NcGRA7 (Lally *et al.*, 1997). This result indicates that NcGRA7 is one of the immunodominant antigens present during natural infection in cattle.

The full-length NcGRA7 gene and the NcGRA7t gene without a signal sequence were expressed as GST fusion proteins in *E. coli* in order to compare the amount of soluble productions between GST-NcGRA7 and GST-NcGRA7t. The result showed that the latter was ten times greater, indicating that the removal of the hydrophobic signal sequence had improved its hydrophilicity. The recombinant GST-NcGRA7t purified from the soluble fraction of *E. coli* extracts was recognized by the sera from either dogs experimentally infected with *N. caninum* (Fig. 2) or mice experimentally infected with *N. caninum* (data not shown) but not by the sera from mice experimentally infected with *T. gondii* (data not shown). This observation indicates that the recombinant GST-NcGRA7 has a specific immunoreactivity against antibodies to *N. caninum*.

During an acute infection, tachyzoites rapidly multiply and disseminate to other tissues, resulting in an increased antibody response. It has been demonstrated that an NcGRA7 homologue, TgGRA7, is a good marker for recently acquired infections (Nigro *et al*., 2003), which could also be true for NCGRA7t. Conversely, bradyzoites release antigens following cyst rupture, liberating the parasites that cause reinfection. The fact that NcGRA7 is expressed in both the tachyzoite and bradyzoite stages, unlike NcSAG1, which is only expressed in tachyzoites, further affirms its reliability as a potential diagnostic antigen capable of detecting antibodies against both stages (Fuchs *et al*., 1998). The present results showed that NcGRA7t is a suitable antigen for the diagnosis of dogs potentially exposed to *N. caninum* proteins ranging from bradyzoites and reactivated-tachyzoite antigens, with either acute or latent infection.

It is deemed necessary to use serum samples from the field in order to evaluate the diagnostic potential of an antigen because the antibody responses of experimentally inoculated animals may differ from those of animals with natural infections. Therefore, dog serum samples collected from different areas in Japan and China were diagnosed using NcGRA7t-ELISA. The results showed that 17.03% (23 of 145) were positive in Japan and 9.47% (9 of 95) were positive in China. These results were confirmed by Western blot analysis with a 90.62% (19 of 32) agreement with NCGRA7t-ELISA (Table 1). To our knowledge, this is the first *N. caninum* dense-granule antigen-based ELISA for the diagnosis of canine neosporosis. Furthermore, this is the first reported survey of canine neosporosis in China and the second in Japan after the work by Sawada *et al*. using IFAT (Sawada *et al*., 1998).

To date, there have been very few reported surveys on bovine neosporosis and practically none on dogs in Southeast Asia (Huong *et al*., 1998; Kashiwazaki *et al*., 2001; Suteeraparp *et al*., 1999). However, field cases in some parts of Asia do not usually reflect its true prevalence but, rather, appear to be the result of poor monitoring, lack of surveillance, and inefficient diagnostic tools. Further serologic surveys on the prevalence of neosporosis in dogs, which serve as both the final and intermediate host in many parts of Southeast Asia, need to be accomplished.

In summary, it has been shown that the truncation of NcGRA7 greatly improved its hydrophilicity and thus facilitated antigen preparation with relative ease. Consequently, the truncated protein was able to retain its antigenic property. It is thus the conclusion of the present study that the recombinant NcGRA7t-ELISA is an improved and reliable method for the detection of *N. caninum* infection and is also applicable for the practical serodiagnosis of canine neosporosis. Similarly, it can also be used in the development of a rapid

immunochromatographic assay to complement the ELISA method to achieve a reliable diagnosis of *N. caninum* infection.

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