Production of MAG1 Antigen of *Toxoplasma gondii* in *Escherichia coli*

Mohammad Talebzadeh, Reyhaneh Mohabati, Jalal Babaie, Samira Amiri, Mojgan Allahyari, *Majid Golkar*

Department of Parasitology, Molecular Parasitology Laboratory, Pasteur Institute of Iran, Tehran, Iran

Received Oct 28, 2013; accepted Dec 14, 2013

**INTRODUCTION**

*Toxoplasma gondii* is an obligate intracellular protozoan parasite that can infect human and other warm-blooded animals. Although usually asymptomatic in immunocompetent individuals, toxoplasmosis may reactivate in immunocompromised patients (e.g. AIDS patients and organ transplant recipients) and cause severe diseases such as toxoplasmic encephalitis. Moreover, primary maternal infection can put the fetus at risk for serious medical problems, e.g., permanent neurological and ocular damage or even for death in utero. On the other hand, occurrence of *T. gondii* infection before pregnancy brings no danger to the fetus, except in immunocompromised individuals, while it is quite harmless in immunocompetent individuals.

TRICHOMONAS Tissue Cyst Matrix Protein (MAG1) induces early humoral and cell-mediated immune responses. Previous studies suggested recombinant MAG1 as a promising antigen for serodiagnosis of *Toxoplasma gondii* infection. Methods: A DNA fragment encoding *mag1*, comprising amino acids 50 to 207, was amplified from *T. gondii* RH strain and cloned in prokaryotic expression plasmid pET-15b(+). Recombinant MAG1 was expressed in *Escherichia coli*, and highly purified in a single step by immobilized metal ion affinity chromatography. Results: The cloned DNA fragment was sequenced and showed 100% similarity with the published sequences available in GenBank Database. The results indicated proper antigenicity of recombinant MAG1 . In Western blot analysis, purified protein showed a much stronger reactivity with sera from patients with acute *Toxoplasma* infection, compared to sera from chronic infection. Conclusion: MAG1 protein, alone or in combination with other acute-phase markers might be useful in discriminating acute/reactivated *Toxoplasma* infections from chronic forms. J Med Microbiol Infec Dis, 2014, 2 (1): 40-44.

**Keyword:** MAG1, *Toxoplasma gondii*, *Escherichia coli*, serodiagnosis.
They were prepared from ten acute-phase or ten chronic-phase serum samples, respectively. Serum samples were assumed to belong to acute infections if they were IgG-and IgM-positive and had low avidity index, as tested by Euroimmun ELISA kits (Euroimmun, Lübeck, Germany). The chronic serum samples were IgG-positive and IgM-negative.

Parasitic and bacterial strains. *T. gondii* RH strain was used for genomic DNA extraction. Tachyzoites were injected into peritoneal cavity of Swiss mice. Three days later, tachyzoites were harvested from peritoneal fluid, washed with phosphate-buffered saline (PBS) and stored at -80°C until used. Escherichia coli (E. coli) Top 10F’ (Invitrogen, Carlsbad, CA), *E. coli* Rosetta (DE3) and B21(DE3) pLysS (Promega, Madison, WI) were used for cloning and expression of recombinant antigen, respectively.

Construction of the recombinant expression plasmid. DNA sequence of magl was obtained from GenBank database (accession no. AF251813). Genomic DNA was extracted from tachyzoites of *T. gondii* RH strain, by a genomic DNA extraction kit (Bioneer, Seoul, Korea) and used as template for PCR amplification of magl gene fragment, encoding amino acids 50-207 using specific primers of 5′-GAGCATATGGATGAAGCGACCGG-3′ (forward) and 5′-CGCTCGAGACCTGTAACCCTTTAGAATATAC-3′ (reverse). For cloning, *Nde*I and *Xho*I restriction sites (underlined) were introduced at 5′ end of the forward and reverse primers, respectively. PCR amplification was performed as follows: 1 cycle of 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s, and a final primer extension for 30 min at 72°C. The 491 bp amplicon was ligated into the T/A cloning vector, pTZ57R/T (Fermentas, Vilnius, Lithuania); the ligation product was transformed into *E. coli* TOP10F’ cells and recombinant clones were selected by blue/white screening. Recombinant plasmid was extracted from culture of a white bacterial colony and digested with *Xho*I and *Nde*I restriction enzymes. The products of restriction digestion were analyzed by agarose gel-electrophoresis and the insert was purified from the gel using DNA gel purification kit (Bioneer, Seoul, Korea). The purified DNA fragment was inserted in frame into expression vector pET-15b (+) previously digested with *Xho*I / *Nde*I restriction enzymes. The presence of MAG1 insert in the recombinant plasmid was checked by restriction analysis using *Afa*I enzyme, and by sequence analysis. The recombinant plasmid was named pET-MAG1.

Expression of MAG1. *E. coli* Rosetta (DE3) bacteria were transformed by pET-MAG1 and grown in Luria Bertani (LB) broth supplemented with Ampicillin (100 mg/ml) and chloramphenicol (34 μg/ml). The culture was grown with vigorous shaking at 37°C to an optical density of 600 nm of 0.6-0.8. Protein production was induced by 0.1 mM isoprropyl-β-D-thiogalactoside (IPTG), and bacteria were incubated with vigorous shaking for an additional period of 4 h.

Induced cells were harvested by centrifugation and expression of recombinant MAG1 was analyzed by SDS-polyacrylamide gel-electrophoresis (SDS-PAGE). In order to assess solubility of MAG1, 1 ml pellet of induced bacteria was lysed in 100 μl of the lysis buffer (20 mMTris-HCl pH 8.5, 0.5 M NaCl, and 1 mg/ml lysozyme) at 4°C for 30 min. The lysate was centrifuged at 16,000 g for 15 min at 4°C, and supernatant removed for further analysis. Densitometry analysis was performed on supernatant by ImageJ1 software (version 1.6, NIH, Bethesda, MD) to compare expression of MAG1 under different experimental conditions.

Purification of MAG1. Pellet of 500 ml induced Rosetta (DE3) bacteria was resuspended in 10 ml of binding buffer (10 mM imidazole, 0.5 M NaCl, 30 mMTris-HCl pH 7.5, 0.1% Triton X-100, protease inhibitors cocktail without EDTA (Roche, Mannheim, Germany) containing 1 mg/ml lysozyme). The cells were sonicated for 6 min using MSE ultrasonic disintegrator (Fisons, Loughborough, UK) at 60% power, centrifuged at 12,000 g and 4°C for 20 min, and the supernatant was passed through a 0.2 μm (pore size) filter. Nickelnitrilotriacetic acid (Ni2+-NTA) resin (3.5 ml), previously equilibrated with binding buffer, was added to the supernatant and stirred gently for 1 h at 4°C. Then, the resin was sequentially washed with 35 ml of washing buffers 1, 2 and 3 (binding buffer containing 50, 75, and 100 mM imidazole, respectively). MAG1 was eluted with elution buffer (binding buffer containing 500 mM imidazole and 0.01% Triton X-100) and dialyzed against PBS overnight at 4°C. Protein concentration was determined using DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Electrophoresis and Western blot analysis. Bacterial lysate, soluble and insoluble proteins and purified MAG1 were analyzed by SDS-PAGE using 12% polyacrylamide gel. Protein bands were transferred onto PVDF membrane. The membrane was incubated with 2% bovine serum albumin in PBS-0.05% Tween 20 and probed with 1/100 diluted pooled sera from pregnant women with either acute or chronic *T. gondii* infection. Bound antibodies were detected using horseradish peroxidase conjugated goat anti-human IgG antibodies diluted 1/20,000 (Sigma, Hilden, Germany). Human sera and secondary antibodies were diluted in blocking buffer. Signals were detected using 3, 3'-diaminobenzidinetetrahydrochloride (DAB) substrate (Sigma, Hilden, Germany).

RESULTS

Cloning of MAG1 DNA. The magl gene fragment corresponding to amino acids 50 to 207 was amplified by PCR from genome of tachyzoites of *T. gondii* RH strain (Figure 1). The amplicon was inserted into pTZ57R/T, and sub-cloned into pET-15b (+) expression vector. Screening of recombinant clones harboring the recombinant pET-MAG1 plasmid was performed by restriction digestion using *Afa*I and also *Nde*IXhol double digestion (Data not shown). Sequence analysis of a positive clone demonstrated 100% similarity between the cloned gene and published sequence of MAG1 (accession no. AF271813) (data not shown).
Production of MAG1 Antigen in E. coli.

Fig. 1. PCR amplification of *T. gondii* mag1 gene fragment by specific primers. Lane 1, mag1 PCR Product; lane 2, DNA size marker (1 Kb DNA Ladder, SinaClon, Iran).

In pET-MAG1, the mag1 gene fragment was inserted with N-terminal fusion of 20 amino acids including a cluster of 6 histidine (6xhis) residues, and C-terminal fusion of 22 vector-encoded amino acids.

**Expression of MAG1.** Induction of *E. coli* Rosetta (DE3) bacteria harboring pET-MAG1 plasmid resulted in expression of a protein band of 42 kDa, which was absent in uninduced bacteria (Figure 2).

Expression of MAG1 was tested under various IPTG concentrations; while considerable expression of the protein was observed at the as low concentration as 0.1 mM, the expression reached its highest at 1 mM of IPTG (Figure 2). Moreover, the expression reached to the maximum level by 2 h after induction, and slightly decreased thereafter (Figure 3). However, the expression remained constant until 16 h after induction suggesting stability of MAG1 against protease degradation. Induced bacteria were lysed and crude bacterial lysate was fractionated into soluble and insoluble fractions. SDS-PAGE analysis showed about half of MAG1 was soluble and the remaining was insoluble (data not shown). Expression of MAG1 in BL21 (DE3) pLysS bacteria produced lower amounts of the protein, compared to *E. coli* Rosetta (DE3) bacteria (data not shown).

**Purification of MAG1.** Soluble MAG1 protein was purified by immobilized metal-ion chromatography (IMAC) as explained in material & methods section. Figure 4 shows complete binding of MAG1 to Ni2+-NTA resin as no protein was seen in the flow through, while elution of MAG1 from the resin began at the imidazole concentration of 50 mM with purity above 95%. High amounts of the highly purified protein were also eluted at 500 mM concentration of Imidazole. About 15 mg of purified MAG1 was obtained from 500 ml culture of induced bacteria. The purification experiment was performed at different pH values with almost the same purity and yield of MAG1.

Fig. 2. Expression of *T. gondii* MAG1 protein at different concentration of IPTG. Lane 1-4, bacteria induced by IPTG concentration of 0.1, 0.4, 1.0 and 2.0 mM; lane 5, bacteria before induction; lane 6, protein size marker.

Fig. 3. Expression of *T. gondii* MAG1 protein at various times after induction. Lane 1, bacteria before induction; lane 2, protein size marker; lane 3-6, bacteria induced for 2, 3, 4 and 16 h, respectively.

Fig. 4. Purification of *T. gondii* MAG1 protein. Lane 1, protein size marker; lane 2, flow through (unbound proteins); lane 3-5, washing buffer 1, 2 and 3 respectively; lane 6-8, eluted MAG1 protein.
Immunoreactivity of MAG1. Antigenicity of MAG1 was assessed in Western-blotting. One µg of the purified protein was transferred onto a PVDF membrane and probed with pooled human sera obtained from pregnant women with acute or chronic *T. gondii* infection. Acute sera strongly reacted with MAG1 but chronic sera failed to react, indicating that IgG antibodies against MAG1 are mostly produced during acute infection and disappear in chronic phase (Figure 5). The acute and chronic pooled sera used above were capable of detecting many proteins in the blots of RH tachyzoites (data not shown).

![Fig. 5. Immunoreactivity of *T. gondii* MAG1 protein with human sera. Purified MAG1 probed with chronic (lane 1) and acute (lane 2) pooled sera. Lane 3, protein size marker.](image)

**DISCUSSION**

We report expression and single-step purification of *T. gondii* matrix antigen 1 (MAG1) (amino acids 50 to 207) in *E. coli* as a soluble and antigenic protein. Western blot analysis showed preferential immune-reactivity of MAG1 with sera from acute infection.

Recombinant MAG1 was seen as a protein band of about 42 kDa in SDS-PAGE; while the calculated size of the protein was about 21.5 kDa (fusion protein contained 201 amino acids). The difference between calculated and observed size of MAG1 was also reported by others [13, 21]. We didn’t perform codon optimization for the MAG1 gene sequence; however, high-level expression was achieved in Rosetta (DE3) host, which has been genetically engineered to co-express tRNAs for rare codons. In fact higher expression of MAG1 in these bacteria, compared to BL21 (DE3) pLysS, was probably due to the presence of some rare codons in the MAG1 sequence. About half of the expressed protein was in insoluble form, which requires solubilization followed by refolding to obtain soluble antigenic protein. The use of a solubility-enhancing fusion protein could increase the solubility, but the fusion might interfere with serological tests. Optimization of expression conditions such as culture temperature, inducer concentration and expression at lower culture density might enhance protein solubility. Purification of MAG1 by IMAC was very efficient and resulted in highly pure protein suitable for ELISA experiments. The observation that acute-phase sera presented much higher reactivity with MAG1 in immunoblotting indicates its usefulness in distinguishing acute from chronic infections, as previously reported by other studies [12, 13].

The most challenging situation encountered in serodiagnosis of *T. gondii* infection in pregnant women is concurrent presence of IgG and IgM antibodies in the first antenatal test. Avidity tests were developed to help discriminate acute from chronic infections in these situations; however, they showed some limitations, *i.e.*, large amounts of low avidity results in chronic infections [3, 6].

Many studies showed that IgG antibodies to some *T. gondii* antigens were primarily produced in acute/reactivated infection, and greatly decreased in chronic infection. They suggested application of these so called “markers of acute infection” would help distinguish between recently-acquired/reactivated and chronic infections [24, 25]. Holec and colleagues applied MAG1 in IgG ELISA and reported sensitivity of 93.7% and 7.5% for detection of acute and chronic infections, respectively [13]. Another study applied two MAG1-derived peptides in ELISA, and showed their usefulness for distinguishing between acute and chronic infections [12]. Conversely, a study showed preferential immunoreactivity of MAG1 with chronic sera [26]. The difference is probably due to MAG1 sequence used in different studies.

We previously reported GRA2 as an acute infection marker; the mean OD value of acute-phase sera, obtained from women seroconverted during pregnancy, in GRA2 IgG ELISA was about ten times more than that of chronic sera [8]. Later, preferential immunoreactivity of GRA2 and GRA2-derived peptide with sera from acute/reactivated infection was reported by other researchers [27, 28]. Interestingly, a study reported that the level of anti-GRA2 and anti-ROP1 IgG antibodies during reactivated toxoplasmosis was much higher than latent infection in CBA/J mice [29]. These studies imply that acute-phase antigens might be useful in detection of reactivated toxoplasmosis where the level of *T. gondii* IgM antibodies is unchanged.

Taken together, it seems that more studies need be done using acute-phase antigen(s) such as MAG1, GRA2, ROP1 and GRA8 to explore their potentials in differentiating acute/reactivated from chronic infections. Such studies should, preferentially, use validated serum samples, *i.e.*, from women seroconverted during pregnancy and from immunocompromised patients presenting reactivated toxoplasmosis.

The availability of highly purified and antigenic MAG1 produced in the present study would facilitate further analysis of diagnostic performance of MAG1. Moreover, regarding the vaccine potential of MAG1 reported in previous studies, it is possible to further study protective immune response of the protein in animal models.

**ACKNOWLEDGEMENT**

This work was supported by the research grants No. 91046905 from Iranian National Science Foundation.
CONFLICT OF INTEREST

The authors do not have any conflict of interests.

REFERENCES