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Cloning of 1183 bp Fragment from Rhoptry Protein I (ROPI) Gene of Toxoplasma gondii (RH) in Expression Prokaryote Plasmid PET32a

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Abstract

***Background*:** *Toxoplasma gondii* is an obligatory intracellular protozoan. Considering to high prevalence of this disease the best way to reduce the raised loses is prevention of human and animal infection, rapid diagnosis, differentiation between acute and chronic disease. Rhoptry protein 1 of *Toxoplasma gondii* is an excretory-secretory antigen that exists in the most stages of life cycle. According to specifications of excretory-secretory antigen that seems this antigen is a suitable candidate to produce recombinant vaccine and diagnostic kit. The main object of the present work was cloning rhoptry protein 1 (ROP1) gene of *Toxoplasma gondii* (RH) in a cloning vector for further production of rhoptry proteins.

***Materials and Methods*:** Genomic DNA was extracted by phenol-chloroform method. The ROP1 fragment was amplified by PCR. This product was approved by sequencing and was cloned between the EcoR1 and Sal1 sites of the pTZ57R/T vector. Then transformed into *Escherichia coli* DH5α strain and screened by IPTG and X-Gal. After isolating of this gene from pTZ57R/T, it was subcloned into pET32a plasmid.

***Results*:** The plasmid was purified and approved by electrophoresis, enzyme restriction and PCR. After isolating of this gene from pTZ57R/T, it was subcloned into pET32a plasmid. After enzyme restriction and electrophoresis a fragment about 1183bp was separated from pET32a.

***Conclusion*:** Recombinant plasmid of ROP1 gene was constructed and ready for future study. That seems the antigen is a suitable candidate to produce recombinant vaccine and diagnostic kit.

Introduction

Toxoplasma gondii (*T. gondii*) is an obligatory intracellular protozoan that causes toxoplasmosis disease. The life cycle of parasite is completed by cat and warm-blooded animals [1]. In acute form of disease the parasite reproduced inside the host blood and other organs' cells and in chronic form of disease the parasite transforms to cysts [1]. As toxoplasmosis could result in abortion, congenital infection and fatal encephalitis [1], it is considerably important from health and medicine points of view. Considering the high rates of toxoplasmosis infection, the most practical way of reducing disease burden could be prevention of infection of warm-blooded beings, rapid and on-time diagnosis of cases and differentiation between acute and chronic cases of disease [2].

Several findings suggested that it is possible inhibiting placental transfer of parasite by suitable vaccination before pregnancy and also could be produced effective vaccine against human toxoplasmosis [3, 4]. In spite of current strategies in vaccine production that are based on using living or non-living agents, but it seems necessary using of novel methods such as molecular methods to improve vaccine production and inhibiting of activation risk of organisms [4-6].

The novel strategies in production of vaccine are based on production of recombinant antigens of micro organisms [7] and diagnosis of toxoplasmosis are based on serological tests and the differentiation between chronic and acute cases of infections is performed by comparison of levels of G, M and A immunoglobulin. Natural antigens of toxoplasma are used to identify parasite antibodies in commercial kits, and considering that the parasite is cultivated in living cell, the possible risk for contamination of parasite antigens with host-cell antigens may lead to diagnostic deviations and also the quality of this kind of antigens are not stable [2]. Considering that the production of these recombinant antigens are possible and such antigens do not have deficiencies listed for natural antigens, so nowadays the production of this kind of antigens are considered [8]. Choosing of the type of antigen to produce recombinant vaccine or diagnostic kits is considerably important because it should be immunogene, trigger of cellular immunity response, proliferator of T lymphocytes and also presented in the most of the parasite life-cycle. Rhoptry protein 1 (ROP1) is one of the excretory-secretary antigens of toxoplasma with the above mentioned specification and it seems that an appropriate

candidate in production of recombinant vaccines and diagnostic kits. In the former studies of Iranian researchers and other countries, limited fragments of ROP1 -single or fused with other genes- were used for cloning [9-13]. In the current study we decided to clone a larger fragment of ROP1 gene, so that it could be used to reach completely expressed ROP1 and constructing of the vaccine or diagnostic kit.

Materials and Methods

In this experimental study, RH strain of *T. gondii* was obtained from parasitology department of Public Health Faculty, Tehran University of Medical Sciences. Then, 2×10^4 fresh and active tachyzoites were injected to mice by peritoan. After 4-5 days, symptoms of infection including snoozing, narcosis and hair changes manifested. Then, mice were killed and after opening its abdomen, sterile PBS buffer containing streptomycin and penicillin was injected to peritoan. In order to isolate parasites from peritoneal macrophages, the peritoneal fluid was passed through a syringe repeatedly. In the next step, parasite-containing-fluid was centrifuged (with 5 ml sterile PBS at 3000-5000 rpm for 5 minutes) and supernatant fluid was discarded. This process was repeated for three times and parasites ready for DNA extraction.

Bacterial strains: DH 5α strain of *E. coli* (obtained from Microbiology Department, Faculty of Medicine, Arak University) was used as host cells to harvest plasmids. Bacterial strain was cultivated in LB media containing 100 mg/ml ampicillin.

Genomic DNA extraction: Genomic DNA was extracted using phenol-chloroform method. To confirm of this product electrophoresis on a 0.8% agarose gel, was performed. The concentration of product was determined by measurement of OD absoption at 260 nm wavelength.

Polymerase Chain Reaction (PCR): For amplification of ROP1 gene, total sequence of gene was obtained from NCBI GenBank ref. no M71274.1. Then two appropriate primers for this gene were designed as below (Fig. 1).

Forward and reverse primers were 25 and 27 length containing a restriction cleavage site for EcoR1 (on nucleotide 198) and SaI1 (on nucleotide 1381 of gene) respectively. Accordingly, an 1183-fragment of gene was constructed. Each PCR reactions comprised of 10 pmol of R and F primers, 10 mM nucleotides mix, 5 units of Taq DNA polymerase, 2.5 µl enzyme buffer, 50 mM Mgcl2 and 8 µl of template DNA (total reaction volume 25 µl). All reactions were subjected to PCR by an initial denaturation (94ºC for 5 min) followed by 35 cycles each consisting denaturation (94ºC for 1 min), primer annealing (59ºC for 30 sec), amplification (72ºC for 1 min) and a final extention (72ºC for 10 min). To verify respective amplicon amplification, PCR products were subjected to electrophoresis on 1% agarose-gel and a final sequencing by Danesh Arian Inc.

Ligation of ROP1: For ligation of ROP1 fragment to plasmid, PCR products were initially agarose-gel electrophorized. Then, according to manufacturer's instructions, they were purified by utilizing a gel-extraction kit (Fermentas Inc.). Finally, the ligation reaction was performed by utilizing a cloning kit according to manufacturer's instructions (using pTZ57R/T plasmid, Fermentas Inc.).

Transformation and screening of recombinant clones: For bacterial transformation of resulting ligation products, DH 5α strain of *E. coli* was initially cultivated in a liquid LB media and then turned into competent bacterial cells ready to accept ligation products by using $Cacl_2$ [14]. According to protocol, the resulting ligation products were transformed into competent cells. After adding 500 µl of antibiotic-free liquid to LB media, the culture was incubated in a shaker for 1-2 hours at 37°C. Then, followed by overnight incubation (14-16 hours) at 37°C, the cells were placed on LB-ampicillin XGal/IPTG agar plates (LB agar plates consisted of 100 mg/ml ampicillin, 20 mg/ml IPTG and 20 mg/ml X-Gal). A quick check method was performed on white-blue colonies. Afterwards the suspicious transformant colonies were cultivated in high volume scale and were subjected to plasmid extraction (Sina clone Inc.).

For verification of correct ligation of DNA fragments and transformation, below mentioned methods were used:

1- Quick check method: this method was used to rapidly analysis white and blue colonies on transformation product plate. The respective colonies was suspended in 12 ml of Rascunis buffer and placed on room temperature for 10 minutes. Then, 2 ml of same-volume phenol-chloroform mixture was added and after gently mixing (2 min) was centrifuged at 14000 rpm. The resulting products were subjected to electrophoresis and corresponding DNA bands of white and blue colonies were compared.

2- Contrast of extracted plasmids from white and blue colonies: 5 ml of respective extracted plasmids were gel-electrophorized on 1% agarose-gel and the resulting bands were precisely compared.

3- PCR reaction: Extracted plasmids of white and blue colonies were used in PCR as template DNA. After agarose-gel electrophoresis, the length of resulting band was measured by referring to molecular weight marker.

4- Enzymatic cleavage: the last confirmatory method for correct ligation was done by using restriction enzymes. The extracted plasmids from white also blue (as negative control) colonies were simultaneously cleaved by Eco R1 and SaI1 (Fermentas Inc.) enzymes. The reactions were consisted of 5-8 µl plasmid, 1 µl of Eco R1 and SaI1 and 2 µl enzyme buffer (total volume of 20 µl by distilled water). These reactions were incubated for 1-3 hours at 37°C. Enzymatic cleavage products were subjected to gel-electrophoresis and resulting fragments were measured by using appropriate molecular weight marker.

Sub-cloning: The gene fragment was sub-cloned in a pET32a expression plasmid (prepared from Microbiology Department, Faculty of Medicine, Arak University). Initially, the gene fragment was separated from previous cloned plasmid by restriction cleavage and then was purified using gel-extraction kit (Fermentas Inc.). In addition, pET32 was cultivated in an antibiotic-containing media. After plasmid extraction its restriction cleavage

sites for Eco R1 and SaI1 were enzymatically cleaved and purified. Then, resulting plasmids were used for ligation and transformation of gene fragment. To verify recombinant ligated and transformed plasmid, previous mentioned methods were used.

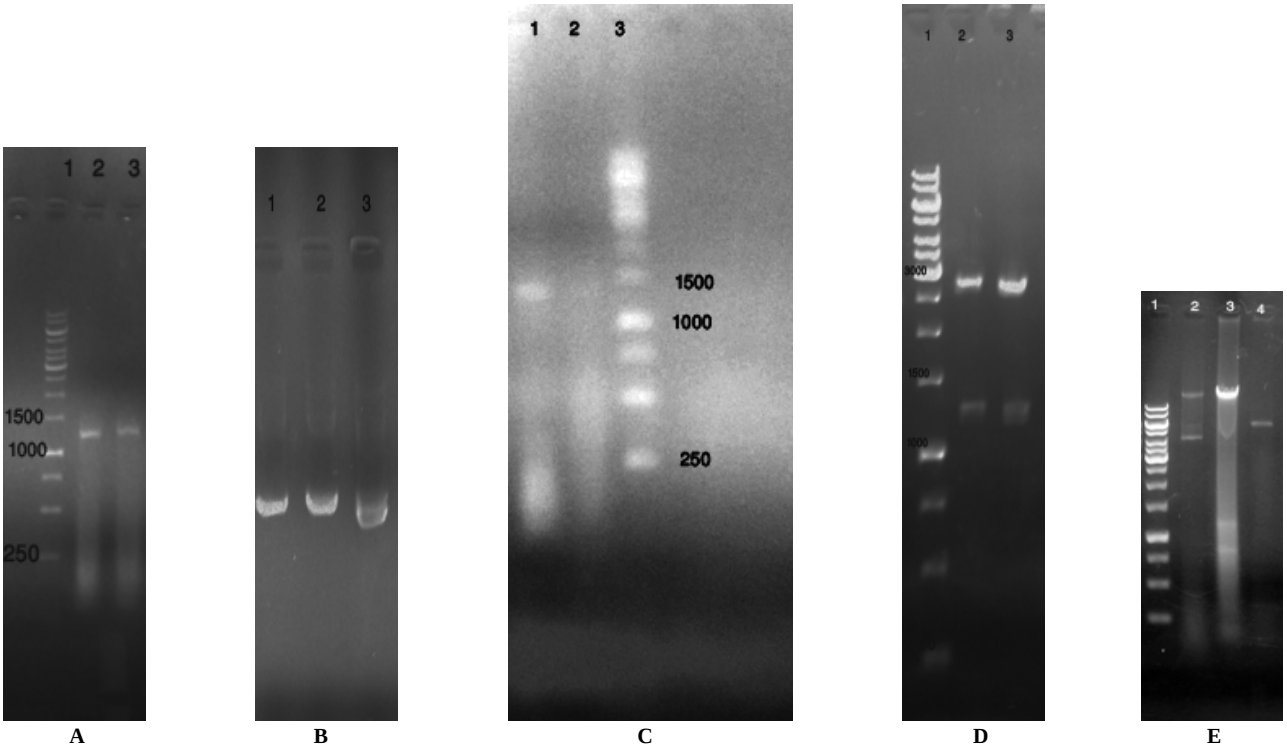
Results

Genomic DNA of RH strain of *T. gondii* was subjected to PCR to amplify ROP1 gene fragment. PCR products revealed an 1183bp band on agarose gel (Fig. 2 A). Before performing next procedures, PCR products were sent to sequencing and resulting sequences were analyzed using BLAST assay on NCBI website. The results showed a hundred-percent homology with RH strain ROP1 sequence (ref. No M71274.1 and AY661790.1). After ligation of gene fragments to plasmids, transformation of plasmids into competent *E. coli* cells and screening of colonies consisted of recombinant plasmid (according to color of colony); a quick check method was used on some of resulting colonies. In this experiment, due to their increased molecular weight, migration of white colonies of recombinant plasmids (pT-ROP1) through the gel was significantly slower than non-recombinant blue colonies (pTZ57R/T). Once extracting plasmids from white and blue colonies, same results were also manifested on gel-electrophoresis (Fig. 2 B).

Then, PCR reaction was performed on plasmids of both blue (as negative control) and white colonies. On agarose-gel electrophoresis, the fragment including 1183bp length plasmids extracted from white colonies was evidently amplified and confirmed by referring to molecular weight marker (Fig. 2 C). For the next confirmatory assay, some white colonies, cultivated on high-scaled volume, were subjected to plasmid extraction. The plasmids were enzymatically cleaved by restriction enzymes and the resulting 1183bp fragment was isolated on gel electrophoresis from white colonies (Fig. 2 D). In the next phase, separated fragments from pTZ57R/T plasmid were sub-cloned into an expression plasmid. After ligation and transformation of recombinant plasmid to competent cells, the resulting colonies were compared by quick check method. Then, colonies of recombinant plasmid (which migrated slower on gel electrophoresis) were cultivated and subjected to plasmid extraction. Finally, the existence of ROP1 fragment in expression plasmids was confirmed by enzymatic cleavage (Fig. 2 E).

Forward: 5`-GGAATTCACCATGGAGCAAAGGCTG-3`
Reverse: 5`-GCGTCGACTTATTGCGATCCATCATCC-3`

Figure 1. Appropriate primers for ROP1 gene



A **B** **C** **D** **E**

Figure 2. A. Gel-electrophoresis of 1183bp PCR product. Lane 1: 1 kbp molecular weight marker, Lanes 2 and 3: PCR products, **B.** Compare of plasmids extracted from white and blue colonies. Lanes 1 and 2: recombinant plasmid containing ROP1 fragment (pT-ROP1), Lane 3: non-recombinant plasmid (pTZ57R/T), **C.** Electrophoresis of PCR product of pT-ROP1 plasmid. Lane 1: PCR product of recombinant pT-ROP1 plasmid showing an approximate 1183bp band, Lane 2: PCR product of non-recombinant pTZ57R/T plasmid extracted from blue colonies (without band), Lane 3: 1Kbp molecular weight marker. **D.** Cleavage of ROP1 fragment from recombinant pT-ROP1 plasmid. Lane 1: 1Kbp molecular weight marker, Lane 2, 3: recombinant pT-ROP1 plasmid subjected to restriction cleavage showing a 1183bp ROP1 fragment. **E.** Cleavage of ROP1 fragment from prokaryotic pET32a expression plasmid. Lane 1: 1 Kbp molecular weight marker, Lane 2: non-recombinant pET32a plasmid before cleavage, Lane 3: recombinant pc-ROP1 plasmid subjected to restriction cleavage showing a 1183bp ROP1 fragment, Lane 4: non-recombinant pET32a plasmid subjected to restriction cleavage and absence of 1183 ROP1 fragment

Discussion

Toxoplasma gondii is the cause of toxoplasmosis; that it is worldwide distribution and it is one of the most prevalent parasitic infections around the world. It has been estimated that 500 million to 1 billion of human population have been asymptomatic and chronic form of the infection [15]. The treatment of this infection is difficult because current drugs have toxic side effect. Therefore on-time and rapid diagnosis of infection, rapid and precise differentiation between acute and chronic cases and also construction of effective vaccine are considerably important [15].

In recent years, many progresses have been achieved in identifying candidate antigens of vaccine which are capable to inducing protective immune response. A number of these antigens were expressed in prokaryotic organisms and after purifying used in diagnostic studies [15].

In the performed study, the 1183bp fragment of *T. gondii* ROP1 gene was cloned into pET32a expression plasmid and ready to produce protein and using of that in diagnostic studies, recombinant vaccine production and immunologic surveys in animal models. ROP1 gene is one of the major antigens of *T. gondii* by unusual gene structure. The major part of this gene has similar sequence in genomic DNA, cDNA and mRNA so it is possible directly isolating gene fragment from DNA [9]. This protein is approximately 33.5 KDa but because of amino acids conformations, moves slowly and similar to 60 KDa protein during SDS-PAGE [16]. This protein is presented in tachizoytes, bradizoytes and sporozytes forms of parasite and plays a role in invasion to host cells [17]. The ROP1 is expressed in many phases of parasitic life cycle; it confers a suitable target for production of clinical diagnostic kits and recombinant vaccines [18].

Gue et al. cloned a 760bp ROP1 gene fragment into pBV220 expression plasmid and assessed the immune responses of Balb/c mice vaccinated by this plasmid [11]. In another study carried out by this group, encoding plasmids for a 760bp fragment of ROP1 gene and a 600bp fragment of IFN-δ gene were generated and immunologically assessed in Balb/c mice [12]. Chen et al. cloned a 750bp fragment of *T. gondii* ROP1 gene into pUC18 plasmid and then transferred it to pcDNA3 eukaryotic expression plasmid [9]. Another group directed by Chen constructed a fusion-based recombinant plasmid consisting two fragments of ROP1 (760bp) and SAG1 genes (pSAG1-ROP1). Recombinant plasmids were injected to Balb/c mice via liposome-entrapped particles and immune responses were analyzed [10]. Eslami et al. cloned a 760bp fragment of *T. gondii* ROP1 gene into eukaryotic expression plasmid [13]. In the study, the used vector was pTZ57R, that is different from vectors used in other studies.

Also the used gene fragment could only encode part of ROP1 protein that was expressed at very low levels in eukaryotic CHO cells and hardly traceable in vivo. However, the results reflected relatively satisfactory immunogenicity of plasmids in animal models [19]. Aubert et al. produced a number of *T. gondii* antigens such as ROP1 in fusion with Cks protein of *E. coli* and used these recombinant antigens to identify anti-toxoplasmosis specific IgM and IgG antibodies in human serum [20]. Meeka et al. cloned fragments of ROP1 and SAG1 genes into pRP261 expression plasmid and used the resulting expressed proteins to serological diagnosis of toxoplasmosis in patients suffering from acute and chronic toxoplasmosis [21]. In a study Pfreper et al. also investigated the impact of some recombinant protein fragments of toxoplasma including ROP1 on seroactivity and avidity [22]. Holec-Gasior et al. generated a 940bp recombinant fragment of ROP1 gene and utilized it for serological diagnosis of specific anti-toxoplasma IgG antibodies. They concluded that these recombinant proteins can be used as specific markers for differentiation of acute and chronic cases of patients [18].

By review of limited studies performed on ROP1, in most of them carried out on a limited fragment of ROP1 gene or the expressed protein was fused with other proteins. It seems consequently that identification and production of other fragments of ROP1 protein with higher level of antigenicity could have many applications. To achieve to such aim, this study was designed and implemented. As a result of this study, an 1183bp fragment of ROP1 gene of *T. gondii* with capability of expressing complete ROP1 protein was provided. This study showed that if it fulfills expectation of high-thorough expression of ROP1 protein in expression plasmid system, the mentioned fragment could be extensively used in design and production of recombinant vaccine and production of diagnostic kits and nanotechnology research.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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References

1. Weiss LM, Kim K. Toxoplasma gondii: The model apicomplexan, Perspectives and methods. 1st ed. USA: Academic Press; 2007.
2. Jacobs D, Vercammen M, Saman E. Evaluation of recombinant dense granule antigen 7 (GRA7) of Toxoplasma gondii for detection of immunoglobulin G

antibodies and analysis of a major antigenic domain. Clin Diagn Lab Immunol 1999; 6(1): 24-29.
3. Letscher V, Pfaff AW, Abou-Bacar A, et al. Vaccination with Toxoplasma gondii SAG-1 protein is protective against congenital toxoplasmosis in BALB/c mice but not in CBA/J mice. Infect Immun 2003; 71(11): 6615-19.
4. Bhopale GM. Development of a vaccine for toxoplasmosis: Current status. Microbes Infec 2003; 5(5): 457-62.
5. Dalton J, Mulcahy G. Parasite vaccines: A reality? Vet Parasitol 2001; 98(1-3): 149-67.
6. Garcia JL, Gennari SM, Navarro IT, et al. Partial protection against tissue cysts formation in pigs vaccinated with crude rhoptry proteins of Toxoplasma gondii. Vet Parasitol 2005; 129(3-4): 209-17.
7. Crampton A, Vanniasinkam T. Parasite vaccines: The new generation. Infect Genet Evol 2007; 7(5): 664-73.
8. Li S, Maine G, Suzuki Y, et al. Serodiagnosis of recently acquired Toxoplasma gondii infection with a recombinant antigen. J Clin Microb 2000; 38(1): 179-84.
9. Chen G, Guo H, Lu F and Zheng H. Construction of a recombinant plasmid harbouring the rhoptry protein 1 gene of Toxoplasma gondii and preliminary observations on DNA immunity. Chin Med J (Engl) 2001; 114(8): 837-40.
10. Chen H, Chen G, Zheng H and Guo H. Induction of immune responses in mice by vaccination with liposome-entrapped DNA complexes encoding Toxoplasma gondii SAG1 and ROP1 genes. Chin Med J (Engl) 2003; 116(10): 1561-6.
11. Guo H, Chen G, Zheng H, et al. [Immune responses in mice vaccinated with recombinant plasmid pcDNA3 containing ROP1 gene from Toxoplasma gondii] Chinese [Abstract]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 1999; 17(6): 334-7.
12. Guo H, Chen G, Lu F, et al. Immunity induced by DNA vaccine of plasmid encoding the rhoptry protein 1 gene combined with the genetic adjuvant of pcIFN-gamma against Toxoplasma gondii in mice. Chin Med J (Engl) 2001; 114(3): 317-20.
13. Eslamirad z, Dalimi A, Ghaffarifar F and Sharifi Z. Cloning rhoptry protein1 gene of Toxoplasma gondii(RH) in expression vector. Arch Razi Inst 2008; 63(2): 11-7.
14. Sambrook J, Russell DW. Molecular cloning: A laboratory manual. 4th ed. New York: Cold Spring Harbor Laboratory Press; 2001.
15. Nigro M, Gutierrez A, Hoffer AM, et al. Evaluation of Toxoplasma gondii recombinant proteins for the diagnosis of recently acquired toxoplasmosis by an immunoglobulin G analysis. Diagn Microbiol Infect Dis 2003; 47(4): 609-13.
16. Bradley PJ, Hsieh CL, Boothroyd JC. Unprocessed Toxoplasma ROP1 is effectively targeted and secreted into the nascent parasitophorous vacuole. Mol Biochem Parasitol 2002; 125(1-2): 189-93.
17. Garcia JL, Gennari SM, Navarro IT, et al. Toxoplasma gondii: Isolation of tachyzoites rhoptries and incorporation into Iscom. Exp Parasitol 2004; 108(1-2): 40-6.
18. Holec-Gasior L, Kur J, Hiszczynska-Sawicka E. GRA2 and ROP1 recombinant antigens as potential markers for detection of Toxoplasma gondii-specific immunoglobulin G in humans with acute toxoplasmosis. Clin Vaccine Immunol 2009; 16(4): 510-4.
19. Eslamirad Z, Dalimi A, Ghaffarifar F and Sharifi Z. [Comparison of two aluminum compounds effects by DNA vaccine encoded ROP1 gene of RH strain of Toxoplasma gondii to evaluated immunization and survival time in animal model] Persian. J Modares Med Sci 2011; 14(1): 17-27.
20. Aubert D, Maine GT, Villena I, et al. Recombinant antigens to detect Toxoplasma gondii-specific immunoglobulin G and immunoglobulin M in human sera by enzyme immunoassay. J Clin Microbiol 2000; 38(3): 1144-1150.
21. Meeka B, Jan Diepersloot B, van Goolc T, et al. IgM recognition of recombinant Toxoplasma gondii antigens by sera of acutely or latently infected humans. Diagn Microbiol Infect Dis 2003; 45(1): 45-52.
22. Pfrepper KI, Enders G, Gohl M, et al. Seroreactivity to and avidity for recombinant antigens in toxoplasmosis. Clin Vaccine Immunol 2005; 12(8): 977-82.

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