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Cloning rhoptry protein 1 (ROP1) gene of *Toxoplasma gondii* (RH) in expression vector

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ABSTRACT

Toxoplasma gondii contain various immunogenic antigens. The most important *Toxoplasma* antigens are somatic and excreted/secreted antigens. Rhoptry proteins are known as excreted/secreted antigens. These antigens have been proposed as a vaccine candidate against toxoplasmosis. The main objective of the present work was cloning rhoptry protein1 (ROP1) Gene of *Toxoplasma gondii* (RH) in a cloning vector for gene analysis and further production of rhoptry proteins. Tachyzoites of the RH strain of *T. gondii* were harvested from the peritoneal fluid of mice that has been experimentally infected with the parasites. Genomic DNA was extracted by phenol- chloroform method. The ROP1 fragment amplified with specific primers. The purified PCR products were ligated between the EcoR1 and BamH1 sites of the pTZ57R/T cloning vector and transformed into *Escherichia coli* TG1 strain and screened by IPTG and X-Gal. The plasmid was purified and visualized under UV transilluminator. The amplified fragment was cloned in pTZ57R vector successfully. The correct orientation of the ROP1 fragment was identified by restriction enzyme analysis and sequencing of constructed plasmid. A fragment about 760bp was separated from PTZ57R following digestion and demonstrated on agarose gel electrophoresis.The sequence of this amplified gene showed homology up to 96% with target gene in GenBank database (Accession no. M71274). Recombinant plasmid of ROP1 gene was constructed. It is ready for future study.

Keywords: *Toxoplasma gondii*, Cloning, Rhoptry Protein1 (ROP1) Gene

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan belonging to Apicomplexa. This parasite is prevalent throughout the world and it is estimated, about one third of people are infected with this zoonotic parasite. *Toxoplasma gondii* almost can infect any kind of vertebrate cells. Infection during pregnancy may lead to abortion and congenital transmission. Latent infection in immune-compromised individuals may cause encephalitis which is often lethal (Leyra 2001). Natural infection with *Toxoplasma gondii* usually leads to a type of cellular immunity, which both CD4+ and CD8+ T-cells are affects in it (Leyra 2001, Martin 2004). Like other unicellular organisms *Toxoplasma gondii* is composed of various antigens. The most important *Toxoplasma* antigens are somatic and excreted/secreted antigens. Some surface antigens like SAG1 is proposed as candidate for vaccine production against toxoplasmosis (Bhopale 2003). Numerous studies have been performed on the

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structure, function and immunity of SAG1. Moreover various DNA vaccines against *Toxoplasma* compose of single or cocktail antigens have been investigated (Hafid 2005). Nowadays interest to the somatic antigen has been reduced and the studies focused on the antigens known as excreted/secreted antigens or exoantigens. *Toxoplasma gondii* secretory proteins are effective antigens that can activate strong immune responses. One of these antigens is rhoptry protein1 (ROP1) that secreted from rhoptry organelle. So far, nine rhoptry proteins have been described. The time of the release of these molecules, as well as their targeting to the host cell surface or parasitophorous vacuole, suggests their role in invasion of parasite (Reichman 2002, Bradley 2002). Rhoptry proteins may facilitate formation of the vacuole and mediate its clustering with host cell organelles. ROP1 has been associated with a molecularly activity that can enhance invasion *in vitro*. This property makes ROP1 as vaccine candidate (Reichman 2002, Bradley 2002). The main objective of the present work is cloning Rhoptry Protein1 (ROP1) gene of *Toxoplasma gondii* (RH) in appropriate vector for production of rhoptry proteins.

MATERIALS AND METHODS

Parasite. The RH strain of *Toxoplasma gondii*, was provided by Department of Parasitology, Faculty of Health, Tehran Medical Sciences University. Tachyzoites of parasite were harvested from peritoneal fluid of Balb/c mice which 3-4 days earlier had been experimentally infected.

Bacterial strain. *E.coli* strain TG1 was used as host cell for all plasmid manipulations. The bacteria were propagated in Luria Bertani broth or on Luria Bertani agar supplemented with ampicillin (100 m*g/ml*) where appropriate.

Genomic DNA extraction. Standard methods were used for DNA manipulation (Sambrook 2001). About 10-30×10^6 of tachyzoite were harvested from peritoneal fluid, washed with phosphate buffer saline (PBS) and lysed in 1ml lysis buffer contain 50mM Tris-HCl, pH 8, 0.1M NaCl, 10mM EDTA, 2% sodium dodecyl sulfate, and 0.2 µ*g/ml* proteinase k and incubated at 56 ºC for 2 hours. DNA was extracted with phenol-chloroform method. The DNA was removed and then mixed with 1/10 volume of 3M sodium acetate and two volumes of absolute ethanol and incubated at -70 ºC for 30 min. Then the mixture was centrifuged at 13000 rpm for 30 min. The DNA pellet was washed with 70% ethanol and air-dried after washing then dissolved in sterile distilled water and stored at -20 ºC. The DNA concentration assessed by both UV absorbance at 260 nm and electrophoresis on a 0.8% agarose gel.

PCR amplification. The ROP1 gene of *Toxoplasma* was amplified by PCR. The reaction was performed in 25 µl volume contain: 2.5 µl of 10X PCR buffer, 0.5 µl of dNTP (10 mM), 0.75 µl of $MgCl_2$ (50 mM), 0.25 µl of Taq DNA polymerase (5 u/µl), 0.25 µl of Primer forward (10 pmol / µl), 0.25 µl of primer reverse (10 pmol / µl), 8 µl of Template DNA and 14.75 µl of distilled water. Primers were designed according to the DNA sequence of the ROP1 gene which was obtained from the GenBank database (Accession number M71274).

Forward: 5´- CA **GAA TTC** ATG GAC TTC GCC TCC GAC GAC - 3´

Reverse: 5´- CG **GGA TCC** TTA CAG ACT GGC ACC ACT TGT - 3´

The upstream primer for the ROP1 fragment contains an EcoR1 site and the downstream primer contains a BamH1 site, so they are as recognition sites. PCR procedure include: Initial denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 95 ºC for 1 min, annealing at 56º C for 30 s, extension 72 ºC for 1 min then final extension at 72 ºC for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel and the size of them were /compared with 1kb DNA ladder (size

marker). The PCR products were isolated and purified from agarose gel using DNA Extraction Kit (Roche®).

Ligation of ROP1 gene. The purified PCR products were ligated into *pTZ57R/T* plasmid vector (InsT/A clone™ PCR product cloning Kit, Fermentas ®) according to the manufacturing protocol.

Transformation and screening. Competent cell was prepared from *E. coli* TG1 strain by calcium chloride method (Sambrook 2001). The ligation product was transformed in competent cell according to the protocol (Sambrook 2001) and recovered in Luria-Bertani (LB) broth medium free antibiotic by incubated at 37 ºC for 1-2 h with shaking. These cells were plated onto LB agar plates contain: ampicillin 100 mg/ml, IPTG 200 mg /ml and X-Gal 20 mg/ml. These plates were incubated at 37 ºC for 16-18h to screening blue and white colonies. The selected blue and white colony passage in LB broth or LB agar and incubated at 37 ºC for 16-18 h. The plasmid was extracted by Accuprep Plasmid Extraction Kit (Roche®) according to the protocol.

Electrophoresis. Five µl of plasmid extracted from blue (pTZ57R/T) and white (pT-ROP1) colonies of bacteria electrophoresis on a 0.8% agarose gel and their bands were compared.

PCR amplification of ROP1 fragment with plasmid. The pT-ROP1 was used as template to amplify the ROP1 fragment by PCR. The reaction was performed in 25 µl volume according to the protocol. The PCR product was analyzed by electrophoresis on a 1% agarose gel and size of product was estimated with 1kb DNA ladder.

Digestion of plasmid with enzyme: The pT-ROP1 and the pTZ57R/T (negative control) were digested by *EcoR1* and *BamH1* enzymes. The reaction was performed separately for each enzyme in 20 µl volume contains: 15 µl (1-3 µg) of plasmid (pT-ROP1 or pTZ57R/T), 1 µl of one enzyme, 2 µl of 10 x buffers, 2 µl of DW. The mixture was incubated overnight at 37 ºC. The product was analyzed by electrophoresis on a 1% agarose gel and compared with plasmids without digestion. The product was purified using a DNA Extraction Kit from agarose gel (Roche®). Then the reaction was performed with second enzyme and the product analyzed by electrophoresis on a 1% agarose gel.

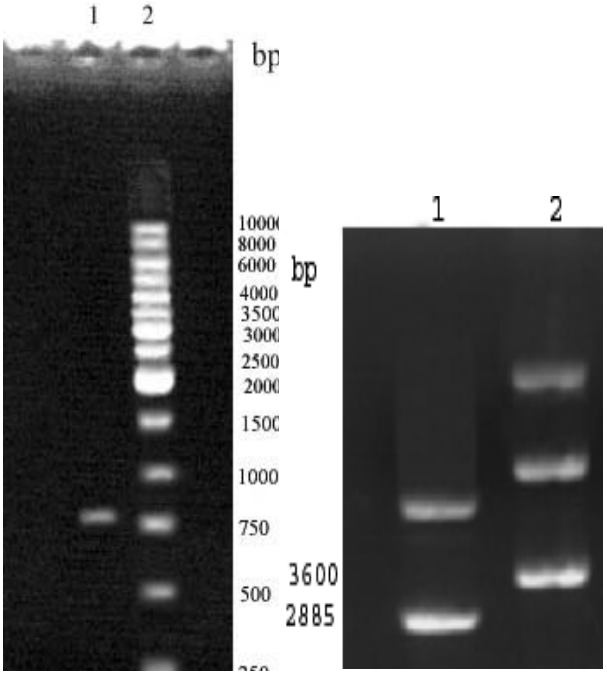
Sequencing. The extracted plasmids from the white colonies (pT-ROP1) were sequenced by Genfanavaran Company, Iran.

Subcloning of pT-ROP1 in expression vector. The pcDNA3 used as expression plasmid and ROP1 fragment subcloned into this plasmid. The pcDNA3 and the pT-ROP1 were digested by EcoR1 and HindIII. Then digested plasmids were isolated and purified from agarose gel using DNA Extraction Kit (Roche®). The digested pT-ROP1 ligated into the digested pcDNA3 according to the protocol (Sambrook 2001). The ligation product was transformed in competent cell according to the protocol (Sambrook 2001) and recovered in Luria-Bertani (LB) broth medium free antibiotic by incubated at 37 ºC for 1-2 h with shaking. These cells were plated onto LB agar plates contain ampicillin 100 mg/ml. These plates were incubated at 37 ºC for 16-18h to screening colonies. The selected colony passage in LB broth or LB agar and incubated at 37 ºC for 16-18 h. The plasmid was extracted by Accuprep Plasmid Extraction Kit (Roche) according to the protocol. For identifying the pcROP1 recombinant plasmid used electrophoresis, PCR amplification, restriction digestion.

RESULTS

The ROP1 gene was amplified by PCR using extracted genomic DNA from RH strain of *Toxoplasma gondii* as template. The size of amplified fragment is similar to predict size of fragment, 760 bp (Figure 1A). The PCR product has ligated successfully into pTZ57R/T plasmid and

then has transformed in TG1 strain of *E. coli*. The extracted plasmid from white colonies place above of blue colonies then the ROP1 gene has cloned into pTZ57R/T (Figure 1B).



A **B**

Figure 1. (A). Electrophoresis of PCR product for ROP1 fragment of *Toxoplasma gondii* amplification. From left to right *Lane1:* ROP1 gene (760 bp), *Lane2:* 1kb ladder. **(B)**. Comparison of pTZ57R/T and pT-ROP1 plasmids by electrophoresis. *Lane 1:* Plasmid extracted from blue (pTZ57R/T) colony and *Lane2:* Plasmid extracted from white (pT-ROP1) colony.

The extracted plasmids were digested with EcoR1 and BamH1 restriction enzymes. After digestion, the extracted plasmid from white colonies shows two bands where ROP1 fragment placed on 760bp (Figure 2), but the extracted plasmid from blue colonies shows one band. Thus the ROP1 gene has cloned into pTZ57R/T (Figure3). The cloned plasmid was sequence and compared with ROP1 gene of Toxoplasma *gondii* in GenBank. The result shows homology of 97% with *Toxoplasma gondii* rhoptry protein (ROP1) mRNA, complete cds, accession number M71274 (Figure 4), 96% with *Toxoplasma gondii ROP1* gene, partial sequence accession number AF350261 and 97% with *Toxoplasma gondii* rhoptry 1 (ROP1) mRNA, complete cds accession number AY661790. The digested pT-ROP1 has ligated successfully into pcDNA3 plas mid and then has transformed in TG1 strain of *E. coli*.

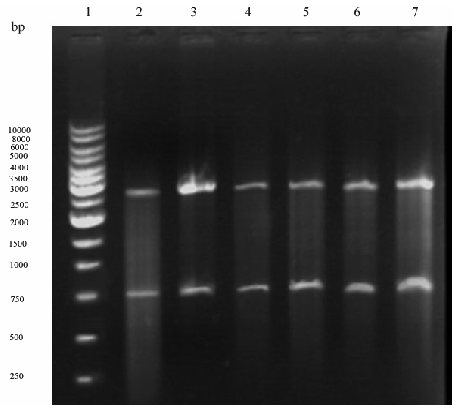


Figure 2. Digestion of extracted pT-ROP1 after ransformation: *Lane1:* ladder 1kb, *Lanes 2-7:* pT-ROP1 after enzyme digestion with 2 enzyme (ROP1 760 bp, pTZ57R/T 2885 bp).

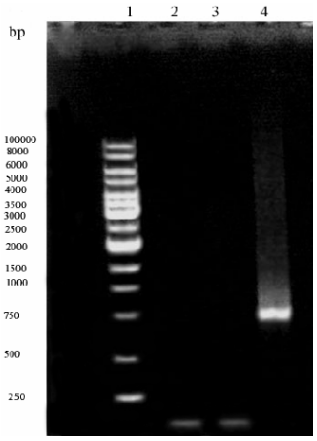


Figure 3.
PCR amplification of pT-ROP1: *Lane1:* ladder, *Lane 2, 3:* pTZ57R/T, *Lane 4:* pT-ROP1.

The extracted plasmid from this colonies placed above of pcDNA3 then the ROP1 gene has cloned into pcDNA3 (Figure 5). The extracted plasmids were digested with EcoR1 and HindIII restriction enzymes. After digestion, the extracted plasmid from inserted colonies shows two bands where ROP1 fragment placed on almost 760bp, but the

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gb|M71274.1|TOXROP1A Toxoplasma gondii rhoptry protein (ROP1) mRNA, complete cds
Length=2122

Query  299   TGCCCCCACCAAATTCACAGGAGCTGCCCCCACCAAATGCACAGGAGCTGCTCCCACCAA  358
              ||| |||||||||| ||||||||||||||||||||||| |||||||||| ||||||||
Sbjct  655   TGCGCCCACCAAATGCACAGGAGCTGCCCCCACCAAATGTACAGGAGCTGCCCCCACCAA  714

Query 359   CTGAACAGGATCTGCCCCCACCAACTGAACAGGAGCTGCCCCCACCAGTGGGCGAAGGTC  418
              |||||||||| || |||||| ||||||||||||||||||||||||||||||||||||||
Sbjct 715   CTGAACAGGAGCTACCCCCATCAACTGAACAGGAGCTGCCCCCACCAGTGGGCGAAGGTC  774

Query 419   AAGGTCTGCAAGTCCCTGGGGAACATGGACCACAGGGGCCCCCAGATGATGATCAGCAGC  478
              || |||||||||||||||||||||||| ||||||||||||| | ||||||||||||||
Sbjct 775   AACGTCTGCAAGTCCCTGGGGAACATGGGCCACAGGGGCCCCCATACGATGATCAGCAGC  834

Query 479   TGCTTTTAGAGCCTACGGAAGAGCAACAGGAGGGCCCTCAGGAGCCGCTGCCACCGCCGC  538
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 835   TGCTTTTAGAGCCTACGGAAGAGCAACAGGAGGGCCCTCAGGAGCCGCTGCCACCGCCGC  894

Query 539   CGCCCCCGACTCAGGGCGAACAACCCGAAGGACAGCAGCCGCAGGGACCAGTTCGTCGAA  598
              |||||||||||| ||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct 895   CGCCCCCGACTCGGGGCGAACAACCCGAAGGACAGCAGCCGCAGGGACCAGTTCGTCAAA  954

Query 599   ATTTTTTTCGTCGGGCGTTGGGGGCCGCAAGAAGCCGATTCGGAGGTGCACGACGCCATG  658
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 955   ATTTTTTTCGTCGGGCGTTGGGGGCCGCAAGAAGCCGATTCGGAGGTGCACGACGCCATG  1014

Query 659   TCAGTGGGGTGTTCCGAAGAGTCAGAGGTGGTTTGAACCGTATAGTAGGTGGAGTGAGAA  718
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct 1015  TCAGTGGGGTGTTCCGAAGAGTCAGAGGTGGTTTGAACCGTATAGTAGGTGGAGTGAGGA  1074

Query 719   GTGGTTTCAGGCGTGCAAGAGAAGGTGTCGTTGGGGGAGTCCGTCGTTTAACAAGTGGTG  778
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1075  GTGGTTTCAGGCGTGCAAGAGAAGGTGTCGTTGGGGGAGTCCGTCGTTTAACAAGTGGTG  1134

Query 779   CCAGTCTG  786
              ||||||||
Sbjct 1135  CCAGTCTG  1142
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Figure 4. Comparison of ROP1 gene amplified in the present study (A) with ROP1 gene under accession number M71274.

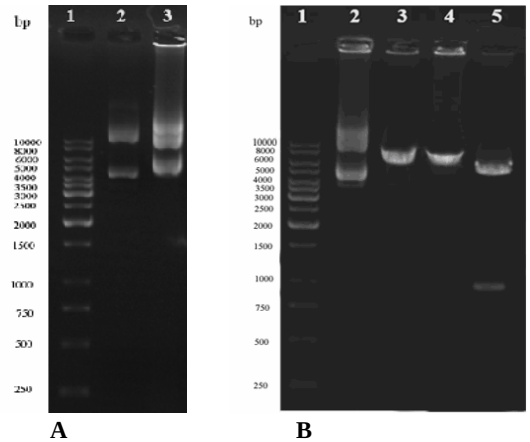

Figure 5. (A): Comparison of pcDNA3 and pc-ROP1 plasmids by electrophoresis.
Lane 1: ladder (1 kb), *Lane 2:* pcDNA3, *Lane 3:* pc-ROP1

Figure 6. (B): Digestion of extracted pc-ROP1 after transformation: *Lane1:* ladder 1kb, *Lane2:* pcROP1, *Lane 3-4:* pc-ROP1 after digestion with 1 enzyme , *Lane5:* pcROP1 after digestion with, enzyme (ROP1˜ 760 bp).

extracted plasmid from empty colonies shows one band. Thus the ROP1 gene has cloned into pcDNA3 (Figure 6).

DISCUSSION

Numerous studies have been carried out to investigate on new effective vaccine against toxoplasmosis. In these vaccines either killed parasites or crude extracts of *Toxoplasma* have been used. Some of these antigens are capable to increase only survival rate of vaccinated animals. A veterinary vaccine against toxoplasmosis has been reported be able to reduced the incidence of abortion in sheep and goats (Bhopale 2003). In the present work, the ROP1 gene was cloned successfully into pTZ57R vector and then subcloned in eukaryotic expression vector, pcDNA3. This plasmid is ready to express in eukaryotic cell and carry out the research of DNA vaccination against toxoplasmosis.

ROP1 is an important molecule of Toxoplasma antigens its gene structure is unusual and the major part of the gene from genomic DNA, cDNA and mRNA possesses the same base sequence. Therefore, ROP1 gene fragments could be derived directly from genomic DNA (Chen 2001). ROP1 is a single copy gene encoding a 2.1 kb transcript (Ossorio 1992). The mature protein has mass ~33.6 kDa but migrates as if ~60 kDa on SDS-PAGE (Bradley 2002) probably because of the octa peptide repeats which are rich in proline-glutamic acid residues (Soldati 1998). This protein is expressed in tachyzoite, bradyzoite and sporozoite (Garcia 2004). The molecular weight of rhoptry protein1 from full length gene is ~ 46 kDa but because of amino acids composition it has strong charge asymmetry Bradley 2002) and migrates unusually slowly on SDS-Page (Garcia 2004). In the natural *Toxoplasma gondii* infection, Th1 immune response is predominant. CD8+ T cells are major effectors cells responsible for protection against *Toxoplasma gondii* and CD4+ T cells playing a synergistic role (Reichman 2002). Therefore, a vaccination protocol that directs immune response to the Th1 type and CD8+ is desirable. DNA vaccine is a powerful method for induction of specific humoral and cellular immune responses. This kind of vaccines are known to induce CD8+ T-cell responses and in recent years a substantial effort to determine their effectiveness against toxoplasmosis. Some of *Toxoplasma* antigens that used for DNA vaccine are: SAG1, SAG2, SAG3, P30, GRA1, GRA4, GRA7, ROP2, HSP30, HSP70, MIC1, MIC2, MIC3, MIC4 (Hafid 2005). Recently, ROP1 (Haifag 2003), ROP2 (Bhopale 2003, Jenkins 2001), ROP4 (Jenkins 2001) antigens and HSP30, HSP70 (Mohamed 2003) and HSP90 (Rojas 2000) have also been proposed as vaccine candidates against toxoplasmosis. Immunization of mice with ROP1 DNA vaccine induces proliferation of T-cell and increase of IgG antibody (Chen 2001, Chen 2003, Gue 1999 & Gue 2001).

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