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Title: Apical membrane antigen 1 is a cross-reactive antigen between Neospora caninum and Toxoplasma gondii, and the anti-NcAMA1 antibody inhibits host cell invasion by both parasites

Article Type: Research Paper

Keywords: Neospora caninum; Toxoplasma gondii; Apical membrane antigen 1; Cross-reactive; Invasion

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Abstract: The cross-reactive antigens of Neospora caninum and Toxoplasma gondii are important in the exploration to determine the common mechanisms of parasite-host interaction. In this study, a gene encoding N. caninum apical membrane antigen 1 (NcAMA1) was identified by immunoscreening of a N. caninum tachyzoite cDNA expression library with antisera from mice immunized with recombinant T. gondii apical membrane antigen 1 (TgAMA1). NcAMA1 was encoded by an open reading frame of 1,695 bp, which encoded a protein of 564 amino acids. The single-copy NcAMA1 gene was interrupted by seven introns. NcAMA1 showed 73.6% amino acid identity to TgAMA1. Mouse polyclonal antibodies raised against the recombinant NcAMA1 (rNcAMA1) recognized a 69-kDa native parasite protein by Western blotting. Immunofluorescence analysis showed that NcAMA1 was localized to the apical end of tachyzoites. Two-dimensional electrophoresis and Western blotting indicated that an approximately 57-kDa cleavage product was released into the excretory/secretory products of N. caninum. Preincubation of free tachyzoites with anti-rNcAMA1 IgG antibodies inhibited the invasion into host cells by N. caninum and T. gondii. These results indicated that AMA1 is a cross-reactive antigen between N. caninum and T. gondii and a potential common vaccine candidate to control two parasites.

Cover Letter

Dear Dr. Andrew Paul Waters,

Thank you very much for your constructive and useful comments. We have revised our manuscript in line with the comments and suggestions. The detailed changes made and responses to the comments are shown on the attached sheet papers.

We hope that these revisions are satisfactory and the revised version will be acceptable for publication in Molecular & Biochemical Parasitology.

Yours sincerely

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*** Response to Reviewers**

Responses to reviewer

General comments:

The manuscript should be condensed with special attention to the Materials and Methods section (provide refs and short descriptions). Also, figures could be combined (e.g. fig 1-4 could easily be presented as 1 figure with one legend).

Re: We agree with the comments. We have condensed the Materials and Methods section as highlighted below. We have combined the Figures 1-4 as Fig. 1. In addition, we have combined the Figures 5-7 as Fig. 2, according to the editor's comment.

2. Materials and methods

2.8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis (2-DE), immunoblotting and protein identification

For the determination of the native NcAMA1, the *N. caninum* lysates and crude ESP were separated on 12% SDS-PAGE under reducing conditions as described by Liao et al [20]. 2-DE and protein identification by mass spectrometry were performed as described by Lee et al [32]. The proteins separated on 1-DE and 2-DE were visualized with colloidal Coomassie Brilliant Blue (CBB) G-250 or transferred onto a membrane (Immobilon™-P, Millipore, Bedford, USA) for immunoblotting. The immunoblotting was performed as described by Lee et al [32]. After spot image analysis, the trypsin digested peptide mixture of the protein spot was analyzed using Voyager-DE STR MALDI-TOF mass spectrometry (PerSeptive Biosystems, Framingham, MA, USA) and FineMod software (http://us.expasy.org /tools/findmod/).

2.9. Immunofluorescence analysis (IFA)

IFA of NcAMA1 in both intracellular and extracellular *N. caninum* tachyzoites were performed as described previously [20]. Fluorescent signals were visualized using a laser microdissection system (TCS NT, Leica, Germany).

Figure legends

Fig. 1. Characterization of the NcAMA1 gene. (A) Multiple-sequence alignment of AMA1 proteins from *N. caninum* (Nc), *T. gondii* (Tg), *B. bovis* (Bb), *B. gibsoni* (Bg), *P. vivax* (Pv), and *P. falciparum* (Pf). Strictly conserved residues are indicated with a black background, and four different cysteine residues with species of *Plasmodium* in domain III are indicated with a gray background. The cleavage site of NcAMA1 putative signal peptide is indicated by an arrowhead, and the transmembrane region is indicated by (*—*); cysteine residues that formed disulfide bonds in *P. falciparum* AMA1 are indicated by domain (I,II, and III) and bond (a, b, and c) designations. (B) Phylogenetic tree of AMA1 proteins from *N. caninum* (Nc), *T. gondii* (Tg), *B. bovis* (Bb), *B. gibsoni* (Bg), and *Plasmodium* species AMA1 (*P. vivax* (Pv), *P. falciparum* (Pf), *P. berghei* (Pb), *P. yoelii* (Py), *P. chabaudi* (Pc), *P. knowlesi* (Pk)). The sequence identities were calculated using DNASTAR software. (C) Southern blot analysis. The *N. caninum* genomic DNA was digested with *Kpn*I (lane 1), *Sph*I (lane 2), *Hind*III (lane 3), and *Sac*I (lane 4); the separated DNA fragments were hybridized with the NcAMA1 cDNA probe. (D) Schematic view of the NcAMA1 gene. White rectangles indicate exons, and black bold lines indicate the introns. The numeric numbers indicate the number of nucleotides.

Fig. 2. Characterization of the NcAMA1 protein. (A) Two-dimensional electrophoresis of the excretory/secretory product (ESP) under reducing conditions. ESP proteins were resolved by isoelectric focusing (pH 3~10) in the first dimension followed by SDS-PAGE in the second dimension on a 10% gradient gel. A spot corresponding to the NcAMA1 is indicated. (B) Western blot analysis of NcAMA1 in ESP. The arrow indicate specific spots recognized by the antiserum against rNcAMA1 in ESP. (C) Western blot analysis of *N. caninum* tachyzoite lysates, *T. gondii* tachyzoite lysates, and excretory/secretory products (ESP) of *N. caninum*. The blots were probed with anti-rNcAMA1 antibodies after 1D SDS-PAGE. Lane 1, Vero cell lysates; lane 2, *N. caninum* lysates; lane 3, *T. gondii* lysates; lane 4, ESP of *N. caninum*. (D) Immunofluorescence microscopy analysis. The extracellular and intracellular tachyzoites were stained with anti-rNcAMA1 antibodies followed by Alexa Fluor 488-conjugated goat anti-mouse IgG.

In some instances, references are lacking or are not correct, as detailed below. For some data not enough detail is provided to make a judgement, also detailed below.

Specific comments:

*Page 3, line starting with "Previously,..." does not make sense.

Re: The sentence "Previously, we demonstrated that six mAbs against cross-reactive antigens between *N. caninum* and *T. gondii*, the six mAbs could inhibit the two parasites invasion to host cells [20]." has been changed as "Previously, we have identified three cross-reactive antigens between *N. caninum* and *T. gondii* by using ten mAbs in six groups, and demonstrated that four groups of the mAbs could inhibit the host cell invasion by the two parasites [20]."

*Page 4, top paragraph is discussing *Plasmodium* AMA1, but refs 21-23 are from different species; In addition, ref 25 is not a correct ref for micronemal localization.

Re: We have deleted the refs 21-23 and ref 25, and cited new references as follows:

[21] Thomas AW, Deans JA, Mitchell GH, Alderson T, Cohen S. The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit *Plasmodium knowlesi* invasion of erythrocytes. Mol Biochem Parasitol 1984;13:187–99.

[22] Narum DL, Thomas AW. Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of *Plasmodium falciparum* merozoites. Mol Biochem Parasitol 1994;67:59–68.

*Page 5, last paragraph, a ref for rTgAMA1 antisera is lacking.

Re: We have added the description about "Expression of recombinant TgAMA1" and "Production of anti-rTgAMA1 sera" as follows:

2.3. Expression of recombinant TgAMA1

The cDNA fragment of TgAMA1 without a signal peptide was amplified by PCR using primers with the introduced *EcoR*I and *Xho*I sites (underlined), P1 (5'-CTGAATTCCTCAAGCACAAGGTCTCGCGAGTCG-3') and P2 (5'-CTCTCGAGTT CTGACTCTAGTAATCCCCCTCGAC-3'). The product was inserted into *E. coli*

expression vector pGEX-4T-3 (Pharmacia Biotech, USA). The recombinant TgAMA1 (rTgAMA1) fused with a glutathione *S*-transferase (GST) tag was expressed in the *E. coli* BL21 strain according to the manufacturer's instructions. The denaturing and refolding of insoluble rTgAMA1 with urea and subsequent purification were performed as described previously [30].

2.4. Production of anti-rTgAMA1 sera

Six-week-old ddY mice (Clea, Japan) were immunized intraperitoneally with 100 µg of purified rTgAMA1 in an equal volume of Freund's complete adjuvant (Sigma) for the first injection. Mice were immunized with 50 µg of the antigen in Freund's incomplete adjuvant (Sigma) at 2 and 4 weeks post-primary injection. The anti-rTgAMA1 sera were collected two weeks after the last immunization.

*Page 6, 2.4 ref is lacking.

Re: We have cited the ref 31 for southern blot analysis as "……using the standard procedure [31]."

[31] Sambrook J, Russell DW. Molecular Cloning: a Laboratory Manual. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press 2001.

2.5, one primer is underlined, what does that mean?

Re: We have corrected the sentence as "……. introduced *Xho*I sites (underlined), P1 (5'-TTCTCGAGCAGTTTGCTCGCAACCGGAATTAAC-3')"

Please give more detail on the fragment that was expressed. Does this include the TM and cytoplasmic tail? TM is notably difficult to express, so what were expression levels?

Re: In this study, the cDNA fragment of NcAMA1 without a signal peptide was expressed in *E. coli*. It included TM and cytoplasmic tail. The expression levels were not so high and the amount expressed was around 1000 µg per liter culture.

Was the protein refolded before being used as immunogen? It is known for *Plasmodium* AMA1 that natively folded protein is essential for generating inhibitory antibodies. How is

that in *N. caninum*? This is not at all discussed in the manuscript.

Re: We have discussed it in Discussion section according to the comments as "The correct folding of *Plasmodium* AMA1 has been shown to be critical for its immunological activity [42]. In this study, the cDNA fragment of NcAMA1 without a signal peptide was expressed in *E. coli*. The rNcAMA1 was initially insoluble, however, after refolding and purification, the rNcAMA1 becomes to be soluble. Moreover, the antibodies raised against rNcAMA1 were reactive with parasites, and inhibited the host cell invasion by the two parasites *in vitro*. These observations suggested that the refolded rNcAMA1 has immunological activity.

[42] Hodder AN, Crewther PE, Anders RF. Specificity of the protective antibody response to apical membrane antigen 1. Infect Immun 2001;69:3286–94.

*Page 10, Fig 1, TM is not correctly indicated for *Plasmodium* AMA1.

Re: The sentence "The putative signal peptide cleavage site is indicated by an arrow, and the transmembrane region is indicated by (*—*);" in the Figure legends has been changed as "The cleavage site of NcAMA1 putative signal peptide is indicated by an arrowhead, and the transmembrane region is indicated by (*—*);".

*Page 11, ref 33 is wrongly cited.

Re: We have cited new ref as: "….intramolecular disulfide bridges in AMA1 [35].

[35] Hodder AN, Crewther PE, Matthew ML, et al. The disulfide bond structure of *Plasmodium* apical membrane antigen-1. J Biol Chem 1996;271:29446–52.

Fig. 2, data on how the tree was calculated are lacking.

Re: We have corrected the sentence "…..between NcAMA1 and *B. bovis* AMA1 (13.5%) (Fig. 2)." as "…… between NcAMA1 and *B. bovis* AMA1 (13.5%), as calculated using DNASTAR software (NetWell Corporation, Japan) (Fig. 1B).

The data provided with the Southern blot are not sufficient to demonstrate that ama1 is a single-copy gene. The localization of the restriction sites must be provided and they should be far enough outside the ama1 locus to be sure that there is no gene duplication. Q-PCR

would be another way to demonstrate this.

Re: We have corrected the sentence as: "Southern blot analysis indicated that NcAMA1 is a single copy gene. The NcAMA1 cDNA probe hybridized to a single band with restriction enzymes that do not cut within the gene (Fig. 1C, lanes 1 and 2), and two bands with enzymes that cut once within the gene (Fig.1C, lanes 3 and 4).

*Page 12, fig 5, the resolution of the blot does not allow to see a 2 kDa difference, especially not since lane 3 contains much more protein than lane 2.

Re: The sentence "Additionally, the mouse antiserum against rNcAMA1 reacted with the 67-kDa protein in the lysates of *T. gondii* (Fig. 5)." has been changed as "Additionally, the mouse antiserum against rNcAMA1 cross reacted with a specific protein band in the lysates of *T. gondii* that migrated at the same position (Fig. 2C)."

As in the IFA procedure the parasites were permeabilised, how was it established that the fluorescence is on the surface of the parasites?

Re: We made a mistake here. The sentence "The specific fluorescence was observed on the surface of the apical part of both intracellular and extracellular parasites (Fig. 7)." has been changed as: "The specific fluorescence was observed on the apical part of both intracellular and extracellular parasites (Fig. 2D)."

*Page 13, discussion 1st paragraph, the genomic organisation of ama1 should also be considered in the discussion on evolutionary relationships.

Re: We have changed the position of the sentence "The genomic analysis indicated that the NcAMA1 gene existed as a single copy in the genome of *N. caninum* and was interrupted by seven introns, similarly to TgAMA1 [37]." in the 1st paragraph as highlighted below.

In the present study, we identified NcAMA1 as a cross-reactive antigen between *N. caninum* and *T. gondii*. Phylogenetic analysis of the deduced amino acid sequence of the NcAMA1 gene with the amino acid sequences of AMA1 proteins from other apicomplexan parasites, *Plasmodium* species, *B. bovis*, *B. gibsoni*, and *T. gondii*, demonstrated that each of the AMA1 sequences was clustered depending on their genus (Fig. 1B). The AMA1 proteins of *Babesia* were closer to those of *Plasmodium* than *Toxoplasma* and *Neospora*.

Moreover, the NcAMA1 and TgAMA1 had higher amino acid sequence identities than other *Plasmodium* and *Babesia* AMA1 proteins (Fig. 1B). In addition, the genomic analysis indicated that the NcAMA1 gene existed as a single copy in the genome of *N. caninum* and was interrupted by seven introns, similarly to TgAMA1 [37]. These results suggested that *N. caninum* had a closer evolutionary relationship with *T. gondii* than others.

*Page 14, top paragraph, it is not really clear what is meant by "invariant and high conservation" and by "although they were more distantly related in the phylum Apicomplexa". Please explain.

Re: We have changed the sentence "A total of 10 cysteine residues of the putative domains I and II of NcAMA1, TgAMA1, BbAMA1, and BgAMA1 showed invariant and high conservation with the AMA1 proteins of *Plasmodium* parasites, although they were more distantly related in the phylum Apicomplexa." as "Although the AMA1 proteins of *Plasmodium spp* and *Babesia spp* were more distantly related to those of *N. caninum* and *T. gondii*, all the AMA1 proteins contained highly conserved 10 cysteine amino acid residues in the putative domains I and II."

Title

Apical membrane antigen 1 is a cross-reactive antigen between *Neospora caninum* and *Toxoplasma gondii*, and the anti-NcAMA1 antibody inhibits host cell invasion by both parasites✧

Authors

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Abbreviations: AMA1, apical membrane antigen 1; PAGE, polyacrylamide gel electrophoresis; IFA, immunofluorescence analysis; ESP, excretory/secretory products

✧*Note:* The nucleotide sequence data reported in this paper are available in the DDBJ, GenBank and EMBL databases under the accession number **AB265823**.

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Abstract

The cross-reactive antigens of *Neospora caninum* and *Toxoplasma gondii* are important in the exploration to determine the common mechanisms of parasite-host interaction. In this study, a gene encoding *N. caninum* apical membrane antigen 1 (NcAMA1) was identified by immunoscreening of a *N. caninum* tachyzoite cDNA expression library with antisera from mice immunized with recombinant *T. gondii* apical membrane antigen 1 (TgAMA1). NcAMA1 was encoded by an open reading frame of 1,695 bp, which encoded a protein of 564 amino acids. The single-copy NcAMA1 gene was interrupted by seven introns. NcAMA1 showed 73.6% amino acid identity to TgAMA1. Mouse polyclonal antibodies raised against the recombinant NcAMA1 (rNcAMA1) recognized a 69-kDa native parasite protein by Western blotting. Immunofluorescence analysis showed that NcAMA1 was localized to the apical end of tachyzoites. Two-dimensional electrophoresis and Western blotting indicated that an approximately 57-kDa cleavage product was released into the excretory/secretory products of *N. caninum*. Preincubation of free tachyzoites with anti-rNcAMA1 IgG antibodies inhibited the invasion into host cells by *N. caninum* and *T. gondii*. These results indicated that AMA1 is a cross-reactive antigen between *N. caninum* and *T. gondii* and a potential common vaccine candidate to control two parasites.

Key words

Neospora caninum; *Toxoplasma gondii*; Apical membrane antigen 1; Cross-reactive; Invasion

1. Introduction

Neospora caninum, a pathogenic intracellular protozoan parasite, is a major cause of bovine neonatal mortality and abortion [1–3]. In phylogenetic analysis, *N. caninum* was placed as a sister group to *Toxoplasma gondii* in the phylum Apicomplexa, which includes *Plasmodium*, *Cyptosporidium*, *Eimeria*, *Babesia*, and *Theileria* [4,5]. *N. caninum* and *T. gondii* are known to have highly similar biological and morphological characteristics. *T. gondii* has long been recognized as an important pathogen in humans and animals; in particular, it is known to be responsible for congenital neurological defects in the fetus and opportunistic infections in immunocompromised patients [6,7]. In addition, both neosporosis and toxoplasmosis have been regarded as economically important diseases, as they have considerable impact on the livestock industry.

Recently, a number of reports have been published about the biological, morphological, and molecular differences between two parasites [8]. In addition, major antigens of *N. caninum* are genetically and immunologically distinct from those of *T. gondii* [9–11]. However, antigenic cross-reactivity between *N. caninum* and *T. gondii* has been reported with immunoblotting and the enzyme-linked immunosorbent assay (ELISA) using crude tachyzoite extracts as the antigen [12–17] and with an immunohistochemical test using anti-*T. gondii* polyclonal and monoclonal antibodies (mAbs) [11,18,19]. Previously, we have identified three cross-reactive antigens between *N. caninum* and *T. gondii* by using ten mAbs in six groups, and demonstrated that four groups of the mAbs could inhibit the host cell invasion by the two parasites [20].These results suggested that the conserved antigens of the two parasites could be useful in the development of vaccines or drugs for controlling the diseases caused by the two

parasites.

Apical membrane antigen 1 (AMA1) is identified as a conserved antigenic protein in the *Plasmodium* species; it is essential for host-cell invasion, but its role is not completely understood [21]. *Plasmodium* AMA1 has been shown to be localized to the micronemes of developing intracellular parasites and to the apical surface of extracellular parasites just prior to invasion [22]. Vaccination with recombinant *Plasmodium* AMA1 has been demonstrated to induce protective immunity against a homologous parasite challenge in animal malarial models [23–26]. Recently, AMA1 homologues were identified from other apicomplexan parasites, such as *Babesia bovis*, *B. gibsoni*, and *T. gondii*, as immunogenic proteins [27–29].

In this study, we reported the cloning and characterization of an AMA1 of *N. caninum* (NcAMA1) as a cross-reactive antigen between *N. caninum* and *T. gondii* and showed that the anti-NcAMA1 antibody inhibited host-cell invasion by both the *N. caninum* and *T. gondii* parasites. The NcAMA1 provided additional insights into the host-parasite interactions and may be useful in the development of a novel vaccine candidate.

2. Materials and methods

2.1. Parasite culture and purification

N. caninum tachyzoites (Nc-1 strain) and *T. gondii* tachyzoites (RH strain) were maintained in African green monkey kidney (Vero) cells cultured in a minimum essential medium (MEM, Sigma) supplemented with 8% heat-inactivated fetal bovine

serum (FBS) and 50 μg/ml kanamycin at 37℃ in a 5% CO_2 air environment. For the purification of tachyzoites, parasites and host cell debris were washed in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS and syringed three times with a 27-gauge needle. The parasites were then filtered through a 5.0 μm pore filter (Millipore, USA), washed twice with 10 ml of PBS, and pelleted at 1,500 rpm for 10 min.

2.2. Preparation of lysates and excretory/secretory products (ESP)

For lysate preparation, *N. caninum* tachyzoites were harvested and purified as described above. Parasites were then resuspended in PBS, disrupted three times by a freeze-thaw cycle in liquid nitrogen, and then sonicated in an ice slurry. For ESP preparation, purified *N. caninum* tachyzoites were resuspended in MEM, incubated at 37℃ for 30 min, and then centrifuged at 1,500 rpm for 10 min. The supernatant was used as ESP. The protein concentration of the lysates and ESP was determined with a BCA protein assay kit (PIERCE, USA), and the lysate and ESP were stored at -80℃ until use.

2.3. Expression of recombinant TgAMA1

The cDNA fragment of TgAMA1 without a signal peptide was amplified by PCR using primers with the introduced *EcoR*I and *Xho*I sites (underlined), P1 (5'-CTGAATTCCTCAAGCACAAGGTCTCGCGAGTCG-3') and P2 (5'-CTCTCGAG TTCTGACTCTAGTAATCCCCCTCGAC-3'). The product was inserted into *Escherichia coli* expression vector pGEX-4T-3 (Pharmacia Biotech, USA). The recombinant TgAMA1 (rTgAMA1) fused with a glutathione *S*-transferase (GST) tag

was expressed in the *E. coli* BL21 strain according to the manufacturer's instructions. The denaturing and refolding of insoluble rTgAMA1 with urea and subsequent purification were performed as described previously [30].

2.4. Production of anti-rTgAMA1 sera

Six-week-old ddY mice (Clea, Japan) were immunized intraperitoneally with 100 µg of purified rTgAMA1 in an equal volume of Freund's complete adjuvant (Sigma) for the first injection. Mice were immunized with 50 µg of the antigen in Freund's incomplete adjuvant (Sigma) at 2 and 4 weeks post-primary injection. The anti-rTgAMA1 sera were collected two weeks after the last immunization.

2.5. Cloning of the NcAMA1 gene

A cDNA expression library was constructed as described previously [20]. The plaques were transferred onto nitrocellulose membranes and screened with the anti-rTgAMA1 sera from mice immunized with rTgAMA1 expressed in *E. coli*. Ten clones were excised for isolation of the phagemid inserts as described in the manufacturer's instructions and sequenced with M13 forward and reverse primers by using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, USA). A full-length NcAMA1 cDNA including the noncoding 5' end was obtained with a GeneRacer kit (Invitrogen, USA) using a specific primer (5'-GGTTCGGCTGATGAAGTTGAAT GT-3') according to the manufacturer's instructions, and the clone obtained was sequenced on both strands.

2.6. Southern blot analysis

Southern blot analysis was performed using the standard procedure [31]. Genomic

DNA was extracted from *N. caninum* tachyzoites. The DNA was digested overnight with *Kpn*I, *Sph*I, *Hind*III, and *Sac*I, separated through a 1.5% agarose gel, and transferred onto a Hybond^{TM}-N^{+} membrane (Amersham Pharmacia, USA) through capillary action. The membrane was probed with an NcAMA1 fragment labeled with an Alkphos direct labeling kit (Amersham Biosciences, UK).

2.7. Expression of recombinant NcAMA1 and production of anti-rNcAMA1 sera

The cDNA fragment of NcAMA1 without a signal peptide was amplified by PCR using primers with the introduced *Xho*I sites (underlined), P1 (5'-TTCTCGAGCAGTT TGCTCGCAACCGGAATTAAC-3') and P2 (5'-TTCTCGAGACATCCTATGACTAAT AATCGCCCT-3'). Expression, purification of rNcAMA1 and production of anti-rNcAMA1 sera were performed as described above.

2.8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis (2-DE), immunoblotting and protein identification

For the determination of the native NcAMA1, the *N. caninum* lysates and crude ESP were separated on 12% SDS-PAGE under reducing conditions as described by Liao et al [20]. 2-DE and protein identification by mass spectrometry were performed as described by Lee et al [32]. The proteins separated on 1-DE and 2-DE were visualized with colloidal Coomassie Brilliant Blue (CBB) G-250 or transferred onto a membrane (Immobilon™-P, Millipore, Bedford, USA) for immunoblotting. The immunoblotting was performed as described by Lee et al [32]. After spot image analysis, the trypsin digested peptide mixture of the protein spot was analyzed using Voyager-DE STR MALDI-TOF mass spectrometry (PerSeptive Biosystems, Framingham, MA, USA) and FineMod software (http://us.expasy.org /tools/findmod/).

2.9. Immunofluorescence analysis (IFA)

IFA of NcAMA1 in both intracellular and extracellular *N. caninum* tachyzoites were performed as described previously [20]. Fluorescent signals were visualized using a laser microdissection system (TCS NT, Leica, Germany).

2.10. In vitro *inhibition of host-cell invasion*

Fluorescence-based quantification of the inhibition of host-cell invasion by both *N. caninum* and *T. gondii* expressing the green fluorescent protein (GFP) *in vitro* was carried out as described previously [33,34]. Vero cell monolayers were grown in 12-well tissue culture plates. The anti-rNcAMA1 and anti-GST IgG antibodies were sterilized by filtration through a 0.20-μm filter (Pall, USA). The tachyzoites (2.5×10^5) were resuspended in 500 μl of MEM containing 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.1 mg/ml of anti-rNcAMA1 or anti-GST IgG antibodies and incubated 1 hr at 37℃/5% CO_2 before the Vero cells were infected. Parallel wells without the antibodies were used as controls. Thereafter, the mixtures were added to the monolayers of Vero cells and allowed to invade for 1 hr at 37℃/5% CO_2. Uninvaded parasites were removed by rinsing with MEM. After 30 hrs of culture, the percentage of fluorescent parasites in each sample was determined using flow cytometry. The percentage of invaded tachyzoites was calculated by comparison with those obtained from parasites incubated with an anti-GST antibody and without antibodies as the control.

3. Results

3.1. Identification and characterization of the NcAMA1 gene

In a preliminary study, the antiserum against rTgAMA1 showed cross-reactivity with *N. caninum* tachyzoite lysates in the immunoblot analysis (data not shown). Subsequently, to identify the NcAMA1 gene, the *N. caninum* cDNA expression library was immunoscreened with anti-rTgAMA1 serum, and a total of 10 positive clones were obtained. The full-length cDNA of NcAMA1 was 2,666 bp (GenBank accession number **AB265823**), including a single open reading frame of 1,695 bp, encoding a polypeptide of 564 amino acid residues with a molecular mass of 62,808 Da and a theoretical pI of 5.3, as calculated with MacVector software (Oxford Molecular Group, Inc.). The hydrophobic region at the N-terminus of NcAMA1 clearly showed the characteristics of a signal peptide (SignalP3.0), and the most likely cleavage site was predicted to be between 39 and 40 amino acids (Fig. 1A). The NcAMA1 included an N-terminal cysteine-rich (12 conserved cysteine residues) ectodomain and a C-terminal transmembrane domain (from 479 to 501, as predicted using TMHMM2.0) (Fig. 1A).

To determine the phylogenetically conserved functional regions in NcAMA1, the deduced amino acid sequence of NcAMA1 was aligned with AMA1 amino acid sequences from six *Plasmodium* species (*P. berghei* [U45969], *P. yoelii* [U45971], *P. chabaudi* [U49743], *P. vivax* [AF063138], *P. knowlesi* [P21303], *P. falciparum* [320941]), *B. bovis* (AF486101), *B. gibsoni* (DQ368061), and *T. gondii* (AF010264). The result revealed the highest amino acid sequence homology between NcAMA1 and TgAMA1 (73.6% identity) and the lowest homology between NcAMA1 and *B. bovis* AMA1 (13.5%), as calculated using DNASTAR software (NetWell Corporation, Japan) (Fig. 1B). Of the 16 cysteine residues with invariant positions among *Plasmodium*

species, 12 cysteine residues were conserved in *N. caninum* and *T. gondii*; 14 cysteine residues were conserved in *B. bovis* and *B. gibsoni* (Fig. 1A). These cysteines are known to form intramolecular disulfide bridges in AMA1 [35].

Southern blot analysis indicated that NcAMA1 is a single copy gene. The NcAMA1 cDNA probe hybridized to a single band with restriction enzymes that do not cut within the gene (Fig. 1C, lanes 1 and 2), and two bands with enzymes that cut once within the gene (Fig.1C, lanes 3 and 4).

The complete sequence of the PCR-amplified genomic DNA containing the NcAMA1 gene was acquired by primer walking, cloned into a pGEM-T vector (Promega, USA), and subjected to DNA-sequencing analysis. The sequence was completely identified and revealed seven introns within the NcAMA1 coding region ranging from 238 to 630 bp in length. The schematic genome organization of NcAMA1 is shown in Fig. 1D.

3.2. Characterization of the NcAMA1 protein

To characterize the antigenicity of NcAMA1, rNcAMA1 was expressed in *E. coli* as a GST fusion protein. The sera from mice and dogs experimentally infected with live *N. caninum* tachyzoites could recognize the rNcAMA1 (data not shown).

To investigate the size difference of between the tachyzoites and crude ESP, we analyzed the protein profile of crude ESP via 2-DE and immunoblot analyses with the mouse antiserum against rNcAMA1. The NcAMA1 was clearly recognized to have at least two isoforms in an immunoblot image, similarly to TgAMA1[36], and the corresponding protein spots were subsequently analyzed with a MALDI-TOF mass

spectrometry. Of these, the PMF of one spot (arrow in Fig. 2A) was successfully acquired and matched to NcAMA1 with a sequence coverage of 29.1%. The matched peptide mass and corresponding amino acid sequences of peptide fragments are shown in Table 1. The mouse anti-GST sera were used as control to probe the *N. caninum* lysates and ESP, but no reacted bands or spots were observed (data not shown).

In immunoblot analysis, the mouse antiserum against rNcAMA1 recognized a specific protein band in the lysates and crude ESP of *N. caninum* tachyzoites that migrated at approximately 69 kDa and 57 kDa, respectively. Additionally, the mouse antiserum against rNcAMA1 cross reacted with a specific protein band in the lysates of *T. gondii* that migrated at the same position (Fig. 2C).

To analyze the localization of NcAMA1 in *N. caninum*, a mouse antiserum against rNcAMA1 was used to perform IFA with the intracellular and extracellular parasites. The specific fluorescence was observed on the apical part of both intracellular and extracellular parasites (Fig. 2D).

3.3. Anti-NcAMA1 antibodies inhibited the host-cell invasion by both N. caninum *and* T. gondii *tachyzoites in vitro*

Both the *N. caninum* and *T. gondii* tachyzoites expressing GFP were grown in Vero cells in the presence of anti-rNcAMA1 antibodies. The anti-NcAMA1 antibodies significantly inhibited the host-cell invasion by *N. caninum*, with an inhibition percentage of 68.7±3.6% at 1 mg/ml IgG, whereas anti-GST antibodies did not have a significant effect on invasion (9.6±1.5% inhibition) (Fig. 3A). In addition, the anti-rNcAMA1 antibodies cross-inhibited the host-cell invasion by *T. gondii*, with an inhibition percentage of 64.7±3.0% at 1 mg/ml IgG, whereas anti-GST antibodies did

not have a significant effect on invation (9.8±2.6% inhibition) (Fig. 3B).

4. Discussion

In the present study, we identified NcAMA1 as a cross-reactive antigen between *N. caninum* and *T. gondii*. Phylogenetic analysis of the deduced amino acid sequence of the NcAMA1 gene with the amino acid sequences of AMA1 proteins from other apicomplexan parasites, *Plasmodium* species, *B. bovis*, *B. gibsoni*, and *T. gondii*, demonstrated that each of the AMA1 sequences was clustered depending on their genus (Fig. 1B). The AMA1 proteins of *Babesia* were closer to those of *Plasmodium* than *Toxoplasma* and *Neospora*. Moreover, the NcAMA1 and TgAMA1 had higher amino acid sequence identities than other *Plasmodium* and *Babesia* AMA1 proteins (Fig. 1B). In addition, the genomic analysis indicated that the NcAMA1 gene existed as a single copy in the genome of *N. caninum* and was interrupted by seven introns, similarly to TgAMA1 [37]. These results suggested that *N. caninum* had a closer evolutionary relationship with *T. gondii* than others.

Many of the conserved and polymorphic residues in AMA1 proteins across the *Plasmodium* species have been reported [35]. Of these, most notably, the ectodomain of mature *Plasmodium* AMA1 proteins contains 16 conserved cysteine residues that have been shown to form a secondary structure of three domains stabilized by disulfide bridges [35]. In this study, NcAMA1 also contained 16 cysteine residues and exhibited identical localization of cysteine residues with TgAMA1. On the other hand, BbAMA1 and BgAMA1 included 14 cysteine residues in their sequences and also showed identical localization between two proteins (Fig. 1A). Although the AMA1 proteins of

Plasmodium spp and *Babesia spp* were more distantly related to those of *N. caninum and T. gondii*, all the AMA1 proteins contained highly conserved 10 cysteine amino acid residues in the putative domains I and II. Thus, these results revealed that the number and localization of cysteine residues on the sequences of the AMA1 proteins might be conserved depending on their genus.

According to the recently determined crystal structures of *Plasmodium* AMA1 proteins, the core of domains I and II of the ectodomain is intimately related with the PAN-folding motif that is commonly associated with carbohydrate or protein receptor-binding functions [38,39]. Putative domain III in NcAMA1 as well as TgAMA1, although including 6 cysteine residues, shows significant sequence differences to *Plasmodium* AMA1, in particular, the absence of the pair of CXC motifs [37]. Even though AMA1 proteins seem to play identical or very similar functions at the invasion of the host cell, the differences in domain III might reflect the different interaction against different host-cell receptors or genus-specific interactions.

The predicted signal peptide of NcAMA1 was longer than those of the other AMA1 proteins. AMA1 proteins are type I integral transmembrane (TM) proteins that are highly conserved and present in all *Plasmodium* species [21], *Toxoplasma* [29], and *Babesia* [27,28]. Analyses of the amino acid sequences revealed that full-length NcAMA1 contained a predicted TM region (from 479 to 501) near the C terminus, as other AMA1 proteins. The anti-rNcAMA1 antibodies recognized a 69-kDa band in *N. caninum* tachyzoite extracts and an approximately 57-kDa band in the ESP after incubation of extracellular tachyzoites in a serum-free medium (Fig. 2C). The size difference between the tachyzoite lysates and excreted-secreted forms of NcAMA1

suggests that there is a cleavage site in the C-terminal of the TM domain, which also occurs on several other transmembrane proteins, such as TgMIC6, TgMIC12, TgMIC8 [40], and TgAMA1 [29,36]. Recent research revealed that the microneme proteins were cleaved in their TM domains by intramembrane-cleaving serine proteases [41]. The removal of a short segment at the C terminus apparently occurs at some stage during transit to the surface. In extracellular parasites, at least, the mature ectodomain is eventually shed into the supernatant, apparently as a result of cleavage off of the surface. When considered together, these data suggest that the NcAMA1 protein is proteolytically processed before secretion into a medium and that 69-kDa and 57-kDa proteins might correspond to the entire NcAMA1 and processed NcAMA1, respectively.

The correct folding of AMA1, as in case of *Plasmodium* antigens, has been shown to be critical for its immunological activity [42]. In this study, the cDNA fragment of NcAMA1 without a signal peptide was expressed in *E. coli*. The rNcAMA1 was initially insoluble, however, after refolding and purification, the rNcAMA1 becomes to be soluble. Moreover, the antibodies raised against rNcAMA1 were reactive with parasites, and inhibited the host cell invasion by the two parasites *in vitro*. These observations suggested that the refolded rNcAMA1 has immunological activity.

Immunoblot analysis and a cross-invasion inhibition assay revealed that AMA1 was a cross-reactive antigen between *N. caninum* and *T. gondii*. Anti-NcAMA1 antibodies inhibited the host-cell invasion of *N. caninum* and *T. gondii* parasites. The inherent ability to block *in vitro* parasite invasion is slightly lower on *T. gondii* than on *N. caninum*. These results demonstrated that anti-rNcAMA1 antibodies could cross-inhibit parasite invasion of the host cell; thus, further evaluation of the usefulness of NcAMA1

as a potential common vaccine against both neosporosis and toxoplasmosis will be performed.

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Figure legends

Fig. 1. Characterization of the NcAMA1 gene. (A) Multiple-sequence alignment of AMA1 proteins from *N. caninum* (Nc), *T. gondii* (Tg), *B. bovis* (Bb), *B. gibsoni* (Bg), *P. vivax* (Pv), and *P. falciparum* (Pf). Strictly conserved residues are indicated with a black background, and four different cysteine residues with species of *Plasmodium* in domain III are indicated with a gray background. The cleavage site of NcAMA1 putative signal peptide is indicated by an arrowhead, and the transmembrane region is indicated by

(*—*); cysteine residues that formed disulfide bonds in *P. falciparum* AMA1 are indicated by domain (I,II, andIII) and bond (a, b, and c) designations. (B) Phylogenetic tree of AMA1 proteins from *N. caninum* (Nc), *T. gondii* (Tg), *B. bovis* (Bb), *B. gibsoni* (Bg), and *Plasmodium* species AMA1 (*P. vivax* (Pv), *P. falciparum* (Pf), *P. berghei* (Pb), *P. yoelii* (Py), *P. chabaudi* (Pc), *P. knowlesi* (Pk)). The sequence identities were calculated using DNASTAR software. (C) Southern blot analysis. The *N. caninum* genomic DNA was digested with *Kpn*I (lane 1), *Sph*I (lane 2), *Hind*III (lane 3), and *Sac*I (lane 4); the separated DNA fragments were hybridized with the NcAMA1 cDNA probe. (D) Schematic view of the NcAMA1 gene. White rectangles indicate exons, and black bold lines indicate the introns. The numeric numbers indicate the number of nucleotides.

Fig. 2. Characterization of the NcAMA1 protein. (A) Two-dimensional electrophoresis of the excretory/secretory product (ESP) under reducing conditions. ESP proteins were resolved by isoelectric focusing (pH 3~10) in the first dimension followed by SDS-PAGE in the second dimension on a 10% gradient gel. A spot corresponding to the NcAMA1 is indicated. (B) Western blot analysis of NcAMA1 in ESP. The arrow indicate specific spots recognized by the antiserum against rNcAMA1 in ESP. (C) Western blot analysis of *N. caninum* tachyzoite lysates, *T. gondii* tachyzoite lysates, and excretory/secretory products (ESP) of *N. caninum.* The blots were probed with anti-rNcAMA1 antibodies after 1D SDS-PAGE. Lane 1, Vero cell lysates; lane 2, *N. caninum* lysates; lane 3, *T. gondii* lysates; lane 4, ESP of *N. caninum*. (D) Immunofluorescence microscopy analysis. The extracellular and intracellular tachyzoites were stained with anti-rNcAMA1 antibodies followed by Alexa Fluor

488-conjugated goat anti-mouse IgG.

Fig. 3. *In vitro* inhibition of host-cell invasion. (A) *In vitro* inhibition assay for *N. caninum* invasion by IgG of anti-rNcAMA1 antibodies. The value for the invasion of *N. caninum* tachyzoites in the absence of antibodies was considered to be a 100% value. Values for the host-cell invasion of *N. caninum* tachyzoites after preincubation with anti-GST IgG antibodies (▲) and anti-rNcAMA1 IgG antibodies (⬧). (B) *In vitro* cross-inhibition assay for *T. gondii* invasion by anti-rNcAMA1 IgG antibodies. Values for the host-cell invasion of *T. gondii* tachyzoites after preincubation with anti-GST IgG antibodies (▲) and anti-rNcAMA1 IgG antibodies (⬧). The results are representative of five individual experiments, and the error bars indicate standard deviations. (*) According to the Student's t-test, the differences between the treatment with anti-GST antibodies and anti-rNcAMA1 antibodies at the same IgG concentration were significant ($P< 0.05$).

Table(s)

Table 1. Identification of NcAMA1 from ESP of *N. caninum* tachyzoites using peptide mass fingerprinting

Peptide mass*	Position	Matched peptide
2275.092	52-73	SGGSQTLSASTPQNPFQTPELK
1653.875	117-129	YIQLHQPNRPPYK
1810.841	130-144	NNFLEDIPTEAEYQK
2076.983	145-164	SGNPLPGGFNMNFVTPAGQR
1335.701	165-175	ISPYPMELLEK
967.458	201-208	SNQATQYR
1018.492	209-216	YPFVYDSK
1385.676	219-229	LCYILSVSMQR
2663.351	255-279	SPTANHNLIFGSAYVGKDPDAFLTK
1479.671	322-334	TFTNDEVASDQPR
2104.939	431-448	SSEFPETFGSCDVQACKR

* Peptide mass as $[M+H]^+$.

Figure 1

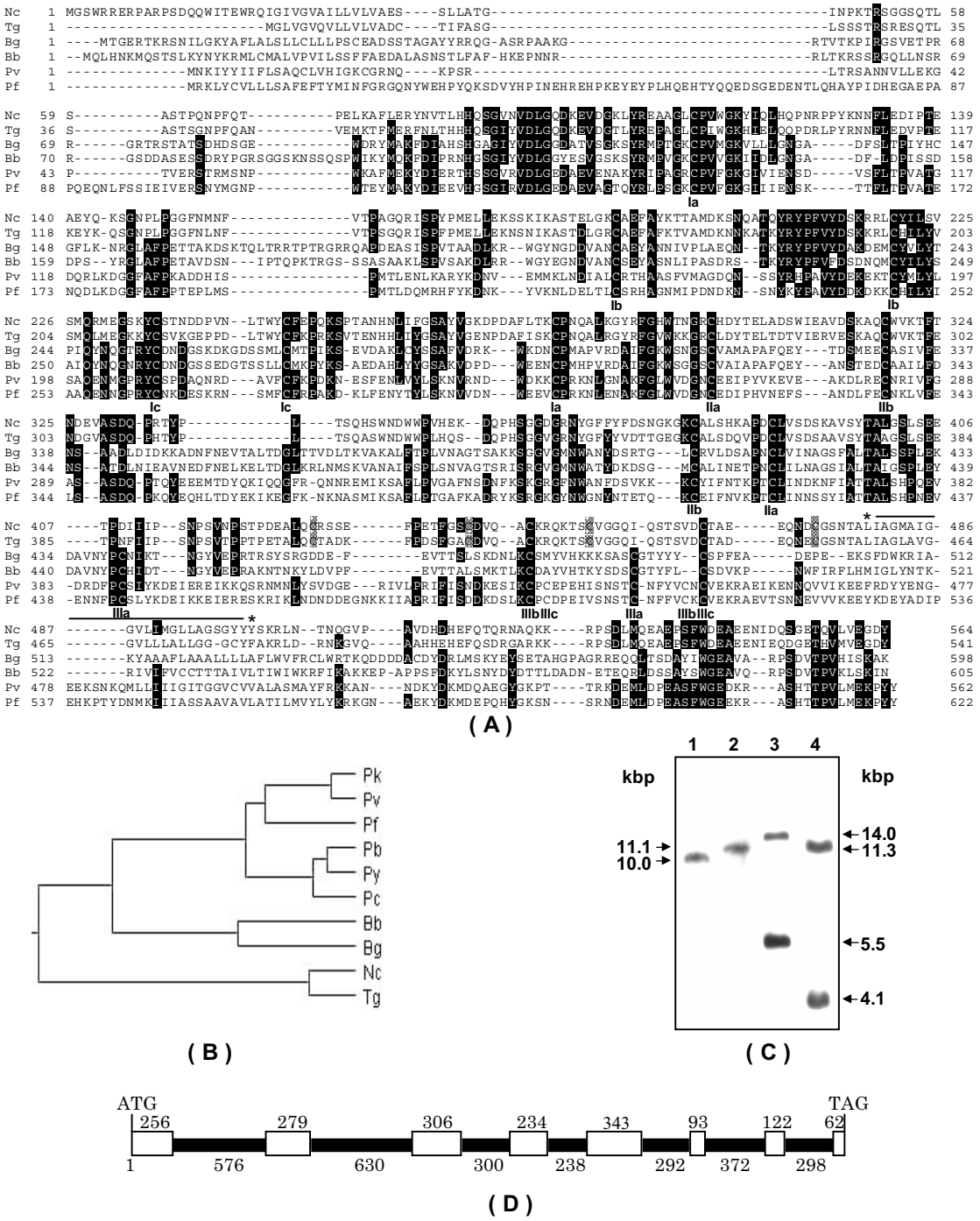
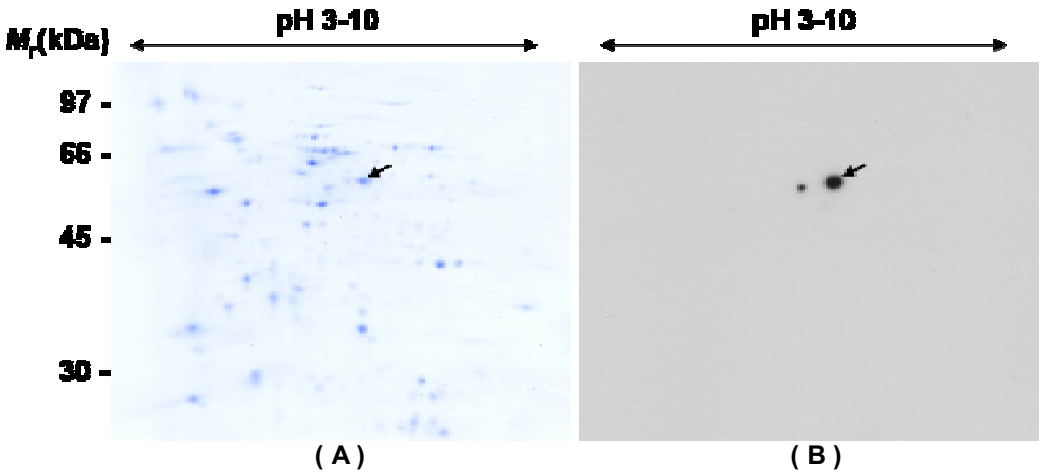
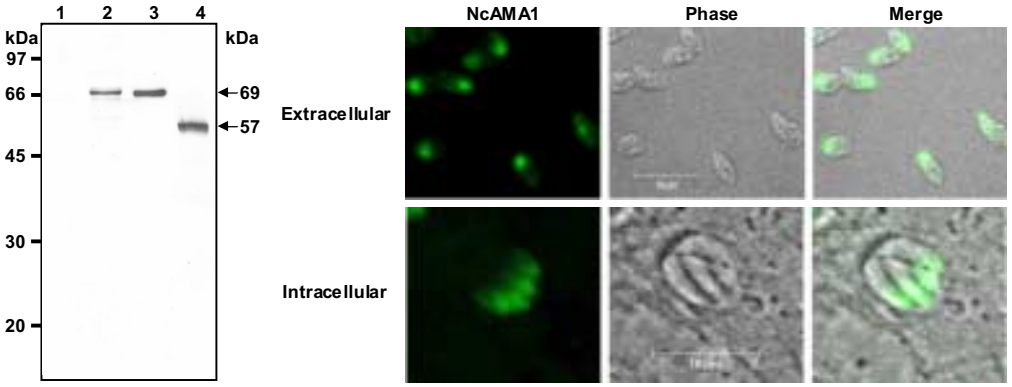

(A)
(B)
(C)
(D)
Pk
Pv
Pf
Pb
Py
Pc
Bb
Bg
Nc
Tg
kbp
kbp
1 2 3 4
11.1
10.0
14.0
11.3
5.5
4.1
ATG
TAG
256
279
306
234
343
93
122
62
1
576
630
300
238
292
372
298

Figure 2

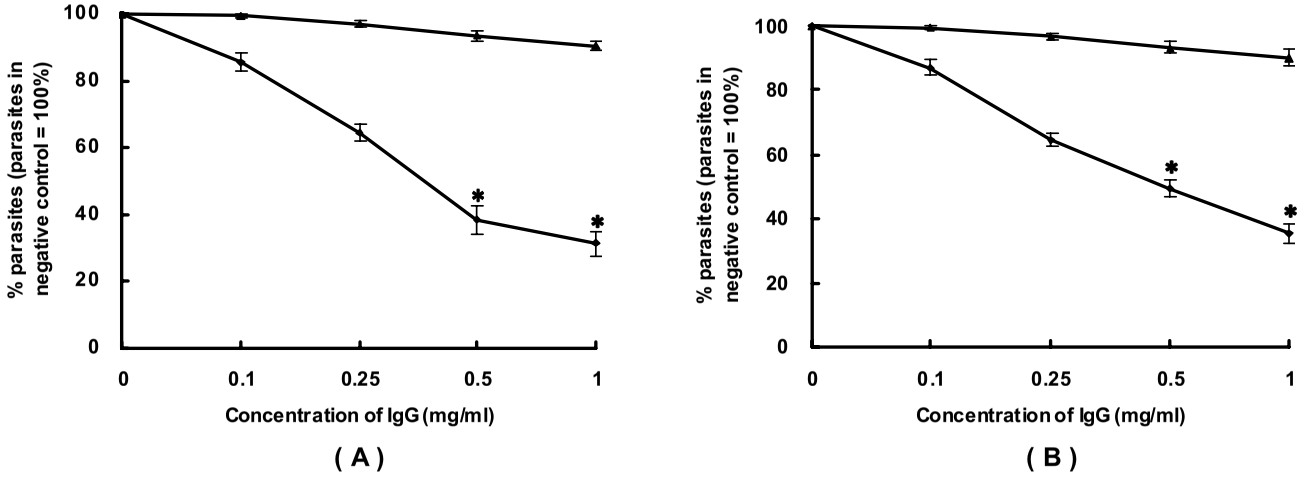


(A) (B)



(C) (D)

Figure 3


100
80
60
40
20
0
% parasites (parasites in negative control = 100%)
0
0.1
0.25
0.5
1
Concentration of IgG (mg/ml)
*
*
(A)
100
80
60
40
20
0
% parasites (parasites in negative control = 100%)
0
0.1
0.25
0.5
1
Concentration of IgG (mg/ml)
*
*
(B)