Original Article

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

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Introduction: Toxoplasmosis is a parasitic infection caused by the protozoan *Toxoplasma gondii*; it leads to serious medical problems in congenitally-infected and immunocompromised individuals, while it is quite harmless in immunocompetent individuals. *Toxoplasma* 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* 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.

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 mothers [1]. Laboratory diagnosis of the infection mainly relies on detection of specific anti-*T. gondii* antibodies, most commonly IgG and IgM antibodies, by a variety of serologic tests [2, 3]. The current methods for discrimination of acute from chronic infections, *i.e.*, IgM and IgG avidity serologic tests, have their own limitations, *e.g.*, low specificities and high amounts of false-positive or indeterminate results [4-7]. Several studies showed antibody response to some antigens of *T. gondii* including GRA2 [8], GRA6 [9], GRA7 [10], GRA8 [11], and response to MAG1 [12, 13] was stronger or much stronger during acute infection. Application of these antigens in serological tests might be useful, along with other tests, in distinguishing between acute and chronic infections.

Once acute infection is detected, chemotherapy can prevent, to some degree, transmission of the parasite to the fetus and development of clinical signs [14, 15]. On the other hand, accurate diagnosis of infection, prevents chemotherapy and unnecessary concern in pregnant women misdiagnosed with acute *T. gondii* infection.

Matrix Antigen 1(MAG1) was originally described as being expressed specifically during bradyzoite development [16], however, it was subsequently showed by molecular and serological methods that MAG1 is expressed during both tachyzoite and bradyzoite stages [17-20]. Several studies showed MAG1 is capable of inducing specific immune response and protection against *T. gondii* infection [21-23]. Moreover, many researchers applied MAG1 in serological tests for detection of *T. gondii* infection or discriminating acute from chronic infection [12, 13, 20]. Interestingly, the N-terminal of MAG1 or MAG1-derived peptides showed to be efficient proteins to trigger IgG antibodies produced during acute infections, and the ELISA based on these protein/peptides turned to be useful for detection of acute infection [12, 13].

In this study, recombinant MAG1 antigen was produced in bacteria, highly purified and its immunoreactivity with sera from patients with acute and chronic *T. gondii* infections was investigated.

MATERIALS AND METHODS

Pooled sera. The pooled serum samples used in Western blot analysis were prepared from pregnant women with assumed acute or chronic *T. gondii* infection.

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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 Bl21(DE3) pLysS (Promega, Madison, WI) were used for cloning and expression of recombinant antigen, respectively.

Construction of the recombinant expression plasmid. DNA sequence of *mag1* 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 *mag1* gene fragment, encoding amino acids 50-207 using specific primers of 5'-GAGCATATGGATGAAGTAGGCACGGG-3' (forward) and 5'-CGCTCGAGACCCTGAACCCTT-AGAATATAC-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, Vilnious, 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-MAG1and 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 isopropyl-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 *mag1* 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*I/*Xho*I 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).

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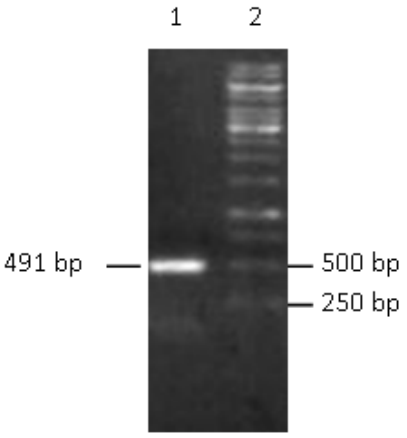

Fig. 1. PCR amplification of *T. gondii mag1* gene fragment by specific primers. Lane 1, *mag1* PCR Product; lane 2, DNA size marker (1 Kb DNA Ladder, (SinaClon, Iran).

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.

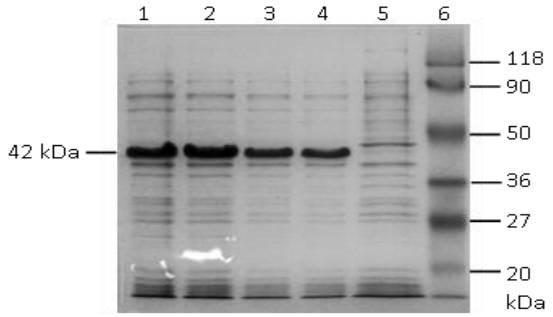

Fig. 2. Expression of *T. gondii* MAG1 protein at different concentration of IPTG. Lane 1-4, bacteria induced by IPTG concentration of 0.1, 0.4, 1.0 and 2.0 mM; lane 5, bacteria before induction; lane 6, protein size marker.

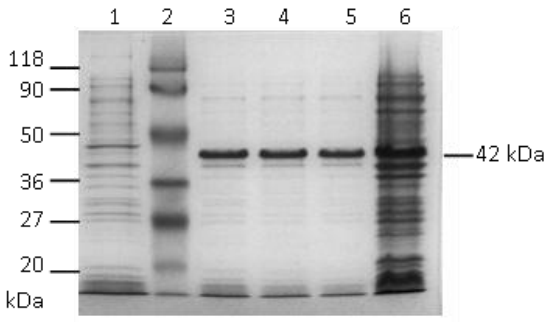

Fig. 3. Expression of *T. gondii* MAG1 protein at various times after induction. Lane 1, bacteria before induction; lane 2, protein size marker; lane 3-6, bacteria induced for 2, 3, 4 and 16 h, respectively.

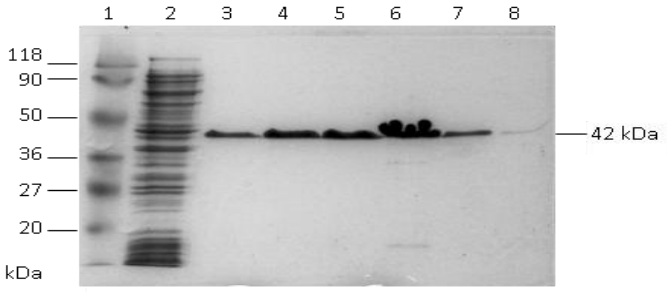

Fig. 4. Purification of *T. gondii* MAG1 protein. Lane 1, protein size marker; lane 2, flow through (unbound proteins); lane 3-5, washing buffer 1, 2 and 3 respectively; lane 6-8, eluted MAG1 protein.

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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).

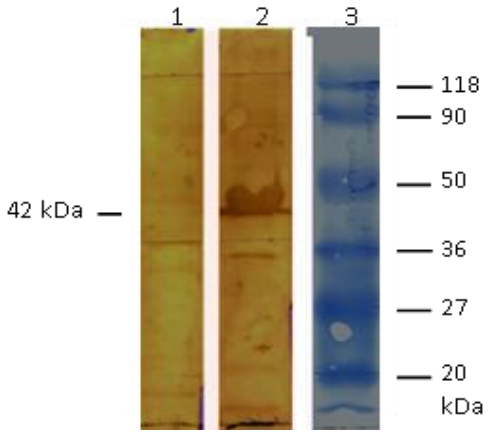

Fig. 5. Immunoreactivity of *T. gondii* MAG1 protein with human sera. Purified MAG1 probed with chronic (lane 1) and acute (lane 2) pooled sera. Lane 3, protein size marker.

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.

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CONFLICT OF INTEREST

The authors do not have any conflict of interests.

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