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Design of a dual-promoter expression vector harboring *Sag1* and *Gra7* genes from *Toxoplasma gondii* (RH strain)

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Abstract. Toxoplasmosis, a parasitic disease caused by *Toxoplasma gondii*, has possible irreparable consequences in immunocompromised patients and fetuses. Finding an effective method of prevention, such as vaccination, is crucial because of the global distribution of the parasite and the lack of effective anti-toxoplasmosis drugs. The Sag1 and Gra7 antigens of *T. gondii* can induce strong humoral and cell-mediated immune responses. Therefore, to develop a novel DNA vaccine against toxoplasmosis, we prepared a eukaryotic construct expressing the *Sag1* and *Gra7* genes of *T. gondii* (RH strain). We then verified the ability of this construct to produce the corresponding Sag1 and Gra7 antigens in mammalian cells. Using specific primers, the complete coding sequences of *Sag1* and *Gra7* genes were amplified by polymerase chain reaction (PCR) from the genomic DNA of *T. gondii*. Then, both genes were subcloned into pVitro2-neo-mcs plasmid. The pVitro-*Sag1–Gra7* construct was subjected to colony PCR, enzymatic digestion, and sequencing to confirm successful subcloning. *Sag1* and *Gra7* expression in HeLa cells was investigated. *Sag1* and *Gra7* were successfully subcloned in pVitro2-neo-mcs plasmid. The expression of Sag1 and Gra7 in HeLa cells was confirmed through Western blot analysis. The recombinant pVitro-*Sag1–Gra7* construct that simultaneously produces *Sag1* and *Gra7* antigens in one mammalian cell may be used to develop a novel protective vaccine against toxoplasmosis.

INTRODUCTION

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii*, an obligate, intracellular coccidian protozoa with global distribution. The infection is life threatening to fetuses and immunocompromised patients (Montoya & Remington, 2008; Pereira-Chioccola *et al.*, 2009) and is particularly disastrous in the livestock industry because it may cause abortion and stillbirths (Tenter *et al.*, 2000). The seroprevalence of toxoplasmosis is approximately 10%–80% in the global human population and 39.5% in the general Iranian population (Robert-Gangneux & Darde, 2012; Daryani *et al.*, 2014). Chemotherapy is the only treatment strategy for toxoplasmosis. However, the drug treatment of choice, sulphadiazine plus pyrimethamine, fails to eradicate infection while inducing toxic side effects (Rodriguez & Szajnman, 2012).

Considering the above adverse aspects, designing a new effective vaccine that prevents toxoplasmosis is a priority (Henriquez *et al.*, 2010). No effective vaccine against toxoplasmosis exists, however, except for one based on a live attenuated strain (Toxovax) that is exclusively for veterinary use (HiszczyŃSka-Sawicka *et al.*,

2014). Several studies have evaluated various anti-toxoplasmosis vaccination strategies that are based on inactivated or attenuated strains, recombinant proteins, and DNA vaccines (Zhang *et al.*, 2013). DNA vaccines effectively protect against toxoplasmosis by eliciting long-lasting humoral and cell-mediated immunity (Alarcon *et al.*, 1998).

Various antigens, including surface antigen glycoproteins (Sag), excretory-secretory dense granule proteins (Gra), rhoptry proteins, and micronemal proteins have been evaluated as potential anti-toxoplasmosis vaccine candidates (Kur *et al.*, 2009; Zhang *et al.*, 2013). Sag1 is abundantly expressed on the surface of *T. gondii* tachyzoites. It is highly immunogenic and activates humoral and cellular immunity. In addition, it plays a crucial role in the adhesion and invasion capabilities of the parasite (Wang & Yin, 2014). Gra7, which is expressed at all stages of toxoplasmosis infection, has a key role in protective immune responses against the disease by triggering significant humoral and cellular immune responses (Jongert *et al.*, 2007; Hiszczyńska-Sawicka *et al.*, 2011; Verhelst *et al.*, 2011; Min *et al.*, 2012). Therefore, Sag1 and Gra7 are attractive vaccine candidates against toxoplasmosis.

In this study, we cloned the *Sag1* and *Gra7* genes from *T. gondii* (RH strain) into a dual-promoter plasmid vector (pVitro2-neo-mcs) and confirmed their co-expression in HeLa cells.

MATERIAL & METHODS

Parasite

To propagate parasites, the tachyzoites of *T. gondii*, (RH strain) were intraperitoneally injected into Swiss mice (outbred). After 5 days, peritoneal fluid was aspirated from the mice. The tachyzoites were collected and then washed with phosphate buffer saline (pH = 7.2).

Preparation of Eukaryotic expression constructs

DNA extraction and gene amplification

QIAamp DNA Mini kit (Qiagen, Germany) was used to extract and purify genomic DNA from tachyzoites in accordance with the manufacturer's instructions.

The extracted DNA was employed as a template for amplifying the entire protein-coding sequence of *T. gondii* (RH strain). *Sag1* (1187 bp; sequence position 161 to 1168; GeneBank accession No. GQ253075.1) and *Gra7* (762bp; sequence position 25 to 732; GeneBank accession No. DQ459443) genes were amplified through polymerase chain reaction (PCR) using specific primers (Table 1).

PCR amplification was performed with a 20 µl reaction volume containing 10 µl of 2x Master Mix RED with 1.5 mM Mgcl2 (Ampliqon, Denmark), 1 µl of each primer at the concentration of 10 pmol, 7 µl of distilled water, and 1 µl of template DNA for each gene.

Table 1. Primers for PCR amplification of *Sag1* and *Gra7* genes

Primer name	Sequence	Restriction enzyme
Sag1 forward primer	-GAAGATCTCATTGTCGTGTAAACACACGG-	BglII
Sag1 reverse primer	-GCTAGCCGCGACACAAGCTGCGAT-	NheI
Gra7 forward primer	-GGATCCATGGCCCGACACGCAAT-	BamHI
Gra7 reverse primer	- ATCGATCTGGCGGGCATCCTC-	ClaI

The underlining sequences are corresponding restriction enzyme sites

Amplification was conducted under the following thermocycler conditions: initial denaturation of 94°C for 5 min, 30 cycles of denaturation at 94 for 1 min, annealing at 56 for 1 min, extension at 72 for 1 min, and final extension at 72 for 15 min. PCR products were analyzed through agarose gel (1%) visualization.

Cloning of *Sag1* and *Gra7* genes from *T. gondii*

PCR products were separately cloned into pTZ57R/T vectors (Thermo Scientific InsTAclone PCR cloning kit) in accordance with the manufacturer's instructions. Then, the generated recombinant constructs were transfected into chemically transformed ($CaCl_2$, 100 mM) competent *E. coli* $DH_5\alpha$ cells (Sambrook & Russell, 2001).

The transformed bacteria were cultured on Luria–Bertani (LB) agar medium containing 50 µg/ml ampicillin. The colonies were screened using isopropyl-β-D-thiogalacto pyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. White colonies were considered positive for transformation. Successful insertion in white colonies was confirmed through colony PCR, enzymatic digestion, and bidirectional sequencing.

Subcloning of *Toxoplasma gondii Sag1* and *Gra7* genes in pVitro2-neo-mcs

Extracted and purified (Bioneer AccuPrep® Nano-Plus Plasmid Mini Extraction Kit, Korea) pTZ-*Sag1* and its recombinant constructs were digested with BglII and NheI, whereas pTZ-*Gra7* and its recombinant constructs were digested with BamHI and ClaI. The cleaved gene fragments were extracted (Bioneer AccuPrep® Gel Purifractoin kit, Korea) from 0.8% agarose gel after electrophoresis. Extracted gene fragments were separately subcloned in digested pVitro2-neo-mcs eukaryotic expression vectors (Invivogen, USA) using T4 DNA Ligase enzyme (Fermentas, Lithuania). To produce a construct that harbors both genes, the pVitro-*Gra7* construct was extracted from transformed *E. coli*, digested with Bg1II and NheI to produce sticky ends, and then purified from 0.8% agarose gel. The *Sag1* fragment was digested with the same enzymes and then cloned into a digested pVitro-*Gra7* construct. The fragment was ligated into competent *E. coli* $DH_5\alpha$ cells. The transformed *E.coli* $DH_5\alpha$ cells were then cultured on LB agar medium containing 70 µg/ml kanamycin. The colonies were screened by colony PCR, enzymatic digestion, and bidirectional sequencing to verify the successful construction of pVitro-*Sag1*, pVitro-*Gra7*, and pVitro-*Sag1–Gra7* recombinant plasmids.

Plasmids were purified from transformed *E. coli* $DH_5\alpha$ cells using Endofree Plasmid Mega Kit (Qiagen, Germany) following the manufacturer's instructions. The purified plasmids were then dissolved in sterile endotoxin-free H_2O. The plasmid concentration for every construct was determined using Thermo scientific NanoDrop 2000 spectrophotometer (Thermo, USA). All constructs were stored at -20°C until further use.

Transient Transfection

HeLa cells were cultured in 6-well plates (Nunc, Denmark) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, streptomycin (100 mg/ml), and penicillin (100 IV/ml) at 37 in the presence of 5% CO_2.

Using lipofectamin 2000 (Invitrogen, USA), 80%–90% confluent cells were transfected with recombinant plasmids following the manufacturer's instructions. The negative control was prepared by transfecting HeLa cells with empty pVitro2-neo-mcs vectors.

A mixture of every single plasmid with lipofectamin 2000 reagent at the concentration of 10 µg/ml was prepared in DMEM (without FCS or antibiotics). The mixtures were incubated at room temperature for 30 min and then added dropwise to a HeLa cell culture. After 6 h of incubation in a cell incubator, the spent culture medium was replaced with fresh cell culture medium, and incubation was continued for 72 h. Afterwards, modified SDS-PAGE and Western blot analysis were used to confirm the individual expression of *Sag1* and *Gra7* and the simultaneous expression of *Sag1* and *Gra7*

in transfected cells (Rassouli *et al.*, 2013; Selseleh *et al.*, 2012).

RESULTS

Construction of pVitro-*Sag1–Gra7*

The PCR amplification of *Sag1* and *Gra7* genes using specific primers produced the expected 1010-bp fragment for *Sag1* and 750-bp fragment for *Gra7* (Figure 1). After cloning the purified PCR products into pTZ57R/T vector, colony PCR showed the expected 1010-bp fragment in *Sag1* colonies and 750-bp fragment in *Gra7* colonies. Restriction enzyme digestion confirmed the existence of the desired fragments in the constructs.

Both genes were subcloned into pVitro2-neo-mcs vectors. The expected fragments corresponding to *Sag1* and *Gra7* genes were obtained through colony PCR using a single colony as the PCR template. Enzymatic digestion validated confirmed successful subcloning (Figure 2). The alignment of the sequences of the fragments with the annotated sequences of *Sag1* and *Gra7* genes retrieved from GeneBank showed >99% identity and the absence of a gap. Both sequences lacked mutations or deletions (data not shown).

In vitro expression analysis of *Sag1* and *Gra7* genes

Results confirmed that HeLa cells were successfully transfected, and all three constructs expressed their genes properly and without reaction between toxoplasma-specific antibodies and HeLa cell or empty-vector proteins.

In vitro expression was verified through SDS-PAGE and Western blotting. HeLa cells transfected with pvitro-*Sag1* contained a protein with a molecular weight of 30 kDa, which is consistent with the molecular weight of Sag1. HeLa cells transfected with pVitro-*Gra7* expressed a recombinant protein with a molecular weight of 26 kDa, which is consistent with the molecular weight of Gra7. Two protein bands weighing approximately 30 and 26 kDa appeared in the lane of HeLa cells transfected with pVitro-*Sag1–Gra7*, indicating that the pVitro-*Sag1–Gra7* vector co-expresses both genes (Fig. 3). Western

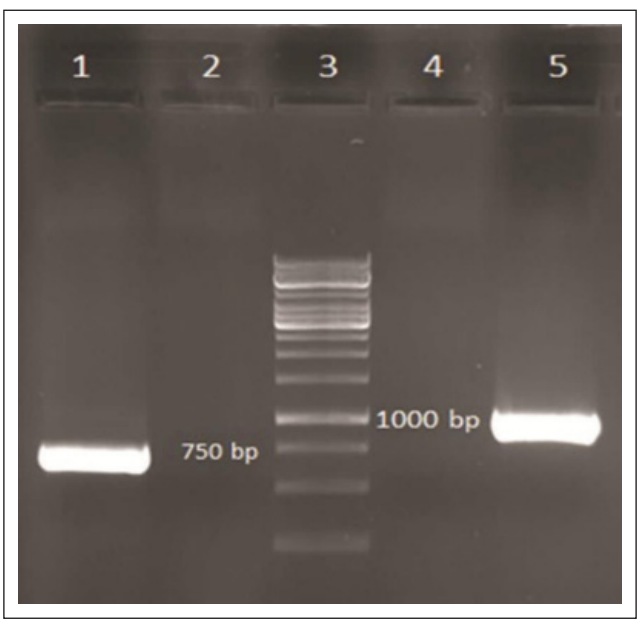

Figure 1. *Sag1 and Gra7* PCR products. Lane 1 illustrates *Gra7* 750 bp PCR product, Lane 2 NC, Lane 3 1kb Fermentas DNA ladder, Lane 4 NC, Lane 5 *Sag1* 1000 bp PCR product (NC=Negative control).

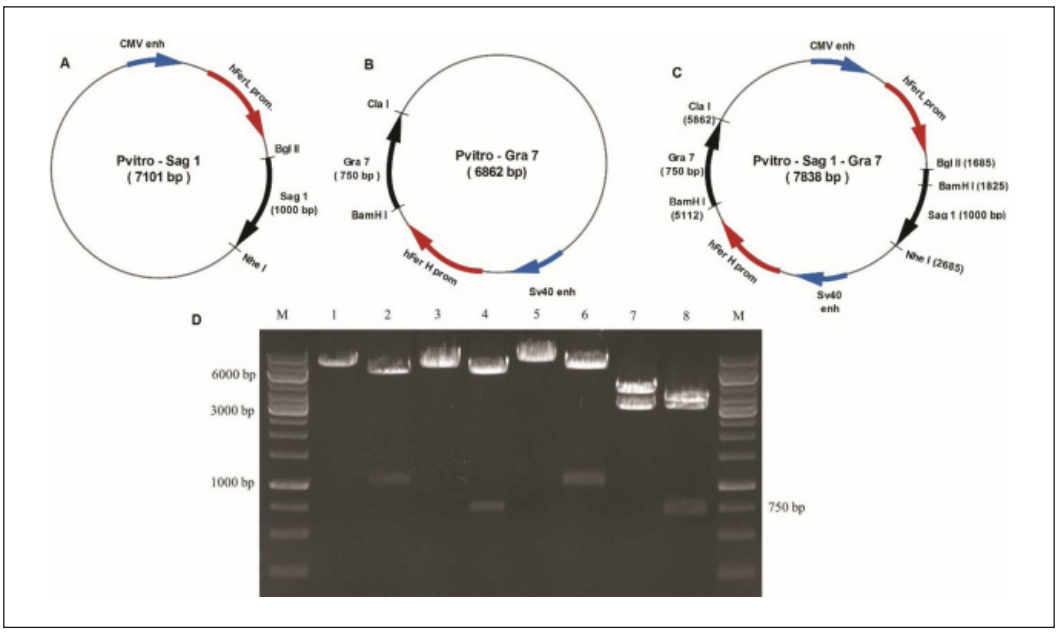

Figure 2. The constructed vectors and their enzymatic digestion pattern. A Pvitro-*Sag1* construct B Pvitro-*Gra7* construct C Pvitro-*Sag1-Gra7* construct D Restriction enzyme digestion pattern of pvitro-Sag1, pvitro-Gra7 and pvitro-Sag1-Gra7 constructs. Lane M Fermentas 1kb DNA ladder, Lane 1 linear pvitro-Sag1 (BglII digested), Lane 2 double digested pvitro-Sag1 (BglII and NheI) depicts 1000bp Sag1 fragment cleaved out from 6kb vector, Lane 3 linear pvitro-Gra7 (BamHI digested), Lane 4 double digested pvitro-Gra7 (BamHI and ClaI) depicts 750 bp Gra7 fragment cleaved out from 6kb vector, Lane 5 linear pvitro-Sag1-Gra7 (BglII digested), Lane 6 double digested pvitro-Sag1-Gra7 (BglII and NheI), Lane 7 pvitro-Sag1-Gra7 digested with BamHI Lane 8 pvitro-Sag1-Gra7 double digested (BamHI and ClaI) and Lane Fermentas 1kb DNA ladder.

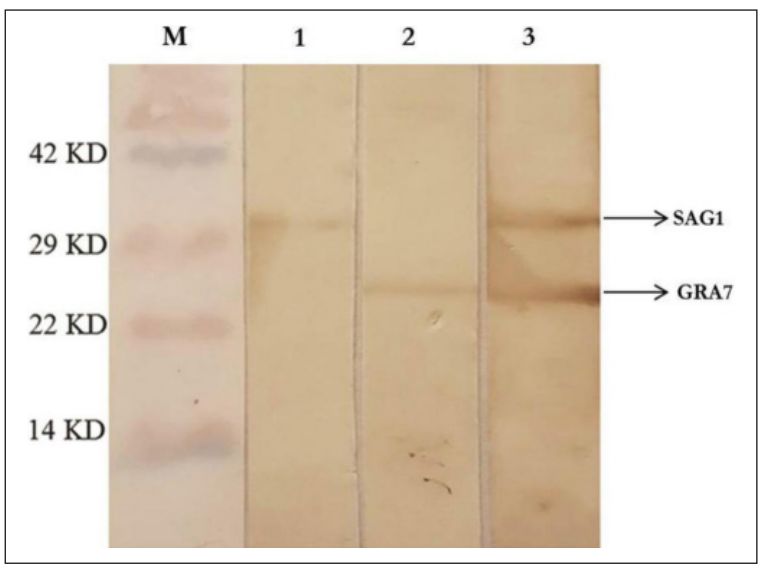

Figure 3. *In vitro* expression western blot analysis. Lane M abm protein plus ladder, Lane 1 Sag1 expression in pvitro-Sag1 construct, Lane 2 Gra7 expression in pvitro-Gra7 construct, Lane 3 Sag1 and Gra7 co-expression in pvitro-Sag1-Gra7 construct.

blot analysis showed no protein bands from the cell lysates of empty HeLa cells and HeLa cells transfected with the empty vector (pVitro2-neo-mcs) (data not shown).

DISCUSSION

Aiming to develop an effective DNA vaccine against toxoplasmosis, this study amplified and subcloned the *Sag1* and *Gra7* genes of *T. gondii* into pVitro2-neo-mcs, a dual-promoter mammalian expression vector. The ability of the recombinant pVitro-*Sag1–Gra7* construct to simultaneously produce Sag1 and Gra7 antigens was validated after the induction of expression in HeLa cells.

The global burden attributed to toxoplasmosis is considerable, and congenital toxoplasmosis infections cause an estimated 1.2 million DALYs (Torgerson *et al.*, 2014). Given the parasite's irreparable consequences, global distribution, and the absence of efficient anti-toxoplasmosis drugs, finding an effective preventative method, such as vaccination, is crucial.

A vaccine for human use has yet to be developed despite numerous efforts to develop an effective vaccine against toxoplasmosis (Zhang *et al.*, 2013). Various production strategies for anti-toxoplasmosis vaccines, including the use of inactivated, live attenuated, recombinant protein, subunit, and DNA vaccines, have been investigated (Jongert *et al.*, 2009). Live attenuated strains provide highly promising results because they significantly decrease oocyst shedding, brain cyst formation, and abortion; these vaccines, however, have not been licensed for human use because the contained strains may revert to the wild strain (Jongert *et al.*, 2009). DNA vaccines induce powerful, long-lasting humoral and cell-mediated immune responses against the antigen of interest and are thus highly efficient vaccine bases. DNA vaccines are also easy to produce, cheap, and are heat resistant; moreover, their immunogenicity can be augmented through vector modification (Liu 2003; Saha *et al.*, 2011). The association between Th1 response and protection against *T. gondii* makes this vaccination strategy preferable. Numerous studies have investigated the preparation and evaluation of DNA vaccines against toxoplasmosis (Zhang *et al.*, 2013). Although different DNA vaccines showed different levels of survival rate increment or cyst burden reduction, none of them provided complete protection against toxoplasmosis (Jongert *et al.*, 2009; Ma *et al.*, 2013).

Several studies have proposed that multivalent DNA vaccines are more efficient than monovalent DNA vaccines (Fachado *et al.*, 2003). The pVitro-*Sag1–Gra7* construct, which contains two genes that are expressed at different stages of toxoplasmosis infection, might provoke an efficient immune response. In contrast to cocktail multivalent DNA vaccines, the pVitro-*Sag1–Gra7* construct assures the equal delivery of both genes to cells that present the host antigen. The construction of this vector also provided the opportunity for comparing our dual-expressing DNA vaccine with fusion DNA vaccines that produce a single fusion protein.

The majority of host immune responses target the surfaces of pathogens. Proteins that originate from the surface of tachyzoites, dense granules, rhoptries, and micronems have been evaluated as anti-toxoplasmosis vaccine candidates (Jongert *et al.*, 2009). Sag1 is the dominant and most abundant surface antigen of tachyzoites (Kasper & Khan, 1993). The *Sag1* sequence is highly conserved among different *T. gondii* types, including pathogenic (type I) and cystogenic (type II/III) types (Hartati *et al.*, 2006; Smith *et al.*, 2007). An initial study showed that direct immunization with the *Sag1* DNA vaccine remarkably increases survival rate but provides only partial protection (Nielsen *et al.*, 1999). The *Sag1* gene has been utilized in monovalent or multivalent DNA vaccines by many researchers (Angus *et al.*, 2000; Couper *et al.*, 2003; Mévélec *et al.*, 2005; Zhang *et al.*, 2007; Liu *et al.*, 2010). Although the majority of these researchers admit that Sag1 is an immunodominant antigen, none has developed Sag1-based vaccines with complete protection against toxoplasmosis because *T. gondii* expresses different antigens in different infectious forms. In

the present study, the *Gra7* gene, which is expressed at all stages of toxoplasmosis infection, was additionally selected because immune responses elicited by Sag1 are only effective during the acute phase of toxoplasmosis infection. Gra7 triggers remarkable humoral and cellular immune responses (Ferguson *et al.*, 1999; Jongert *et al.*, 2007; Verhelst *et al.*, 2011) and is the most immunogenic antigen among Gra proteins (Hiszczyñska-Sawicka *et al.*, 2011). Other studies that have assessed the feasibility of using *Gra7* as a DNA vaccine assert its attractiveness as a vaccine candidate (Jongert *et al.*, 2007; Hiszczyńska-Sawicka *et al.*, 2010).

pcDNA and pVAX are regularly exploited as vectors for DNA vaccines. In this study, pVitro2-neo-mcs, a 6125-bp eukaryotic expression vector, harbors both genes. Although it is generally applied in gene therapy (Wen *et al.*, 2010), it has been utilized as a DNA vaccine vector in several studies (Ma *et al.*, 2013). The Western blot results obtained in the present study demonstrated that pVitro-Sag_1–Gra_7 can express *Sag1* and *Gra7* genes under its ferritin promoter and provides a high level of gene co-expression in transfected HeLa cells. After the expression of both genes in eukaryotic cells, post-translational modifications will occur properly. Thus, both antigens will preserve their functional conformation and seroreactivity. The authors intend to assess the immunogenicity and efficacy of pVitro-Sag_1–Gra_7 as a vaccine in a murine model.

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REFERENCES

Alarcon, J.B., Waine, G.W. & McManus, D.P. (1999). "DNA vaccines: Technology and application as anti-parasite and anti-microbial agents." In *Advances in Parasitology* **42**: 343-410.

Angus, C.W., Klivington-Evans, D., Dubey, J.P. & Kovacs, J.A. (2000). 'Immunization with a DNA plasmid encoding the SAG1 (P30) protein of *Toxoplasma gondii* is immunogenic and protective in rodents', *The Journal of Infectious Diseases* **181**: 317-24.

Couper, K.N., Nielsen, H.V., Petersen, E., Roberts, F., Roberts, C.W. & Alexander, J. (2003). 'DNA vaccination with the immunodominant tachyzoite surface antigen (SAG-1) protects against adult acquired *Toxoplasma gondii* infection but does not prevent maternofoetal transmission', *Vaccine* **21**: 2813-20.

Daryani, A., Sarvi, S., Aarabi, M., Mizani, A., Ahmadpour, E., Shokri, A., Rahimi, M.T. & Sharif, M. (2014). 'Seroprevalence of *Toxoplasma gondii* in the Iranian general population: A systematic review and meta-analysis', *Acta Tropica* **137**: 185-94.

Fachado, A., Rodriguez, A., Angel, S.O., Pinto, D.C., Vila, I., Acosta, A., Amendoeira, R.R. & Lannes-Vieira, J. (2003). 'Protective effect of a naked DNA vaccine cocktail against lethal toxoplasmosis in mice', *Vaccine* **21**: 1327-35.

Ferguson, D.J., Jacobs, D., Saman, E., Dubremetz, J.F. & Wright, S.E. (1999). 'In vivo expression and distribution of dense granule protein 7 (GRA7) in the exoenteric (tachyzoite, bradyzoite) and enteric (coccidian) forms of Toxoplasma gondii', *Parasitology* **119**: 259-65.

Hartati, S., Kusumawati, A., Wuryastuti, H. & Widada, J.S. (2006). 'Primary structure of mature SAG1 gene of an Indonesian *Toxoplasma gondii* and comparison with other strains', *Journal of Veterinary Science* **7**: 263-70.

Henriquez, F.L., Woods, S., Cong, H., McLeod, R. & Roberts, C.W. (2010). 'Immunogenetics of *Toxoplasma gondii* informs vaccine design', *Trends in Parasitology* **26**: 550-55.

HiszczyŃSka-Sawicka, E., Gatkowska, J.M., Grzybowski, M.M. & DŁUgoŃSka, H. (2014). 'Veterinary vaccines against toxoplasmosis', *Parasitology* **141**: 1365-78.

Hiszczyńska-Sawicka, E., Li, H., Xu, J.B., Olędzka, G., Kur, J., Bickerstaffe, R. & Stankiewicz, M. (2010). 'Comparison of immune response in sheep immunized with DNA vaccine encoding *Toxoplasma gondii* GRA7 antigen in different adjuvant formulations', *Experimental Parasitology* **124**: 365-72.

Hiszczyńska-Sawicka, E., Oledzka, G., Holec-Gasior, L., Li, H., Xu, J.B., Sedcole, R., Kur, J., Bickerstaffe, R. & Stankiewicz, M. (2011). 'Evaluation of immune responses in sheep induced by DNA immunization with genes encoding GRA1, GRA4, GRA6 and GRA7 antigens of *Toxoplasma gondii*', *Veterinary Parasitology* **177**: 281-89.

Jongert, E., De Craeye, S., Dewit, J. & Huygen, K. (2007). 'GRA7 provides protective immunity in cocktail DNA vaccines against *Toxoplasma gondii*', *Parasite Immunology* **29**: 445-53.

Jongert, E., Roberts, C.W., Gargano, N., Förster-Wald, E. & Petersen, E. (2009). 'Vaccines against *Toxoplasma gondii*: Challenges and opportunities', *Memorias do Instituto Oswaldo Cruz* **104**: 252-66.

Kasper, L.H. & Khan, I.A. (1993). 'Role of P30 in host immunity and pathogenesis of *T. gondii* infection', *Research in immunology* **144**: 45-48.

Kur, J., Holec-Gąsior, L. & Hiszczyńska-Sawicka, E. (2009). 'Current status of toxoplasmosis vaccine development', *Expert Review of Vaccines* **8**: 791-808.

Liu, M.A. (2003). 'DNA vaccines: A review', *Journal of Internal Medicine* **253**: 402-10.

Liu, Q., Shang, L., Jin, H., Wei, F., Zhu, X.-Q. & Gao, H. (2010). 'The protective effect of a *Toxoplasma gondii* SAG1 plasmid DNA vaccine in mice is enhanced with IL-18', *Research in Veterinary Science* **89**: 93-97.

Ma, J., Trop, S., Baer, S., Rakhmanaliev, E., Arany, Z., Dumoulin, P., Zhang, H., Romano, J., Coppens, I., Levitsky, V. & Levitskaya, J. (2013). 'Dynamics of the Major Histocompatibility Complex Class I Processing and Presentation Pathway in the Course of Malaria Parasite Development in Human Hepatocytes: Implications for Vaccine Development', *PLoS One* **8**: e75321.

Mévélec, M.-N., Bout, D., Desolme, B., Marchand, H., Magné, R., Bruneel, O. & Buzoni-Gatel, D. (2005). 'Evaluation of protective effect of DNA vaccination with genes encoding antigens GRA4 and SAG1 associated with GM-CSF plasmid, against acute, chronical and congenital toxoplasmosis in mice', *Vaccine* **23**: 4489-99.

Min, J., Qu, D., Li, C., Song, X., Zhao, Q., Li, X.A., Yang, Y., Liu, Q., He, S. & Zhou, H. (2012). 'Enhancement of protective immune responses induced by *Toxoplasma gondii* dense granule antigen 7 (GRA7) against toxoplasmosis in mice using a prime-boost vaccination strategy', *Vaccine* **30**: 5631-36.

Montoya, J.G. & Remington, J.S. (2008). 'Management of *Toxoplasma gondii* infection during pregnancy', *Clinical Infectious Diseases* **47**: 554-66.

Nielsen, H.V., Lauemoller, S.L., Christiansen, L., Buus, S., Fomsgaard, A. & Petersen, E. (1999). 'Complete protection against lethal Toxoplasma gondii infection in mice immunized with a plasmid encoding the SAG1 gene', *Infection and Immunity* **67**: 6358-63.

Pereira-Chioccola, V.L., Vidal, J.E. & Su, C. (2009). '*Toxoplasma gondii* infection and cerebral toxoplasmosis in HIV-infected patients', *Future Microbiology* **4**: 1363-79.

Rassouli, H., Nemati, S., Rezaeiani, S., Sayadmanesh, A., Gharaati, M.R., Hosseini Salekdeh, G., Baharvand, H. & Gourabi, H. (2013). 'Cloning, Expression, and Functional Characterization of In-House Prepared Human Leukemia Inhibitory Factor', *Cell Journal (Yakhteh)* **15**: 190-97.

Robert-Gangneux, F. & Darde, M.L. (2012). 'Epidemiology of and diagnostic strategies for toxoplasmosis', *Clinical Microbiology Reviews* **25**: 264-96.

Rodriguez, J.B. & Szajnman, S.H. (2012). 'New antibacterials for the treatment of toxoplasmosis; A patent review', *Expert Opinion on Therapeutic Patents* **22**: 311-33.

Saha, R., Killian, S. & Donofrio, R.S. (2011). 'DNA vaccines: A mini review', *Recent Patents on DNA and Gene Sequences* **5**: 92-96.

Sambrook, J. & Russell, D.W. 2001. *Molecular cloning: a laboratory manual* (Cold Spring Harbor Laboratory Press).

Selseleh, M., Keshavarz, H., Mohebali, M., Shojaee, S., Modarressi, M.H., Eshragian, M.R. & Selseleh, M. (2012). 'Production and evaluation of *Toxoplasma gondii* recombinant surface antigen 1 (SAG1) for serodiagnosis of acute and chronic Toxoplasma infection in human sera', *Iranian Journal of Parasitology* **7**: 1-9.

Smith, T.K., Kimmel, J., Azzouz, N., Shams-Eldin, H. & Schwarz, R.T. (2007). 'The role of inositol acylation and inositol deacylation in the *Toxoplasma gondii* glycosylphosphatidylinositol biosynthetic pathway', *Journal of Biological Chemistry* **282**: 32032-42.

Tenter, A.M., Heckeroth, A.R. & Weiss, L.M. (2000). '*Toxoplasma gondii*: From animals to humans', *International Journal for Parasitology* **30**: 1217-58.

Torgerson, P.R., de Silva, N.R., Fevre, E.M., Kasuga, F., Rokni, M.B., Zhou, X.-N., Sripa, B., Gargouri, N., Willingham, A.L. & Stein, C. (2014). 'The global burden of foodborne parasitic diseases: an update', *Trends in Parasitology* **30**: 20-26.

Verhelst, D., De Craeye, S., Dorny, P., Melkebeek, V., Goddeeris, B., Cox, E. & Jongert, E. (2011). 'IFN-α expression and infectivity of Toxoplasma infected tissues are associated with an antibody response against GRA7 in experimentally infected pigs', *Veterinary Parasitology* **179**: 14-21.

Wang, Y. & Yin, H. (2014). 'Research progress on surface antigen 1 (SAG1) of *Toxoplasma gondii*', *Parasites and Vectors* **7**: 180-188.

Wen, Y., Wang, C.T., Ma, T.T., Li, Z.Y., Zhou, L.N., Mu, B., Leng, F., Shi, H.S., Li, Y.O. & Wei, Y.Q. (2010). 'Immunotherapy targeting fibroblast activation protein inhibits tumor growth and increases survival in a murine colon cancer model', *Cancer Science* **101**: 2325-2332.

Zhang, J., He, S., Jiang, H., Yang, T., Cong, H., Zhou, H., Zhang, J., Gu, Q., Li, Y. & Zhao, Q. (2007). 'Evaluation of the immune response induced by multiantigenic DNA vaccine encoding SAG1 and ROP2 of *Toxoplasma gondii* and the adjuvant properties of murine interleukin-12 plasmid in BALB/c mice', *Parasitology Research* **101**: 331-338.

Zhang, N.Z., Chen, J., Wang, M., Petersen, E. & Zhu, X.Q. (2013). 'Vaccines against *Toxoplasma gondii*: New developments and perspectives', *Expert Review of Vaccines* **12**: 1287-1299.