Cloning and expression of *Neospora caninum* dense-granule 7 in *E. coli*

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Received: 7 October 2012 / Accepted: 5 December 2012 / Published online: 18 December 2012
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Abstract *Neospora caninum* is an obligate intracellular protozoan which induces abortion, still birth and neuromuscular disorders in cattle and is an important problem in dairy and beef industry worldwide. The dense granule protein 7 (GRA7) of *N. caninum* is an immune-dominant protein shared by both tachyzoite and bradyzoites. This study was conducted to produce recombinant GRA7 of *N. caninum* using a plasmid with high level of expression of this protein in *E. coli*. For this purpose, a segment of *N. caninum* DNA corresponding to GRA7 was amplified using PCR. After sequencing, this fragment was cloned into expression vector pMAL-c2X under the control of the *lac* promoter. Expression of this plasmid in *E. coli* strain TG1 was identified by western blotting. In this study, pMAL-c2X had a strong promoter to produce high level expression of NcGRA7. This result revealed that this

recombinant protein with pMAL-c2X vector may be suitable for developing of diagnostic procedures.

Keywords *Neospora caninum* · NcGRA7 · Cloning · Expressing · *E. coli*

Introduction

Neospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) is a cyst forming protozoan parasite that is closely related to *Toxoplasma gondii* and is one of the most important etiological agent of abortion and neonatal mortality in cattle all around the world (Dubey and Schares 2006, 2011; Anderson et al. 2012). First recognition of the parasite was in 1984 when Bjerkas et al. reported unidentified sporozoan protozoa in dogs in Norway (Bjerkas et al. 1984).

Wide range of mammalian species such as cattle, sheep, goat, deer and horse are known as intermediate hosts of the parasite (Dubey and Lindsay 1996). There are three stages of infection in *N. caninum* life cycle: tachyzoites, tissue cysts and oocysts. Transmission of the parasite to the intermediate hosts occurs mainly by ingestion of sporulated oocysts which shed by definitive hosts or vertically from the mother to the fetus via the placenta (Gondim et al. 2002; Davison et al. 2001).

At yet, many diagnostic tools have been described to detect the infection with *N. caninum* in various hosts. Serologic diagnosis in cattle is based on *N. caninum* specific antibodies (Jenkins et al. 2002). Currently, serologic assays have been developed to determine the anti-*N. caninum* antibodies in cattle including immunoblotting (Atkinson et al. 2000), the indirect fluorescent antibody test (Dubey and Lindsay 1996), direct

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agglutination test (Packham et al. 1998) and enzyme-linked immunosorbent assay (ELISA) (Baszler et al. 1996; Björkman et al. 1999; Osawa et al. 1998), using whole tachyzoites or tachyzoite derived antigens.

So far, three important *N. caninum* recombinant antigens have been determined for diagnostic purposes (Chahan et al. 2003) namely, NcSAG1, NcSRS2 (Howe and Sibley 1999) and the dense granule protein NcGRA7 (Alvarez-Garcia et al. 2007; Aguado-Martínez et al. 2010). The aim of this study was to produce recombinant NcGRA7 protein in an efficient prokaryotic expression system, in order to standardize an ELISA based serodiagnostic procedure in Iran in near future.

Materials and methods

Parasite

Neospora caninum tachyzoites, Nc-1 strain (Dubey et al. 1988), were grown in monolayers of Vero cells cultured in RPMI 1640 medium (Sigma Co., USA) supplemented with 2 % of fetal calf serum and penicillin (10,000 U/ml), streptomycin (100 g/ml) and amphotericin B (25 g/ml) at 37 °C with 5 % CO₂ in tissue culture flasks (Furuta et al. 2007). Tachyzoites were harvested by scraping the monolayer when 80 % of the cells were infected and were then purified by sequential passage of the cell monolayer through a 27-gauge needle and centrifuged to remove host cell debris. The supernatant was collected, centrifuged and washed twice in phosphate-buffered saline (PBS, pH 7.2) (Silva et al. 2007).

DNA extraction and PCR

Tachyzoites were prepared at a concentration of 1×10^8 /ml. Extraction of genomic DNA was performed according to the Qiagen kit protocol. The DNA sequences were obtained from the Genbank database and the accession number—U82229 used to design the required primers. The designed and utilized forward and reverse primers were GCGAGAATTCGCTGGAGACTTGG and GCGAGAATTCCTATTCGGTGT respectively.

PCR was carried out in a total volume of 50 µl, containing 5 µl of DNA template, 1 µl of each primers at 20 pmol, 1 µl of 10 mM dNTPs mix, 5 µl of 10× PFU PCR buffer, 1.5 µl of 50 mM MgCl₂ and 5 u PFU DNA polymerase (BioNeer, Korea). PCR condition was as follow: pre denaturing at 94 °C for 5 min, denaturing at 94 °C for 1 min, annealing at 51 °C for 1 min, extension at 72 °C for 90 s (35 repeats) and final extension at 72 °C for 7 min in a thermal cycler. The amplified DNA was visualized on

1.0 % agarose gel stained with cyber safe. Sequence analysis was performed on the PCR product.

Cloning and expression of NcGRA7

PCR product was purified from gel with the Vivantis gel extraction kit (Malaysia). The purified PCR product and pMAL-c2X plasmid (New England Biolabs, USA) were digested with *Eco*R1 restriction enzymes (Fermentas) at 37 °C for 2.5 h and were extracted from agarose gel using the gel extraction kit. Ligation process was performed on digested DNA fragment and plasmid vector at 22 °C for 2 h by T4 DNA ligase (Fermentas), followed by overnight incubation at 4 °C. *Escherichia coli* host strain TG1 was transformed by the ligation product using Chung transforming method (Chung et al. 1989).

The transformed colonies appeared on the selective Luria–Bertani (LB) agar, were grown in LB broth containing ampicillin. Extraction of plasmid was performed by Vivantis plasmid extraction kit and presence of GRA7 inserts were confirmed by restriction digestion of recombinant plasmids with *Eco*R1.

Expression of GRA7 protein in *E. coli* strain TG1

Transformed *E. coli* with pMAL-c2X-NcGRA7 was cultured in LB broth media containing 20 mM glucose at 37 °C until the OD₆₀₀ nm of 0.5. Synthesis of recombinant MBP-NcGRA7 was then induced with isopropyl-1-β-D—thiogalactopyranoside (IPTG) at 37 °C for 3 h. Maltose binding protein (MBP) is a protein of about 42.5 kDa which is encoded by pMAL-c2X plasmid. Thereafter, samples of bacteria were harvested, centrifuged (7,000×g for 5 min) and boiled for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl, Ph6.8, 2 %SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 0.02 % bromophenol blue) for electrophoresis in a 10 % acrylamide gel.

Western blotting

After SDS-PAGE, the protein bands were transferred onto nitrocellulose membrane. The membrane was blocked with 0.2 % Tween 20 in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂PO₄, 1.4 mM KH₂PO₄ plus 0.05 % Tween) at room temperature for 2 h. The blots were washed three times with PBST (0.05 % Tween 20 in PBS) and incubated with 1/100 dilution of an ELISA positive bovine serum for 1 h. The membranes washed three times with PBST and incubated with 1/1,000 dilution of a peroxidase conjugated rabbit anti-bovine IgG (Sigma, USA) for 1 h. The membranes were washed with PBST and PBS and developed in 4-chloro-1-naphthol and hydrogen peroxide (H₂O₂).

Fig. 1 The PCR products revealing 600 bp band

Fig. 2 SDS-PAGE electrophoresis on 10 % polyacrylamide gel. 1 *E. coli* strain TG1 containing pMAL-c2X as control after induction. 2 *E. coli* extracts expression of pMAL-c2X-GRA7

Results

An expected PCR product of 600 bp was amplified using the designed primers (Fig. 1) and its identity was confirmed by sequencing.

The PCR product was purified and digested with restriction enzyme *Eco*R1. The fragment was then inserted into the expression vector pMAL-c2X and the construct

Fig. 3 Western blotting analysis. Lane 1 reveals marker protein and lane 2 reveals the reactivity positive serum with bacteria expressing MBP and MBP-GRA7

was used to transform the *E. coli* strain TG1. Sixty clones appeared on LB agar. Screening was performed with digesting plasmids by *Eco*R1 enzyme. Finally, three clones carried the recombinant plasmid and expressed the MBP-NcGRA7 protein. Expression of a protein of about 66 kDa, corresponding to predicted molecular weight of MBP-NcGRA7 (GRA7:24 kDa and MBP: 42 kDa) was indicated by SDS-PAGE (Fig. 2).

Reaction of the expressed protein in Western blotting (Fig. 3), with a bovine serum, positive in *N. caninum* ELISA confirmed that NcGRA7 was successfully cloned and expressed in *E. coli*.

Discussion

Neosporosis is one of the serious economical problem in dairy and beef industry worldwide (Dubey 2003). At yet, many researchers have studied on different aspects of *N. caninum* and many studies were reported the prevalence of bovine infection with *N. caninum* in Iran, such as 37 % of cattle in Ahwaz (Haji Kolaei et al. 2008), 15.7–19.6 % of cattle in Mashhad (Sadrebazzaz et al. 2004), 46.29 % of dairy cattle of Mashhad (Razmi et al. 2006), 12.6 % of cattle in Kerman (Nourollahi Fard et al. 2008), 32 % of rural and industrial cattle in North of Iran (Youssefi et al. 2009) and 38.8 % of pregnant cattle in Tehran (Salehi et al. 2009). These data reveal the importance of this parasite in Iran.

It is necessary to find a specific antigen for detection of antibodies against *N. caninum* with the least cross-reactions with closely related parasites such as *T. gondii* (Björkman

et al. 1999; Chahan et al. 2003; Conrad et al. 1993; Dubey and Lindsay 1996).

GRA proteins involve a highly immunogenic group which are excreted/secreted during parasite interacellular development by dense granule to the parasitophorous vacuole (Cesbron-Delauw et al. 1996). 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). NcGRA7 is an immunodominant antigen in both of tachyzoite and bradyzoite stages (Vonlaufen et al. 2004; Alvarez-Garcia et al. 2007), unlike NcSAG1, which is only expressed in tachyzoites (Fuchs et al. 1998) and it seems to be a good marker in detecting antibodies against both stages (Huang et al. 2007). The main objective of our study was to clone and express the NcGRA7 gene using an efficient prokaryotic expression vector, in order to produce a sufficient amount of the protein for developing diagnostic kits.

So far, cloning and expression of NcGRA7 has performed in different expression systems such as pCMV-luc1 eukaryotic expression vector (Liddell et al. 2003) and in bacterial expression vectors, pGEX-4T-3 (Hara et al. 2006) and pRSET-C (Aguado-Martínez et al. 2010). In this study we cloned and expressed NcGRA7 for the first time in Iran with new and different primers which have sites for *Eco*R1 restriction enzymes. The pMALc2x used as a vector for gene expression and protein production. This vector has a strong promoter and so, its protein expression will be high compared to the other vectors. The rate of gene cloning into this vector and subsequently transformation was high and noticeable, which enables work with this vector to be done more easily than with the other vectors. The presence of *lac* promoter in this vector increases the protein expression and under IPTG induction this will be enhanced considerably. In addition, the presence of maltose binding protein in recombinant expressed protein will facilitate its purification. Analysis of sequence variation in (24 KDa) showed a very similar molecular mass for protein expressed by *N. caninum*. Hereafter, the obtained recombinant protein will be used in serological methods in our region to study neosporosis more clearly.

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