

Cloning, nucleotide sequencing and bioinformatics study of NcGRA7, an immunogen from *Neospora caninum*

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Abstract

Neospora caninum is an obligate intracellular parasitic protozoa and considered as causal agent of Neosporosis which infect wide variety of hosts. NcGRA7 is an immunodominant antigen recognized by sera from bovines, naturally infected by *N. caninum*, which is used as a powerful target for recombinant or DNA vaccine preparation against neosporosis. There is no study about identifying the molecular structure of *Neospora caninum* in Iran, so as first step, current study tried to identify NcGRA7 gene in this parasite in Iran. After extraction of total RNA from *N. caninum* tachyzoites, cDNA was synthesized and NcGRA7 gene was amplified using cDNA as template. Then the PCR product was cloned into pTZ57R/T vector and transformed into *Escherichia coli* (DH5 α strain), and the resulted recombinant plasmid was submitted for sequencing, followed by bioinformatics analysis. The data obtained from sequencing of native NcGRA7 was recorded in GenBank. The deduced amino acid sequence of NcGRA7 in current study was compared with other *N. caninum* NcGRA7 sequences and showed some identities and differences. NcGRA7 gene of *N. caninum* was successfully cloned into the pTZ57R/T vector and recombination was confirmed by sequencing, colony PCR, and enzymatic digestion, making it ready expression of recombinant protein for further studies.

Keywords: *Neospora caninum*, NcGRA7, Cloning, Sequencing

Introduction

Bovine neosporosis is the most frequently diagnosed cause of bovine abortion worldwide (Monney et al., 2011). *Neospora caninum*, a persistent protozoan parasite capable of infecting almost any warm-blooded vertebrate, is a member of phylum apicomplexa and has a complex lifestyle involving two phases of growth: an intestinal phase in canine hosts, and an extra-intestinal phase in other mammals (Dubey and Schares, 2011). It was originally identified in tissues of paralyzed dogs (Bjerkas and Presthus, 1988; Dubey et al., 1988). As revealed by molecular analyses, *N. caninum* is closely related to other coccidian parasite, *Toxoplasma gondii*, and therefore many of previously described *T. gondii* biological characteristics can be attributed to *N. caninum* so they would employ similar mechanisms for adhesion and invasion processes (Monney et al., 2011). Results of studies on *Neospora caninum* infection in Iran showed that this parasite could be

considered as a cause of economic loss in dairy cattle (Salehi et al., 2009). From several areas in Iran, *Neospora* infection has been reported in cattle (Nematollahi et al., 2011; Nouroollahi Fard et al., 2008; Razmi et al., 2006; Sadrebazzaz et al., 2007; Sadrebazzaz et al., 2004), dogs (Haddadzadeh et al., 2007; Hosseininejad et al., 2010; Malmasi et al., 2007; Yagoob, 2011) and camels (Hosseininejad et al., 2009; Sadrebazzaz et al., 2006).

Current studies on *N. caninum* are mainly focused on the mechanisms and antigens involved in the tachyzoite adhesion, invasion and its proliferation and persistence in the host cell and using these antigens for immunological purposes (Dubey and Schares, 2011). NcSRS2 was one of the most worked targets for developing recombinant vaccines and diagnostic kits against neosporosis (Soltani et al., 2013).

N. caninum exploits different secretory and antigenic proteins to invade a host cell and gain access to its intracellular environment. These proteins originate from distinct organelles termed micronemes, rhoptries, and dense granules. They

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are released at specific times during invasion to ensure the proteins are allocated to their correct target destinations (Howe and Sibley, 1999). Dense granule antigens (GRAs) are secreted by dense granules to the parasitophorous vacuole during parasite intracellular development (Cesbron-delauw, 1994). Dense-granule secretion shares several features with the regulated secretory pathway: (1) packaging in electron-dense vesicles; (2) fusion of these vesicles with the plasma membrane; and (3) calcium-regulated exocytosis. It has been suggested that dense granule antigens stimulate humoral immunity in the host. GRA7 is a highly immunogenic, dense granule protein in both *T. gondii* and *N. caninum* (Lally et al., 1997; Vonlaufen et al., 2004). Moreover, although GRA proteins appear to be related to intracellular parasite development, previous studies revealed that NcGRA7 might be involved in the initial host cell invasion process of *N. caninum* (Augustine et al., 1999; Cho et al., 2005). It has been showed that this immunogenic protein provides some protection against experimental *N. caninum* infection (Jenkins et al., 2004; Liddell et al., 2003; Nishikawa et al., 2009). Thus, the NcGRA7 protein could be considered as a vaccine candidate against neosporosis. Moreover, the immunogenicity of NcGRA7 has led to investigation of this antigen as a diagnostic reagent (Huang et al., 2007).

In the framework of the investigations on designing recombinant vaccines against neosporosis, this work focuses on the cloning and sequencing of NcGRA7 from Iranian isolate of *N. caninum* for the first time and bioinformatics based characterization of the important properties of its deduced protein. This work is first step in an attempt to design vaccine studies against neosporosis using NcGRA7 antigen that will be studied in the future.

Materials and Methods

Production of N. caninum tachyzoites

All cell culture reagents were purchased from Gibco-BRL (Zurich, Switzerland) and chemicals were from Sigma (St. Louis, MO, USA). Vero cells were routinely cultured in 25 cm² tissue culture flasks in 5 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U of penicillin/ml and 50 ug of streptomycin/ml and incubated at 37°C with 5% CO₂. A strain of *Neospora caninum* was kindly provided by Dr. Sadrebazzaz (Razi vaccine and serum research institute, Mashhad branch). *N. caninum* cells were maintained in BALB/c mice by serial

intraperitoneal inoculation of parasites was used for the experiment. *N. caninum* tachyzoites was maintained by serial passages in Vero cells. Cultures were passaged at least once per week. When 80% of the Vero cells that had been infected with *N. caninum* tachyzoites shows cytopathic effect (typically 3-4 days p.i.), the cell monolayers were removed by scraping, twice washed with phosphate buffered saline (PBS) solution, and then centrifuged at 1000 g for 10 min. Purified tachyzoites were checked for viability using trypan blue staining. Infected cells were trypsinized, washed twice in cold RPMI 1640 medium and the resulting pellet resuspended in 2 ml cold RPMI 1640 medium. Cells were repeatedly passed through a 25G needle.

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from 2 × 10⁶ purified *N. caninum* tachyzoites using NucleoSpin® RNA II kit (Machery-Nagel, Germany) according to the manufacturer's instructions using gene specific primers. RNA concentration was measured with the NanoDrop ND1000 (Thermo Scientific, Delaware, US) system.

Single-stranded cDNA was synthesized from isolated total RNA using a cDNA synthesis kit (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas, Germany) according to the standard protocol for first strand cDNA synthesis. Briefly, first strand cDNA synthesis reaction was performed in a 20 µl reaction mixture containing 100 ng of total RNA, 4 µl 5X reaction buffer, 2 µl 10 mM dNTP Mix, 12 µl nuclease-free water, 1 µl RiboLock™ RNase Inhibitor (20 u/µl), 1 µl RevertAid™ M-MuLV Reverse Transcriptase (200 u/µl) and 15 pmol of each gene specific primers. Reaction mixtures were incubated for 5 minutes at 25 °C followed by 60 minutes at 42 °C and the reactions were terminated by heating at 70 °C for 5 minutes.

PCR amplification

A pair of gene-specific primers were designed using Primer Premiere software (Biosoft) based on published NcGRA7 gene sequence in the GenBank to amplify NcGRA7 gene. Primers were synthesized by as follows: NG71-F (5'-CGAGAATTCAAATGGCCCGACAAGC-3') and NG71-R (5'-CGCAGGATCCTAACTATTCGGTGTCTAC-3') (Bioneer, South Korea). PCR reactions were performed using total cDNA as template. Reaction was carried out in 25 µl volume containing approximately 100 ng of cDNA template, 50 mM Tris buffer (pH: 8.3), 1.5 mM MgCl₂, 200 mM of

each ddNTPs, 0.5 U of Pfu DNA polymerase and 100 pM of each primers. Amplification reaction was performed using the following thermal profile: 95°C for 5 min, 35 amplification cycles (94°C for 40 sec, 62.5°C for 40 sec, and 72°C for 40 sec.), followed by a 72°C final extension for 10 min. Furthermore, false-negative results, caused by inhibitory compounds in the PCR reactions, were excluded by performing a simultaneous positive control reaction using the DNA extracted from tachyzoites of the NC-1 strain. The negative control consisted of dH₂O without DNA. A positive and negative control was included in each reaction. Amplified PCR products were analyzed by electrophoresis of 5 µl of each sample on 1% (W/V) agarose gel at a constant voltage of 100 v for 40 minutes, stained with SYBR® Safe DNA Gel Stain (Invitrogen, Paisley, UK). GeneRuler™ 100 bp Plus DNA Ladder (Fermentas) was used to compare the DNA fragment sizes. Agarose gel illuminated under UV, and photographed with an UVidoc Gel Documentation System (UVitec, UK).

Gel extraction of PCR products

The specific amplimers containing desired gene sequence were purified from the agarose gel by QIAquick Gel Extraction Kit (Qiagen, Germany) based on manufacturer's recommendations. This kit follows a simple bind-wash-elute procedure. Gel slices were dissolved in a buffer containing a pH indicator, allowing easy determination of the optimal pH for DNA binding, then mixtures were applied to the QIAquick spin column. Nucleic acids adsorbed to the silica membrane in the conditions provided by the buffer. Impurities were washed away and pure DNA was eluted with a small volume of low-salt buffer provided.

A tailing of PCR products

As exonuclease activity of the proofreading polymerases removes the 3'-A overhangs necessary for TA cloning, 3'-A overhangs must be added to fragments taking advantages of non-template activity of Taq DNA polymerase after PCR amplification since Taq polymerase preferentially adds an A to the 3'-ends in the presence of all four dNTPs. Briefly, a reaction was set up containing 25 µl purified PCR product, 5 µl 10X Taq reaction buffer, 5 µl MgCl₂, 5 µl dNTP (10 mM stock), 1 µl Taq polymerase, 9 µl H₂O. Then the mixture was incubated at 70 °C for 30 min. Finally, 3 µl of reaction mixture was run on a gel to quantify. This reaction product can directly be used in ligation reaction without any need to clean up reaction.

Ligation into pTZ57R/T vector

Tailed PCR products were ligated into pTZ57R/T Vector (Fermentas, Germany) based on TA cloning scheme according to the manufacturer's instructions. A 1:3 (vector to insert) molar ratio was used. Ligation reaction sat up in 30 µl volume containing 3 µl pTZ57R/T plasmid, 10 µl of A tailed PCR product, 1 µl T4 DNA ligase enzyme, 6 µl 5X buffer and 10 µl nuclease free distilled water. After gentle mix and a brief centrifuge, the ligation reaction mixture was incubated overnight at 10°C. The resulting plasmid was designated as pTZ-NcGRA7. Recombinant vector were stored at -20°C until transformation.

Transformation, Screening and Colony PCR

Preparation of competent cells from *Escherichia coli* strain DH5- α was performed by calcium chloride method (Sambrook et al., 1989). Advantages of chemical preparation of competent cells include simple procedure; no special equipment required and gives good transformation efficiencies. In general, it is the best method to use when the transformation efficiencies is not the problem. For transformation, 10 µl of ligation reaction product was added to 150 µl of competent cells and placed on ice for 40 minutes after vortex and spin. Then the mixture was incubated at 42 °C for 90 s and immediately was placed on ice for 5 minutes. Then 1 ml of LB antibiotic free medium was added to the transformed cells and allowed to recover by incubation at 37 °C for 2 hours with shaking. Cells harboring pTZ-NcGRA7 plasmid was plated and grown overnight at 37 °C on a LB agar plate (10 g NaCl, 5 g yeast extract, 10 g bacto tryptone) with ampicillin (100 µg/ml), X-Gal (Fermentas) and IPTG (Fermentas) for blue-white screening. After overnight incubation, plate was placed at 4 °C for 2 hours and cells from white colonies were harvested and cultured on antibiotic containing LB agar plates. After 16 hours incubation at 37 °C, cells harboring the recombinant plasmid grew up. Recombination confirmed by colony PCR with NcGRA7 gene specific primers. This technique was used to determine insert size in the vector. Briefly, a colony was picked with toothpick and swirl into 50 µl of ddH₂O in 1.5 ml microfuge tube. Then the tubes were heated at 95 °C for 10 minutes. Tubes were centrifuged for 5 minutes at top speed in microfuge and 40 µl of supernatant was transferred to 0.5 ml microfuge tubes and 2 µl of it was used as template in PCR reaction. All other PCR reactions conditions were as explained before.

Plasmid Purification

Cells harboring the recombinant plasmid were cultured in antibiotic containing LB medium for 16 hours at 37 °C in a shaker incubator. GeneJET Plasmid Miniprep Kit (Fermentas) was used to purify plasmids from *E. coli* DH5 α following the manufacturer's instructions. Briefly, 4 ml bacterial culture was harvested and lysed. The lysate was then cleared by centrifugation and applied on the silica column to selectively bind DNA molecules. The adsorbed DNA was washed to remove contaminants, and the pure plasmid DNA was eluted in a small volume of elution buffer. Plasmid DNA concentrations were determined by absorbance at 260 nm using NanoDrop ND1000 (Thermo Scientific, Delaware, US) system. The integrity of the DNA plasmids was checked by agarose gel electrophoresis. Also resultant recombinant plasmid (pTZ-NcGRA7) was compared with native plasmid (pTZ57R/T) by electrophoresis of 3 μ l of extracted plasmid on a 1% agarose gel.

Enzymatic Digestion of pTZ-NcGRA7

With regard to presence of BamHI and EcoRI restriction sites on recombinant plasmid extracted from white colonies, the recombinant plasmid was characterized for the presence and size of inserts by double digestion with *Eco*RI and *Bam*HI. Each 20 μ l digestion reaction contained 10 μ l of plasmid, 1 μ l of each restriction enzyme, 2 μ l of 10X buffer (buffer R, based on Fermentas recommendations) and 6 μ l of dH₂O. Digestion was performed by incubation at 37 °C for 2 hours. Digestion products were analyzed by electrophoresis on 1% agarose gel containing SYBR® Safe DNA Gel Stain (Invitrogen, Paisley, UK).

Sequencing of NcGRA7 gene

The nucleotide sequence of the inserts (NcGRA7) in the recombinant plasmid pTZ-NcGRA7 was verified by sequencing in the forward and reverse directions using primer walking approach (Eurofins MWG Operon, Germany). M13 uni (-21) forward primer (5'-TGAAAACGACGGCCAGT-3') and M13 rev (-29) reverse primer (5'-CAGGAAACAGCTATGACC-3') were used for sequencing. DNA Baser v3 (Heracle BioSoft, Romania) was used for sequencing data assembly to produce a consensus sequence for each DNA sample used.

Blast search and bioinformatics study

The nucleotide sequence of NcGRA7 was

submitted to the BLAST search (megablast algorithm) at NCBI server (<http://www.ncbi.nlm.nih.gov/blast/>) to compare with sequences presented in the GenBank. For detailed analysis, all closely related sequences and deduced amino acid sequences between published sequences were aligned by ClustalW2 multiple sequence alignment program (<http://www.ebi.ac.uk/Tools/clustalw2/>) (Larkin et al., 2007).

The sequences were analyzed for signal peptides using SignalP 4.0 (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen et al., 2011), protein domains using Prosite (<http://prosite.expasy.org/>) (Sigrist et al., 2010) and potential transmembrane regions were checked with the ProtScale tool on the Expasy server (<http://expasy.org/tools/protscale.html>).

Hydrophobicity plot of NcGRA7 protein was also drawn which characterizes its hydrophobic and hydrophilic characteristics that may be useful in predicting membrane-spanning domains, potential antigenic sites and regions that are likely exposed on the protein surface (Hopp and Woods, 1981; Kyte and Doolittle, 1982).

Phylogenetic and molecular evolutionary analyses were conducted using CLC main workbench software (CLC bio) by bootstrap test with 1000 replications was applied to estimate the confidence of branching patterns of the UPGMA tree. Also, pairwise comparisons were done to clarify the pairwise distances and percent identities.

Results

Production of *N. caninum* tachyzoites

Vero cells became confluent on day 3 and then were infected with *Neospora caninum* tachyzoites. Tachyzoites grew well in Vero monolayers (Fig. 1). *N. caninum* tachyzoites were maintained in and purified from, Vero cell monolayers and were immediately used for RNA extraction.

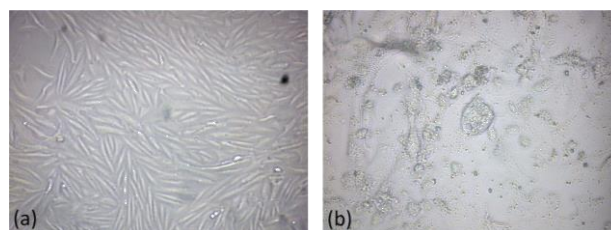


Figure 1. (a) Confluent Vero cells on day 3. (b) *N. caninum* tachyzoite infected Vero cells.

homepage on GeneDB
(<http://www.genedb.org/Homepage/Ncaninum>).

The protein corresponds to the following gene model as NCLIV_021640.

Bioinformatics study

Blast analysis of NcGRA7 gene revealed 100% identity with other recorded NcGRA7 genes in genebank. Similarity of this gene with *Toxoplasma gondii* GRA7 genes varied between 34-45%. Various physico-chemical properties of studied protein were computed using ProtParam program. ProtParam analysis results are shown in Table 1.

Table 1. Physico-chemical properties of NcGRA7 protein derived from ProtParam.

Number of amino acids	217
Molecular weight	22494.9
Theoretical pI	4.54
Negatively charged residues (Asp + Glu)	34
Positively charged residues (Arg + Lys)	19
Formula	C 977 H 1562 N 272 O 330 S 3
Total number of atoms	3144
Extinction coefficients	13075 (12950)
Estimated half-life	30 hours (mammalian reticulocytes, in vitro) >20 hours (yeast, in vivo) >10 hours (<i>Escherichia coli</i> , in vivo)
Instability index	29.87
Aliphatic index	84.56
GRAVY	-0.216

Discussion

In this study, UV spectrophotometry was used in order to take advantage of this method one needs an accurate measure of the protein of interest's extinction coefficient (molar absorption coefficient). The extinction coefficient indicates how much light a protein absorbs at a certain wavelength. This estimation is useful for following a protein with a spectrophotometer when purifying it. Two values are produced by ProtParam, both for proteins measured in water at 280 nm. The first one shows the computed value based on the assumption that all cysteine residues appear as half cystines (i.e. all pairs of Cys residues form cystines), and the second one assuming that no cysteine appears as half cystine (i.e. assuming all Cys residues are

reduced). This measure is estimated using the method of Pace et al., which calculates the sum of (NumberAA x Extinction Coefficient AA) for three amino acids that absorb at 280 nm: tyrosine, tryptophan, and the dimeric amino acid cystine (two cysteine [Cys] residues covalently joined through a disulfide bond). The absorbance of the protein at 280 nm (A₂₈₀, or OD₂₈₀) is calculated by dividing the extinction coefficient by the molecular weight of the protein.

The half-life is a prediction of the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. ProtParam relies on the "N-end rule", which relates the half-life of a protein to the identity of its N-terminal residue; the prediction is given for 3 model organisms (human, yeast and *E. coli*). The N-end rule states that the in vivo half-life of a protein is a function of the nature of its amino-terminal residue (Bachmair et al., 1986). Because the N terminus amino acid of NcGRA7 protein is Methionine, so based on N-end rule, it will be stable more than 10 hours in *E. coli* cells.

The instability index provides an estimate of the stability of protein of interest in a test tube. Values greater than 40 indicate that the protein may be unstable in vitro. This index assigns a weighted instability value to each dipeptide in the protein (Guruprasad et al., 1990). These values were derived from an analysis that found a significant difference in the occurrence of certain dipeptides between stable and unstable proteins. Analysis by ProtParam revealed that NcGRA7 can be classified as a stable protein (Instability index: 29.87).

The protein aliphatic index is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). It may be regarded as a positive factor for the increase of thermo stability of globular proteins. Aliphatic index is calculated using the method of Ikai as the sum of (Molar %AA x Volume AA) for alanine, leucine, isoleucine and valine (where Volume AA is the relative value compared to alanine). Aliphatic index analysis results was consistent with previously described results from instability index while aliphatic index is defined as a measure of thermostability, lower value of instability index for NcGRA7 can be contributed to aliphatic index. In other words, higher aliphatic index is related to lower instability index and higher stability.

A GRAVY (Grand Average of hydropathicity) score can be calculated as the sum of the hydropathy values for all the amino acids in a protein sequence divided by the number of residues in the sequence. In essence, a GRAVY score is the

relative value for the hydrophobic residues of the protein. Although no positional or interaction effects for adjacent residues are taken into consideration by the GRAVY score, it still provides some indication of the physical state of the protein (Kyte and Doolittle, 1982).

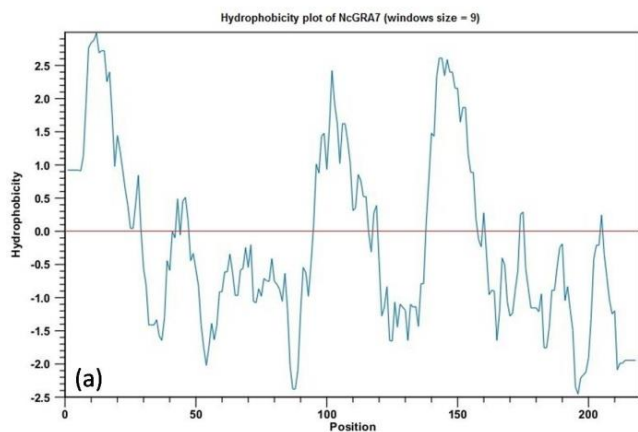


Figure 4. Hydrophobicity plots of the NcGRA7 (a) windows size = 9

This index indicates the solubility of the proteins: positive GRAVY protein is hydrophobic while negative GRAVY protein is hydrophilic (Kyte and Doolittle, 1982). As derived from ProtParam analysis, NcGRA7 gained a negative GRAVY score so it can be inferred that NcGRA7 is a hydrophilic protein. According to Kyte and Doolittle (1982), integral membrane proteins typically have higher GRAVY scores than do globular proteins. Though this score is another helpful piece of information, it cannot reliably predict the structure without the help of hydropathy plots.

There are some methods for evaluation of the degree of interaction of polar solvents such as water with specific amino acids. In these methods a hydrophobicity plot is created that is a quantitative analysis of the degree of hydrophobicity or hydrophilicity of amino acids in a protein (Kyte-Doolittle scale indicates hydrophobic amino acids, while the Hopp-Woods scale measures hydrophilic residues). This measure is implicated to identify possible structure or domains of a protein. Plot shape analysis prepares information about partial structure of the protein of interest. For example, extension of about 20 amino acids with positive shows that these amino acids may be part of alpha-helix spanning across a lipid bilayer, which is composed of hydrophobic fatty acids. On the other hand, stretch of amino acids with negative hydrophobicity indicates that these residues are in contact with solvent or water, and that they are

probably resided on the outer surface of the protein. To elucidate properties of NcGRA7, the hydrophobicity plot of the deduced protein sequence was reproduced based on Kyte and Doolittle (1982) algorithm (Fig 4). Two plots were drawn for NcGRA7; one of them was plotted with windows size of 9 for seeking surface regions and second one was plotted with windows size of 19 to look for transmembrane regions. As shown in Figure 4-a, possible surface regions can be identified as strong negative peaks. In Figure 4-b, transmembrane regions are identified by peaks with scores greater than 1.6.

Protein signature databases are essential tools to identify relationships between sequences, so they can be implicated for protein classification and inferring their function. InterProScan (Zdobnov and Apweiler, 2001) is a tool that combines different protein signature recognition methods into one resource. InterProScan results were summarized in Fig. 5 and Table 2.

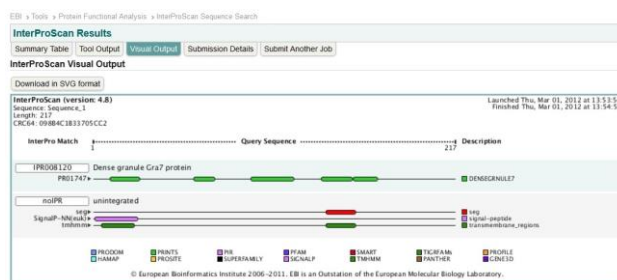


Figure 5. InterProScan visual output for NcGRA7.

Table 2. NcGRA7 confidently predicted domains and features.

Name	Begin	End
signal peptide	1	27
transmembrane	138	160

As mentioned earlier, dense granule antigens (GRAs) are secreted from the *N. caninum* tachyzoite. Gra7 is released by the parasite during intra-vacuolar habitation. NcGRA7 is a 5-element fingerprint that provides a signature for the dense granule Gra7 proteins. The fingerprint was derived from an initial alignment of 2 sequences: motif 1 lies in the putative signal sequence and motif 4 encodes the putative transmembrane domain (Table 2).

Jukes-Cantor distance between each pairs of

sequences was calculated (Fig. 6 – upper diagonal). This number is given as the Jukes-Cantor correction of the proportion between identical and overlapping alignment positions between the two sequences. Also Percent identity calculated as the percentage of identical residues in alignment positions to overlapping alignment positions between each pair of sequences (Fig. 6 – lower diagonal).

	1	2	3	4	5
AF176649	1	0.02	0.02	0.28	0.48
NCU72991	2	98.50	0.01	0.27	0.47
NCU82229	3	98.00	98.83	0.26	0.46
NCU36386	4	76.37	77.05	77.82	0.36
NcGRA7 (Current Study)	5	64.55	64.97	65.73	71.18

Figure 6. Upper diagonal: Calculated pairwise Jukes-Cantor distance, Lower diagonal: Calculated pairwise percent identities.

To determine the phylogenetic position of the NcGRA7 in the current study, its sequence was used for comparative sequence analysis against known NcGRA7 sequences. The NcGRA7 sequence of the current study showed a high relationship to each of known sequences of the NcGRA7 (Fig. 7).

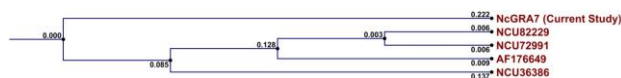


Figure 7: Phylogeny of NcGRA7 sequence of the current study. The tree was constructed using the UPGMA method. Numbers along branches represent length values.

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N. caninum is an obligatory intracellular parasite which has complicated life cycle and almost infects all nucleated cells (Dubey and Schares, 2011). *N. caninum* causes dangerous manifestation in fetus which the most dangerous effect of congenital neosporosis is abortion (Dubey and Schares, 2011; Sadrebazaz et al., 2004). The congenital infection has different symptoms based on the intensity and variety of contamination in the organs. Severity of the disease is related to stage of the pregnancy period which the infection occurs (Salehi et al., 2009). In this study, the NcGRA7 gene of *Neospora caninum* tachyzoites surface antigen was cloned for studying its immunogenic potentials in future. In conclusion, a 679 bp length fragment of a gene corresponding to the 22 kDa protein gene of *Neospora caninum* tachyzoites dense granule protein (NcGRA7) was cloned and verified by sequencing and bioinformatics analysis and expression of this gene is the next step to prepare an effective vaccine formula against neosporosis.

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