

EPI TOPE ANALYSIS AND EFFICACY EVALUATION OF PHOSPHATASE 2C (PP2C) DNA VACCINE AGAINST *TOXOPLASMA GONDII* INFECTION

P. X. Song^{1,4}, S. H. Yao¹, Y. Yao², J. Zhou³, Q. F. Li¹, Y. H. Cao¹, and S. Y. He⁴

¹ Department of Medicine, Quzhou College of Technology, Quzhou, Zhejiang 324000, People's Republic of China.

² Department of Medical Test, Shandong Medical College, Linyi, Shandong 276000, People's Republic of China.

³ Department of Orthopedics, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, People's Republic of China.

⁴ Department of Parasitology, Shandong University School of Basic Medicine, Jinan, Shandong 250012, People's Republic of China.

Correspondence should be sent to S. Y. He at: shenyi.he@hotmail.com; shenyihe@sdu.edu.cn

KEY WORDS ABSTRACT

Toxoplasma gondii
PP2C
Vaccine
Bioinformatics
Toxoplasmosis

Toxoplasma gondii infects almost all warm-blooded animals and negatively affects the health of a wide range of these animals, including humans. Protein phosphatase 2C (PP2C) is a *T. gondii* protein secreted by rhoptry organelles during host cell invasion. However, very little is known about whether this protein can induce protective immunity against *T. gondii*. In this study, bioinformatics analysis of PP2C revealed some useful information in the context of anti-toxoplasmosis treatments and vaccine research. In addition, the PP2C gene was amplified, and a eukaryotic expression vector (pEGFP-PP2C) was successfully constructed to express PP2C. Finally, the constructed pEGFP-PP2C was injected into mice to evaluate whether it could induce immunoprotection. Compared with the control groups, we found that immunizations with the pEGFP-PP2C plasmid could elicit specific IgG antibodies and cytokines against *T. gondii* infection. The survival of mice immunized with the pEGFP-PP2C plasmid was significantly prolonged compared with that of the control group mice. Based on the ability of pEGFP-PP2C to induce specific immune responses against *T. gondii*, we propose that PP2C merits consideration as a potential vaccine candidate against toxoplasmosis.

Toxoplasmosis is one of the most widely prevalent zoonotic diseases in the world, infecting almost one-third of the world population and resulting in a serious public health problem (Dubey and Jones, 2008; Reich et al., 2015). Humans become infected with this parasite by consuming uncooked and undercooked meat or other foods or water contaminated by oocysts from infected definitive hosts (Dubey and Jones, 2008). During pregnancy, *Toxoplasma gondii* parasites can spread to the developing fetus via the placenta and result in miscarriage, ocular complications, and even fetal stillbirth (Reich et al., 2015; Vijaykumar et al., 2016). Toxoplasmosis can also cause significant harm to immunocompromised patients or those infected with human immunodeficiency virus (Aspinall et al., 2003). Hence, researchers are eager to find effective therapeutic methods for toxoplasmosis.

No new drugs have become available in recent years to eliminate *T. gondii* growth in infected animals, but some progress has been made in vaccine research related to *T. gondii* disease in some animal species (Petersen, 2007; Zhang et al., 2013). Nevertheless, there is currently only 1 licensed vaccine for use against toxoplasmosis in sheep and goats and no vaccine for use in humans, likely because of the known side effects and high costs (Kur et al., 2009). To overcome these problems, great effort has

been directed towards DNA vaccine development, which is considered to induce specific antibody and T-cell responses against *T. gondii*, as well as providing protection in animal models (Yuan et al., 2011; Cui et al., 2012). Potential vaccine candidate antigens that have been screened include surface antigens (SAGs) (Angus et al., 2000), dense-granule antigens (Golkar et al., 2007), rhoptry antigens (Mishima et al., 2002), and microneme proteins (MICs) (Liu et al., 2010). The results of these studies are encouraging in that they have shown these proteins can provide long-lasting protection in mice against the *T. gondii* RH strain.

Protein phosphatase 2C (PP2C), a rhoptry secretory protein, is released into the cytoplasm of host cells and then translocated to host nuclei during infection (Gilbert et al., 2007), where it possibly interferes with cell survival and apoptosis regulation (Tamura et al., 2006). Some studies have analyzed the physiological functions of PP2C in host cells and apoptosis regulation in HeLa cells, concluding that it possibly interacts with structure-specific recognition protein 1 to enhance its activity (Gao et al., 2014). However, to date, no published studies have reported on the screening of PP2C as a potential antigen, and therefore a study aimed at confirming its antigenicity is a worthy undertaking. Here, we analyzed the protein structure and biological character-



istics of PP2C based on bioinformatics analyses. We also constructed a recombinant PP2C eukaryotic expression plasmid (pEGFP-PP2C) and assessed its ability to protect mice from infection with the RH strain of *T. gondii*.

MATERIALS AND METHODS

Modular architecture identification

The secondary structure of PP2C (EF450457.1) was analyzed using DNASTAR_Lasergene v. 7.1 software (Lu et al., 2015). The TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) was used for transmembrane domain prediction, SignalP software (<http://www.cbs.dtu.dk/services/SignalP/>) was used for signal peptide prediction, and SWISS-MODEL (<https://swissmodel.expasy.org/>) was used for 3-dimensional (3D) protein modeling.

Predicting potential B-cell epitopes in PP2C

The PP2C B-cell amino-acid sequence was analyzed using the DNASTAR software package. We used the PROTEIN subroutine of the algorithms from Garnier–Robson (Creighton, 1990) and Chou and Fasman (Chou and Fasman, 1978) for predicting alpha, beta, and turn regions, the Garnier–Robson algorithm for predicting coil regions, the Kyte and Doolittle (Kyte and Doolittle, 1982) algorithm for predicting hydrophilicity, the Karplus–Schultz (Wang et al., 2014) algorithm for predicting protein flexibility, the Emini (Emini et al., 1985) algorithm for surface probability prediction, and the Jameson–Wolf (Jameson and Wolf, 1988) algorithm for predicting antigenicity in PP2C. Epitopes with good hydrophilicity, high accessibility, high flexibility, and strong antigenicity were selected as the most advantageous epitopes. DNAMAN software (Lu et al., 2015) was used to predict the binding scores of the linear B-cell epitopes on PP2C for screening.

Identification of the T-cell epitopes

Because *T. gondii* is an intracellular parasite, T-cell-mediated immune responses in its hosts must play an important role against it. Currently, some programs exist for predicting proteins that induce T-cell immune responses. The most common approach for identifying candidate antigens is to predict binding scores. It has been established that the major histocompatibility complex II (MHC II) contains the most polymorphic molecules known to exist (Williams, 2001), and identifying high-affinity peptides that bind specific MHC II alleles is important in vaccine research (Tong et al., 2007). The online Immune Epitope Database (IEDB) service (<http://tools.iedb.org/mhcii/>) was used to predict MHC II molecules for PP2C.

Animal experiments and *T. gondii* parasites

BALB/c mice aged 6–8 wk, purchased from Shandong University Laboratory Animal Center (China), were maintained under standard conventional conditions. *Toxoplasma gondii* tachyzoites of the virulent wild-type RH strain (type I) were harvested from human foreskin fibroblast cells. Soluble tachyzoite antigens were obtained from *T. gondii* tachyzoites after centrifugation and resuspension in sterile phosphate-buffered saline (PBS).

PCR amplification, cloning, and sequencing of the PP2C gene

The coding sequence of the PP2C gene (GenBank: EF450457) was PCR-amplified from genomic parasite DNA using the following specific primer pair: Forward: 5'-CGGGGTAC-CATGGCTTCGTTAAGAGT-3' (*Kpn* I site underlined), reverse: 5'-CG GGATCCTCATGGATAATACAATAAG-3' (*Bam*H I site underlined). Total RNA from RH tachyzoites was extracted using the TRIzol reagent (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. The RNA was reverse transcribed into c-DNA using the RevertAid First Strand c-DNA Synthesis Kit (Han et al., 2017). The whole PP2C gene was amplified using a pair of specific primers according to the manufacturer's instructions, which introduced specific restriction enzyme sites into the gene sequence. PCR amplification was performed under optimized conditions involving 1 cycle of 95 C for 5 min, and 30 cycles of 95 C for 30 sec, 56 C for 30 sec, and 72 C for 30 sec, followed by a 5 min extension at 72 C. The amplification product was electrophoretically detected on a 1.0% agarose gel.

The amplified PP2C cDNA was purified and cloned into the corresponding restriction enzyme sites in the pEGFP-C1 vector (4,731 bp) using T4 DNA ligase, thereby forming the recombinant plasmid pEGFP-PP2C. The pEGFP-PP2C plasmid was transfected into *Escherichia coli* cells, and recombinants were screened on Luria–Bertani medium plates containing ampicillin (100 mg/L) at 37 C for 24 hr. The recombinant plasmid was extracted using a Plasmid Purification kit (TaKaRa Biotechnology, Beijing, China), and its concentration was spectrophotometrically measured and the A260/A280 ratio obtained. The obtained plasmid preparation was diluted to a final concentration of 1 µg/µl in PBS and stored at –20 C.

Recombinant plasmid expression in HEK 293T cells

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml streptomycin/penicillin at 37 C in a humidified 5% CO₂ atmosphere. Before transfection, the HEK 293T cells were transferred to a 6-well plate. When the cell density reached 80–90%, the constructed eukaryotic expression plasmid (pEGFP-PP2C) or pEGFP-C1, an empty plasmid, was separately transfected into the HEK 293T cells with the assistance of lipofectamine TM 2000 reagent (Invitrogen). The cells were incubated in a humidified 5% CO₂ atmosphere at 37 C. After 6 hr, the old medium was exchanged for fresh medium containing 10% FBS, and the 6-well plates were returned to the cell incubator for 48 hr. The expression of green fluorescence-labeled PP2C protein was observed at 48 hr post-transfection under a blue laser.

PP2C protein expression from the HEK 293T cells was analyzed by western blotting experiments as follows. Recombinant protein was isolated on ice with RIPA Lysis Buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing 1 mM PMSF (phenylmethanesulfonyl fluoride) protease inhibitor, and the preparation was centrifuged at 13,000 g for 10 min. The protein-containing preparation was transferred onto a polyvinylidene difluoride membrane by electrophoresis at 60 V for 4 hr. The membrane was saturated for 2 hr with sealing fluid at room

temperature and then coated with anti-soluble tachyzoite antigen (STAg) mouse sera diluted 1:500 in saturation buffer as the first antibody, followed by incubation with horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG antibody (Sigma-Aldrich, St. Louis, Missouri) diluted 1:20,000 in saturation buffer as the secondary antibody.

Immunization and challenge infections

Female BALB/c mice were stochastically assigned to 3 groups (15 mice/group), and these mice were inoculated 3 times with 100 μ l of the purified pEGFP-PP2C vector, the empty vector (pEGFP-C1), or PBS, at 2 intervals weekly. Blood samples were collected from the mice in each group on the day before each immunization and stored at -20°C until further analysis.

Two weeks after the final immunization, 12 mice in each group were infected with *T. gondii* RH strain (1×10^4 tachyzoites) by intraperitoneal injection. The number of mice surviving was recorded every day.

Cytokine production

Three mice from each group were sacrificed 2 wk after the booster immunization, and the spleens were isolated. The splenocytes were cultured in flat-bottomed 96-well microtiter plates, as described in the lymphocyte proliferation assay. The cell-free supernatants were harvested, and the activities of IL-4 at 24 hr, IL-10 at 72 hr, and IFN- γ at 96 hr were assayed using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Minneapolis, Minnesota).

Antibody detection

The IgG, IgG1, and IgG2a antibody levels were measured by ELISA using *T. gondii* RH coating antigen (4 $\mu\text{g}/\text{mL}$) in the plate wells. Serum aliquots obtained from blood samples collected on days 13, 27, and 42 were preserved at -20°C until use. Briefly, 96-well plates were coated with STAg (10 $\mu\text{g}/\text{mL}$) at 4 $^{\circ}\text{C}$ overnight. The plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and blocked with 1% bovine serum albumin at room temperature for 2 hr. After washing, 100 μ l aliquots of HRP-conjugated goat anti-mouse IgG, IgG1, or IgG2a (SouthernBiotech, Birmingham, Alabama), diluted in PBST (1:1,000), were added to the wells, and the plates were incubated for 1 hr at 37 $^{\circ}\text{C}$. Aliquots (100 μ l) of substrate solution (pH 4.0; 1.05% citrate substrate buffer; 1.5% ABTS; 0.03% H_2O_2) were added to the wells, and the plates were incubated in the dark for 20 min. The optical density of each well was measured with an ELISA reader (BioTek, Winooski, Vermont) at 490 nm. All samples were analyzed 3 times.

Statistical analysis

GraphPad Prism 5.0 was used for the statistical analysis. Comparisons between groups were investigated by 1-way ANOVA analysis. The confidence interval data were used to calculate mouse survival times using the Kaplan–Meier method. Data were considered statistically significant when the *p* values were <0.05 and <0.01 .

RESULTS

Gene information

The gene sequence and predicted amino-acid composition of PP2C have been deposited in the National Center for Biotechnology Information Resources (GenBank EF450457). The full-length gene sequence of PP2C, 1,338 bp in length, encodes a predicted protein of 445 amino acids. The physical and chemical properties of the amino acids are as follows. The predicted molecular weight of PP2C is 48.36 kDa. Its theoretical isoelectric point is 9.40, indicating the alkaline nature of the final product. The instability index was calculated at 41.50, which classifies PP2C as an unstable protein (values above 40 predict instability). The aliphatic index was 77.62, and the grand average of hydropathicity (GRAVY) index was -0.203 (negative values indicate hydrophilic proteins).

Epitope prediction

The secondary structure of PP2C was predicted by DNASTAR Lasergene v. 7.1 software. As shown in Figure 1A, PP2C has 161 alpha helices, 90 extended strands, 151 random coils, and 43 beta turns. Coil regions and extended strands account for 53% of the residues in the whole protein. The secondary structure prediction indicated that PP2C is a potentially dominant antigen with possible antibody-binding ability.

The protein sequence was analyzed using bioinformatics tools to identify the presence of transmembrane helices and a signal peptide. The transmembrane helices and exposed regions in PP2C showed that its sequence contains only 1 transmembrane domain, and all the epitopes are located on its outside (Fig. 1B). Furthermore, the bioinformatics analysis indicated that PP2C only had a signal peptide of 22 amino-acid residues and was cleaved off during translocation of the protein across the membrane in the position between 22 and 23 (Fig. 1C). The predicted signal peptide sequence score was 0.9868, strongly indicating that there is a signal-peptide sequence in the amino-acid sequence. The bioinformatics analysis with signal-peptide and 1 transmembrane region suggested that PP2C is likely to be secreted into the extracellular environment.

3D model of PP2C

Regions in proteins that are potentially antigenic can be used for epitope prediction to assist understanding of their 3D structures, which can contribute to elucidating their structure–function relationships (Song et al., 2017). The SWISS-MODEL online service was used to predict the 3D structure of PP2C and offered the highest coverage in all templates (Fig. 1D). The templates with the highest quality were selected for model building. The selected mode showed 100% sequence identity with PP2C. The built template covered 82% of the protein from amino acids 79 to 432. The prediction indicated that the structure might play an important role in the infection process and the pathogenesis of *T. gondii*.

Linear B-cell epitope prediction

With its excellent antigenic index and surface probability index, SAG1 is regarded as the optimum candidate antigen in *T. gondii*. Compared with the hydrophilicity plots, flexible regions, antigenic index, and surface probability of SAG1, PP2C epitopes were respectively obtained using the Karplus–Schulz, Kyte–Doolittle,

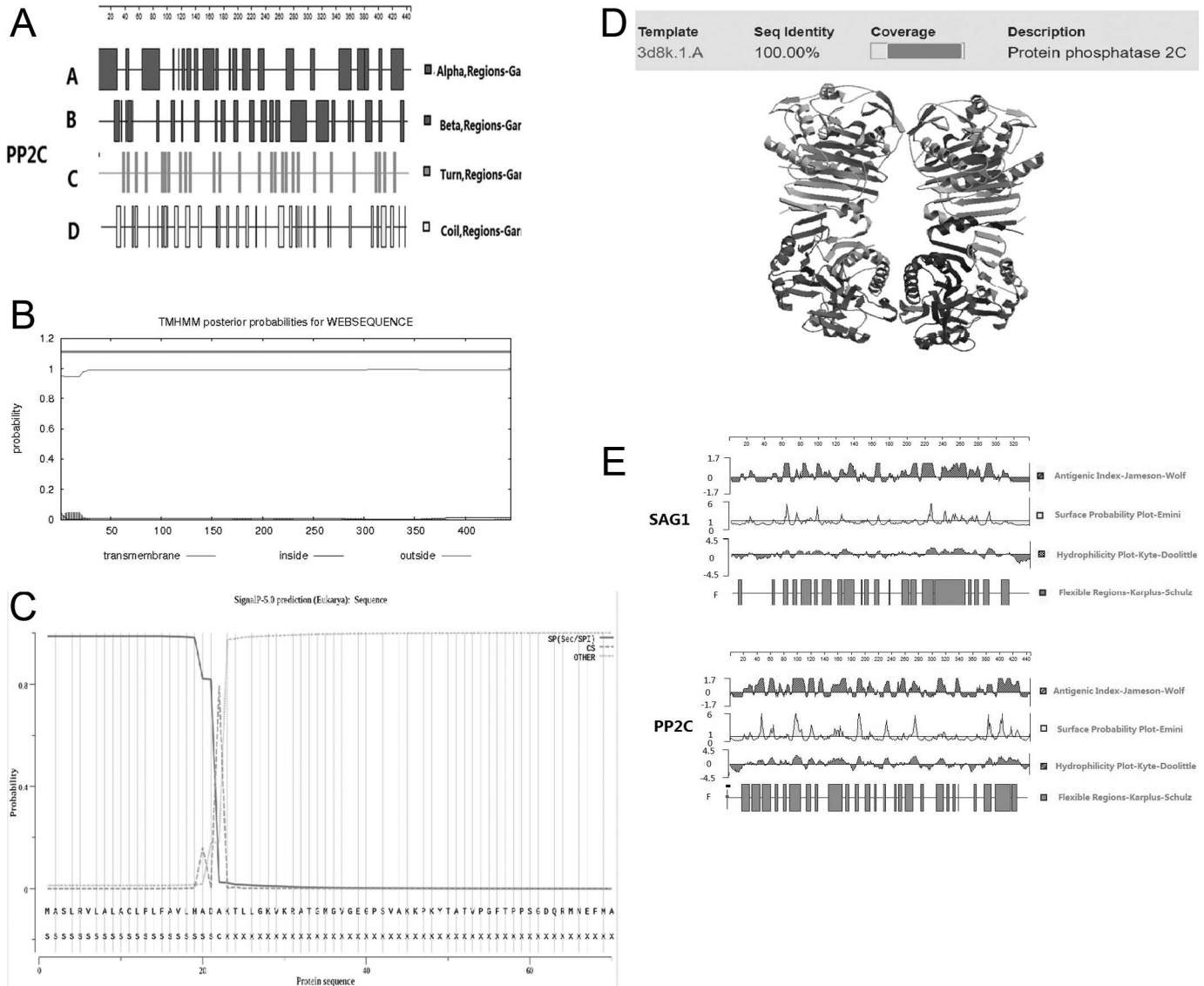


Figure 1. (A) Analysis of the secondary structure of protein phosphatase 2C (PP2C) based on DNASTAR software analysis. (B) Analysis of the transmembrane domain of PP2C. The graphical output from TMHMM shows the posterior probabilities for the transmembrane regions. One transmembrane region is predicted. (C) Signal peptide prediction for PP2C. The cleavage site was predicted to occur between amino-acid positions 22 and 23 in the protein sequence. (D) 3D model of PP2C. The protein sequence was sent to SWISS-MODEL via the website at <https://swissmodel.expasy.org/>. (E) Linear B-cell peptides for SAG1 and PP2C, as predicted by DNASTAR, along with antigenic index, hydrophilicity plots, flexible regions, and surface probability rules.

Emini, and Jameson–Wolf algorithms in DNASTAR software. As shown in Figure 1E, as compared with SAG1, PP2C has a better antigenic index and a greater number of flexible regions. The surface probability and flexible region scores were also higher. DNAMAN software was also utilized to search for potential epitopes in PP2C with the highest antigen index scores, the results of which are shown in Table I.

T-cell epitope identification

The online IEDB (<http://tools.iedb.org/mhcii/>) service was used to evaluate the binding affinity strengths of the PP2C epitopes for the different MHC class II molecules. The half-maximal inhibitory concentration values for the peptides were predicted using the IEDB service. The bioinformatics analyses reported that

the increases in the binding force could effectively promote more T-cell differentiation into Th-1 cells (Singh and Srivastava, 2003). HLA-DRB1×01:01, H2-IAb, H2-IAd, and H2-IEd alleles were screened to compare PP2C with SAG1, and the minimum percentile ranks for each MHC II allele were chosen and are listed in Table II. The percentile ranks of HLA-DRB1×01:01, H2-IAb, H2-Iad, and H2-Ied for PP2C were much lower than those for the SAG1 protein, indicating that PP2C has potential to induce Th-1 cell-mediated immune responses.

Verification of the pEGFP-PP2C eukaryotic expression plasmid

The PP2C gene was ligated to the pEGFP-C1 eukaryotic expression vector to construct a new plasmid using specific

Table I. Analysis of linear B-cell antigen epitopes on Tg-PP2C.*

Order	Position	Sequence	Score†
1	3–22	SLRVLALACLPLFAVLHADA	1.15
2	322–341	QQDLVSAVPDVTTEFAYPGD	1.092
3	352–371	RSHAAIAAAIALYPVSPETV	1.088
4	180–199	SSALIIFVRYEKKPTERAVV	1.069
5	42–61	SVAKKPKYTATVPGFTPPSG	1.035
6	71–90	VDTSGEFMRHLYIEEGRTVC	1.025
7	93–112	ATSRNRRPTSESPHSDDVVV	1.014
8	180–199	SSALIIFVRYEKKPTERAVV	1.069
9	124–143	RVHAMFDGFGQRHSAMWLAQ	1.008

* The prediction was run 3 times. Two or more amino acids condense into a peptide.

† High score = high-level binding.

restriction enzymes. Figure 2A shows the restriction enzyme analysis for the new plasmid. To further verify the sequence of the PP2C gene, the constructed plasmids were sequenced commercially (Shenggong, Shanghai, China) and found to share 100% sequence identity with the wild-type gene.

Expression of the PP2C gene in HEK 293T cells

After successfully transfecting the pEGFP-PP2C expression vector into HEK 293T cells, PP2C protein expression was detected by fluorescence microscopy. As shown in Figure 2B, cells transfected with pEGFP-PP2C or pEGFP-C1 (empty vector control) emitted green fluorescence upon exposure to a blue laser, whereas no fluorescence was observed in the blank control group's cells.

Protein expression of pEGFP-PP2C and pEGFP-C1 in the transfected cells was determined by western blot analysis, and, as shown in Figure 2C, a specific protein band (about 48 kDa) was recognized in the pEGFP-PP2C-transfected cells by incubating them with STAg mouse sera (lane 2), whereas the negative control cells transfected with the empty pEGFP-C1 vector showed no specific bands when incubated with the same mouse sera (lane 1).

Table II. IC₅₀ values for PP2C binding to MHC class II molecules obtained using the immune epitope database.*

MHC II allele†	Start–stop‡		Percentile rank§	
	SAG1	PP2C	SAG1	PP2C
HLA-DRB1*01:01	12–26	345–359	0.88	0.25
	35–49	346–360	2.74	0.47
H2-IAb	26–40	347–361	2.15	1.28
	297–313	348–362	2.81	1.28
H2-IAd	21–35	348–362	0.34	0.01
	168–182	352–366	1.22	0.01
H2-IEd	14–28	375–389	18.45	12.36
	34–48	376–390	30.62	12.91

* The immune epitope database (<http://tools.immuneepitope.org/mhcii>). The prediction was run 3 times.

† H2-IAb, H2-IAd, and H2-IEd alleles are mouse MHC class II molecules; the HLA-DRB1*01:01 allele is a human MHC class II molecule.

‡ We chose 15 amino acids for analysis each time.

§ Low percentile rank = high-level binding, high percentile rank = low-level binding, IC₅₀ values.

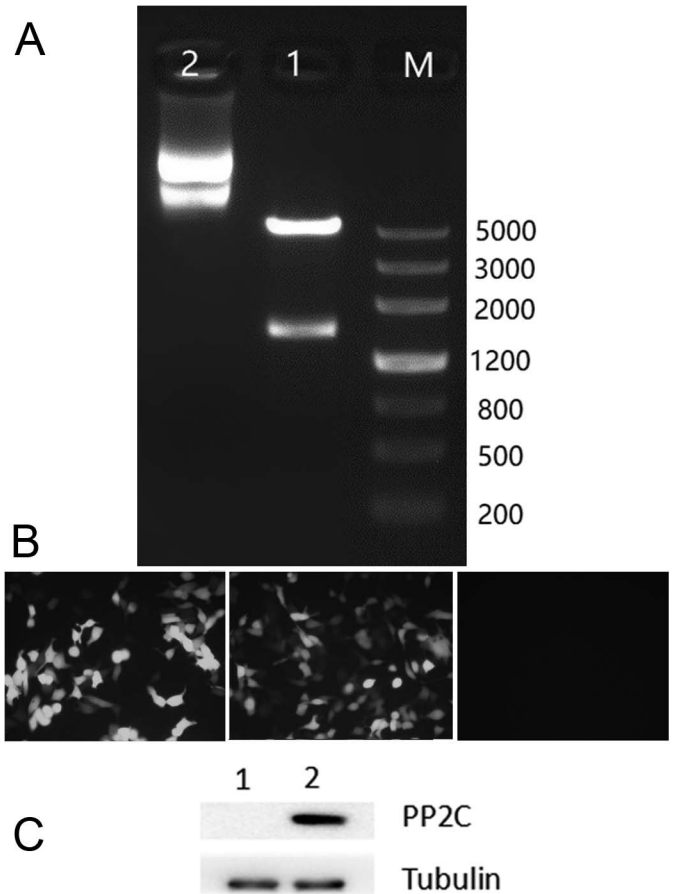


Figure 2. (A) Verification of the pEGFP-PP2C recombinant eukaryotic expression plasmid by PCR amplification and restriction enzyme digestion analysis, with DNA marker (lane M), pEGFP-PP2C digested with *Bam*HI and *Kpn*I (lane 1), and recombinant circular plasmid pEGFP-PP2C (lane 2) indicated. (B) Direct immunofluorescence detection of the pEGFP-PP2C fusion protein in transfected HEK 293T cells. Cells transfected with pEGFP-PP2C (left) were detected under blue light, as were cells transfected with pEGFP-C1 (control, center) and untransfected cells (right). The excitation wavelength was 490 nm. The emission wavelength was 520 nm. (C) Western blotting analysis of protein expression recognized by anti-STAg mouse sera, with protein marker in lane M. The pEGFP-C1 plasmid-transfected cells (control) did not react with anti-STAg mouse sera (lane 1), whereas the recombinant protein from the pEGFP-PP2C plasmid-transfected cells did react with the anti-STAg mouse sera (lane 2).

T. gondii-specific serum antibody response

To evaluate humoral immune response levels against PP2C expressed from pEGFP-PP2C, serum samples were collected from the different groups, and anti-*T. gondii* IgG antibodies were detected by ELISA. As shown in Figure 3A, significantly higher levels of IgG antibodies were observed in the pEGFP-PP2C group than in the other groups ($p < 0.05$). However, there were no differences in the levels of specific antibodies in the pEGFP-C1 and PBS groups ($p > 0.05$).

To investigate the types of immune responses generated in the mice (Th1 and/or Th2), the distribution of the IgG subclasses (IgG1 and IgG2a) was analyzed after the last immunization. As shown in Figure 3B, the serum IgG2a level in the pEGFP-PP2C-

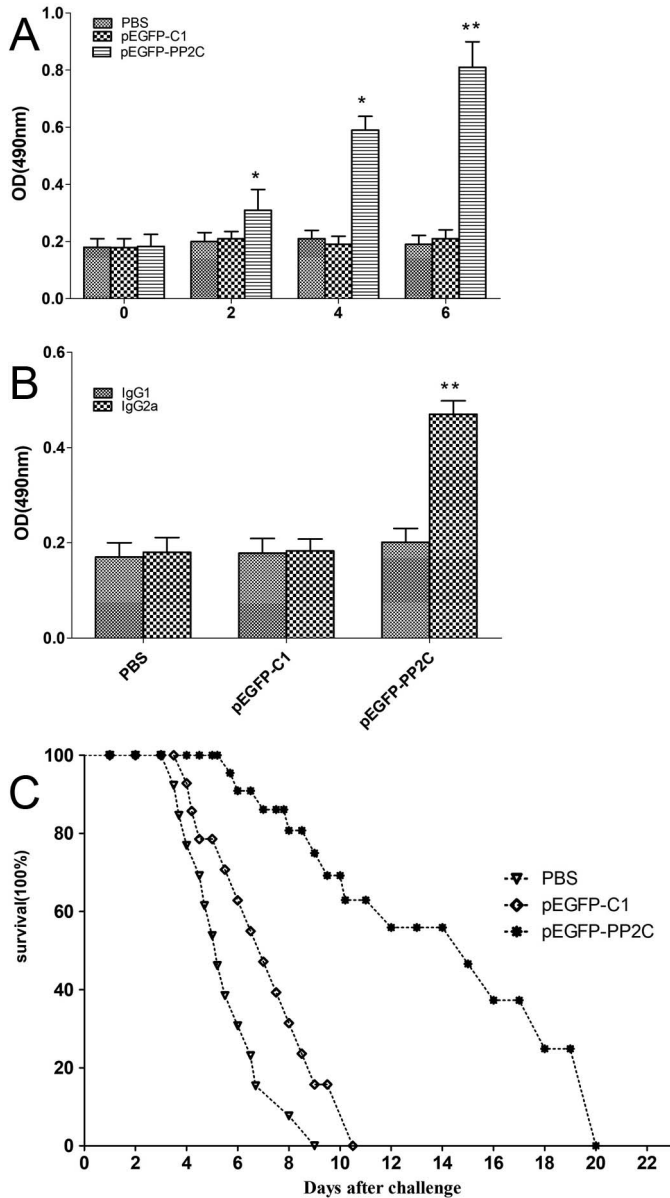


Figure 3. (A) IgG antibody levels were evaluated in serum samples from the immunized mice. Serum samples were collected from the caudal vein of each mouse at 0, 2, 4, and 6 wk post-immunization. Results are expressed as the mean optical density (OD) 490 values from the samples. All the samples were run 3 times. *Compared with PBS or the pEGFP-C1 group, $P < 0.05$. **Compared with PBS or the pEGFP-C1 group, $P < 0.01$. (B) Polyclonal antibody isotypes (IgG1 and IgG2a) in the immunized mice were determined and analyzed by ELISA. **Compared with PBS or pEGFP-C1 group, $P < 0.01$. (C) Three groups of mice were challenged with 1×10^4 tachyzoites of the virulent *Toxoplasma gondii* RH strain 2 wk after their last immunization. Each group contained 12 mice, and the survival time of each group was monitored daily for 20 days post-challenge.

immunized mice was significantly higher in the pEGFP-PP2C group than in the other groups, suggesting that a predominantly Th1-type immune response had been induced. No statistical differences in IgG2a levels between the pEGFP-C1 and PBS groups were found.

Table III. Cytokine production by splenocyte* cultures from immunized BALB/c mice.

Groups	Cytokine production (pg/ml)†		
	IFN- γ	IL-4	IL-10
PBS	36.28 \pm 2.32	39.39 \pm 2.49	41.76 \pm 2.39
pEGFP-C1	39.58 \pm 2.49	49.11 \pm 3.08	45.92 \pm 3.29
pEGFP-PP2C	652.54 \pm 35.72*	59.48 \pm 4.31	51.43 \pm 3.72

* Splenocytes from 3 mice per group 2 wk after the final immunization.
 † Values for IFN- γ at 96 hr, IL-4 at 24 hr, and IL-10 at 72 hr are expressed as mean \pm standard deviation.
 ‡ Compared with PBS, pEGFP-C1 groups, $P < 0.05$.

Cytokine production

To further explore T-cell responses to vaccination, IL-4, IL-10, and IFN- γ cytokines were analyzed by ELISA. As shown in Table III, significant increases in IFN- γ levels were observed in the pEGFP-PP2C-immunized group compared with the 2 control groups ($p < 0.05$). In contrast, IL-4 and IL-10 levels in samples from the pEGFP-PP2C group did not differ statistically from those of the 2 control groups ($p > 0.05$).

Assessing the protective efficacy of PP2C

To investigate the protective efficacy against *T. gondii* infection induced by immunization with pEGFP-PP2C, 12 immunized mice from all the groups were intraperitoneally challenged with 1×10^4 tachyzoites 2 wk after their last immunizations. Figure 3C shows that the survival times of mice immunized with pEGFP-PP2C were prolonged (20 ± 2.89 days) in comparison with the 2 control groups. In contrast, no significant difference was observed between the pEGFP-C1 and PBS groups. All the mice died within 10 days of the challenge.

DISCUSSION

Bioinformatics, which occupies an interdisciplinary field in science, has proved to be useful for analyzing protein structures, epitopes, and potential protein functions with the aid of software and technologies from mathematics, statistics, computer sciences, physics, and, of course, biology and medicine (Romano et al., 2011; Wang et al., 2013a). Compared with traditional methods, bioinformatics offers researchers some distinct advantages in their studies on genes and biological molecules, including removing any preexisting bias as to a protein’s function, improving the accuracy of epitope prediction, and above all else, substantially decreasing the number of laboratory experiments required to characterize such molecules (Bai et al., 2012; Wang et al., 2013b, 2014). In the present study, we conducted a comprehensive analysis of PP2C using bioinformatics. The amino acid sequence of PP2C contains 445 amino acids and a molecular weight (MW) of 48.36 kDa, suggesting it has an antigenic nature because proteins with MWs below 5–10 kDa are considered poorly immunogenic. The aliphatic index and GRAVY value for the PP2C amino-acid sequence were predicted to be 82.90 and -0.391 , respectively, indicating that PP2C is stable across a wide range of temperatures, and its high aliphatic index and hydrophilic properties suggest its antigenic potency.

Alpha helices, one of the spatial conformation characteristics of a protein, have high chemical bond energies and are mainly located within the inner parts of the protein, making them difficult to interact with antibodies. Conversely, random coils and beta turns show more deformational flexibility and are located on the surface of the protein, indicating many as potential epitopes (Zhang et al., 2011). Our analysis showed that 53% of PP2C contained coiled regions and extended strands, again suggesting it has strong antigenic potential. Signal peptides target proteins towards the secretory pathway and can guide their final localization to the cytoplasm, cell membrane, or extracellular space, where their cognate proteins can perform important functions (e.g., structural proteins, virulence factors, and secretory/excretory antigens) (Emanuelson et al., 2007). We identified an N-terminal signal sequence in PP2C, and, with a score of 0.9868, this sequence may be indicative of a transmembrane domain. In addition, the TMHMM Server v.2.0 software analysis showed that the predicted PP2C epitopes are located on the outside of the protein, suggesting that PP2C is an extracellular protein, a finding consistent with the results from previous research (Gilbert et al., 2007).

We used the Garnier–Robson and Chou–Fasman algorithms to predict the secondary structure of PP2C based on the sequence of the PP2C gene. As far as is known, SAG1 can induce effective humoral and cellular immune responses in mice, one of the criteria supporting *T. gondii* DNA vaccine research on this protein domain (Constant et al., 1995). We analyzed B-cell and T-cell epitopes for PP2C using the aforementioned software. Compared with SAG1, PP2C was predicted to contain more potential B-cell epitopes, suggesting its potential as a vaccine candidate. In addition, the T-cell epitopes were analyzed using the online IEDB service; lower numbers were identified but with a suggested higher affinity, indicating they are stronger epitopes. As shown in Table II, PP2C has a high probability to be an excellent T-cell antigen when compared with SAG1. The direction of T-cell differentiation is affected by the binding strength of the interaction: As the binding force increases, cells are more likely to differentiate into Th-1 types (Constant et al., 1995). Our analysis of T-cell epitopes on PP2C indicated that this protein is likely to induce a Th-1 cell-mediated immune response. In short, PP2C appears to have potentially strong antigenicity, with the potential also to induce strong cellular and humoral immune responses, thereby supporting its candidacy as a DNA vaccine against toxoplasmosis.

Based on the results of our bioinformatics analyses, we conducted immunization and challenge infection experiments to investigate immune responses against PP2C in mice. Infection with *T. gondii* generally induces strong cellular and humoral immune responses. Specific IgG antibodies can protect humans from infection and resist secondary invasion (Kang et al., 2000). In our study, IgG was induced at high titers in the mice immunized with pEGFP-PP2C, in contrast with the control groups. High IgG2a to IgG1 antibody ratios were also detected in the samples from the pEGFP-PP2C-immunized mice, illustrating that humoral immunoreactions were oriented towards a Th1 profile in these animals. Cytokines (IFN- γ , IL-4, IL-10) play a key role in targeting *T. gondii* during infections (Benson et al., 2012). Interestingly, IFN- γ has been found to play a major role in resistance to *T. gondii* by contributing to tryptophan degradation

in infected cells and killing the parasites (Sonaimuthu et al., 2016). In the present study, IFN- γ levels were significantly higher in the pEGFP-PP2C-immunized group, unlike IL-4 and IL-10 levels from Th2 cells, which were the same as those of the control groups. Therefore, we conclude that a Th1-type cell immune response was mainly activated in the mice after immunization with pEGFP-PP2C.

Finally, the survival rates of the immunized mice challenged with a lethal dose (1×10^4) of tachyzoites were evaluated. In the control groups, mice died within 10 days post-challenge, while those immunized with pEGFP-PP2C survived for significantly longer (20 days). This shows that immunization with pEGFP-PP2C significantly extended the survival time of the BALB/c mice when compared with the controls. Collectively, these results reveal that the PP2C protein can, as bioinformatically predicted, induce partially effective protection in mice against acute infections with *T. gondii*.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the National Natural Science Foundation of China (Grant 81971968), the State Key Laboratory of Veterinary Etiological Biology (Grant SKLVEB2011KFKT005), the Shandong Provincial Natural Science Foundation (Grant ZR2009CM079), the Quzhou Guiding Projects (Grant 2019122), and Key Scientific Research Projects at school level (Grant QZYZ1905). S.H. conceived the idea for the study and critically revised the manuscript. P.S. drafted and finalized the manuscript. S.Y., J.Z., Y.Y., Y.C., and Q.L. performed the experiments. All authors read and approved the final manuscript.

LITERATURE CITED

- ANGUS, C. W., D. KLIVINGTON-EVANS, J. P. DUBEY, AND J. A. KOVACS. 2000. Immunization with a DNA plasmid encoding the SAG1 (P30) protein of *Toxoplasma gondii* is immunogenic and protective in rodents. *Journal of Infectious Diseases* 181: 317–324.
- ASPINALL, T. V., E. C. GUY, K. E. ROBERTS, D. H. JOHNSON, J. E. HYDE, AND P. F. SIMS. 2003. Molecular evidence for multiple *Toxoplasma gondii* infections in individual patients in England and Wales: Public health implications. *International Journal for Parasitology* 33: 97–103.
- BAI, Y., S. HE, G. ZHAO, L. CHEN, N. SHI, H. ZHOU, H. CONG, Q. ZHAO, AND X. Q. ZHU. 2012. *Toxoplasma gondii*: Bioinformatics analysis, cloning and expression of a novel protein TgIMP1. *Experimental Parasitology* 132: 458–464.
- BENSON, A., S. MURRAY, P. DIVAKAR, N. BURNAEVSKIY, R. PIFER, J. FORMAN, AND F. YAROVINSKY. 2012. Microbial infection-induced expansion of effector T cells overcomes the suppressive effects of regulatory T cells via an IL-2 deprivation mechanism. *Journal of Immunology* 188: 800–810.
- CHOU, P. Y., AND G. D. FASMAN. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Advances in Enzymology and Related Areas of Molecular Biology* 47: 45–148.
- CONSTANT, S., C. PFEIFFER, A. WOODARD, T. PASQUALINI, AND K. BOTTOMLY. 1995. Extent of T cell receptor ligation can

- determine the functional differentiation of naive CD4+ T cells. *Journal of Experimental Medicine* 182: 1591–1596.
- CREIGHTON, T. E. 1990. Prediction of protein structure and the principles of protein conformation. In G. D. Fasman (ed.). Plenum Publishing Corporation, New York, New York, 798 p.
- CUI, X., T. LEI, D. YANG, P. HAO, B. LI, AND Q. LIU. 2012. *Toxoplasma gondii* immune mapped protein-1 (TgIMP1) is a novel vaccine candidate against toxoplasmosis. *Vaccine* 30: 2282–2287.
- DUBEY, J. P., AND J. L. JONES. 2008. *Toxoplasma gondii* infection in humans and animals in the United States. *International Journal for Parasitology* 38: 1257–1278.
- EMANUELSSON, O., S. BRUNAK, G. VON HEIJNE, AND H. NIELSEN. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. *Nature Protocols* 2: 953–971.
- EMINI, E. A., J. V. HUGHES, D. S. PERLOW, AND J. BOGER. 1985. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *Journal of Virology* 55: 836–839.
- GAO, X. J., J. X. FENG, S. ZHU, X. H. LIU, I. TARDIEUX, AND L. X. LIU. 2014. Protein phosphatase 2C of *Toxoplasma gondii* interacts with human SSRP1 and negatively regulates cell apoptosis. *Biomedical and Environmental Sciences* 27: 883–893.
- GILBERT, L. A., S. RAVINDRAN, J. M. TURETZKY, J. C. BOOTHROYD, AND P. J. BRADLEY. 2007. *Toxoplasma gondii* targets a protein phosphatase 2C to the nuclei of infected host cells. *Eukaryotic Cell* 6: 73–83.
- GOLKAR, M., M. A. SHOKRGOZAR, S. RAFATI, K. MUSSET, M. ASSAMAR, R. SADAIE, M. F. CESBRON-DELAUW, AND C. MERCIER. 2007. Evaluation of protective effect of recombinant dense granule antigens GRA2 and GRA6 formulated in monophosphoryl lipid A (MPL) adjuvant against *Toxoplasma* chronic infection in mice. *Vaccine* 25: 4301–4311.
- HAN, Y., A. ZHOU, G. LU, G. ZHAO, L. WANG, J. GUO, P. SONG, J. ZHOU, H. ZHOU, H. CONG, ET AL. 2017. Protection via a ROM4 DNA vaccine and peptide against *Toxoplasma gondii* in BALB/c mice. *BMC Infection Disease* 17: 59.
- JAMESON, B. A., AND H. WOLF. 1988. The antigenic index: A novel algorithm for predicting antigenic determinants. *Computer Applications in the Biosciences* 4: 181–186.
- KANG, H., J. S. REMINGTON, AND Y. SUZUKI. 2000. Decreased resistance of B cell-deficient mice to infection with *Toxoplasma gondii* despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase. *Journal of Immunology* 164: 2629–2634.
- KUR, J., L. HOLEC-GASIO, AND E. HISZCZYNSKA-SAWICKA. 2009. Current status of toxoplasmosis vaccine development. *Expert Review of Vaccines* 8: 791–808.
- KYTE, J., AND R. F. DOOLITTLE. 1982. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 157: 105–132.
- LIU, M. M., Z. G. YUAN, G. H. PENG, D. H. ZHOU, X. H. HE, C. YAN, C. C. YIN, Y. HE, R. Q. LIN, H. Q. SONG, ET AL. 2010. *Toxoplasma gondii* microneme protein 8 (MIC8) is a potential vaccine candidate against toxoplasmosis. *Parasitology Research* 106: 1079–1084.
- LU, G., L. WANG, A. ZHOU, Y. HAN, J. GUO, P. SONG, H. ZHOU, H. CONG, Q. ZHAO, AND S. HE. 2015. Epitope analysis, expression and protection of SAG5A vaccine against *Toxoplasma gondii*. *Acta Tropica* 146: 66–72.
- MISHIMA, M., X. XUAN, N. YOKOYAMA, I. IGARASHI, K. FUJISAKI, H. NAGASAWA, AND T. MIKAMI. 2002. Recombinant feline herpesvirus type 1 expressing *Toxoplasma gondii* ROP2 antigen inducible protective immunity in cats. *Parasitology Research* 88: 144–149.
- PETERSEN, E. 2007. Prevention and treatment of congenital toxoplasmosis. *Expert Review Anti Infection Therapy* 5: 285–293.
- REICH, M., M. RUPPENSTEIN, M. D. BECKER, AND F. MACKENSEN. 2015. Risk of recurrence of preexisting ocular toxoplasmosis during pregnancy. *Ocular Immunology and Inflammation* 23: 240–245.
- ROMANO, P., R. GIUGNO, AND A. PULVIRENTI. 2011. Tools and collaborative environments for bioinformatics research. *Briefings in Bioinformatics* 12: 549–561.
- SINGH, M., AND I. SRIVASTAVA. 2003. Advances in vaccine adjuvants for infectious diseases. *Current HIV Research* 1: 309–320.
- SONAIMUTHU, P., X. T. CHING, M. Y. FONG, R. KALYANASUNDARAM, AND Y. L. LAU. 2016. Induction of protective immunity against toxoplasmosis in BALB/c mice vaccinated with *Toxoplasma gondii* Rhopty-1. *Frontiers in Microbiology* 7: 808. doi:10.3389/fmicb.2016.00808.
- SONG, P., S. HE, A. ZHOU, G. LV, J. GUO, J. ZHOU, Y. HAN, H. ZHOU, Z. HAO, AND H. CONG. 2017. Vaccination with toxofilin DNA in combination with an alum-monophosphoryl lipid A mixed adjuvant induces significant protective immunity against *Toxoplasma gondii*. *BMC Infectious Diseases* 17: 19. doi:10.1186/s12879-016-2147-1.
- TAMURA, S., S. TORIUMI, J. SAITO, K. AWANO, T. A. KUDO, AND T. KOBAYASHI. 2006. PP2C family members play key roles in regulation of cell survival and apoptosis. *Cancer Science* 97: 563–567.
- TONG, J. C., T. W. TAN, AND S. RANGANATHAN. 2007. Methods and protocols for prediction of immunogenic epitopes. *Briefing in Bioinformatics* 8: 96–108.
- VIJAYKUMAR, B. R., S. U. LEKSHMI, R. SAI KANT, D. VAIGUNDAN, A. MAHADEVAN, C. RAJENDRAN, S. K. SHANKAR, AND R. S. JAYSHREE. 2016. Genetic characterization of *Toxoplasma gondii* from autopsy proven cases of AIDS associated cerebral toxoplasmosis in South India. *Infection, Genetics and Evolution* 39: 106–112.
- WANG, Y., G. WANG, J. OU, H. YIN, AND D. ZHANG. 2014. Analyzing and identifying novel B cell epitopes within *Toxoplasma gondii* GRA4. *Parasites & Vectors* 7: 474. doi:10.1186/s13071-014-0474-x.
- WANG, Y., G. WANG, D. ZHANG, H. YIN, AND M. WANG. 2013a. Screening and identification of novel B cell epitopes of *Toxoplasma gondii* SAG1. *Parasites & Vectors* 6: 125. doi:10.1186/1756-3305-6-125.
- WANG, Y., G. WANG, D. ZHANG, H. YIN, AND M. WANG. 2013b. Identification of novel B cell epitopes within *Toxoplasma gondii* GRA1. *Experimental Parasitology* 135: 606–610.
- WILLIAMS, T. M. 2001. Human leukocyte antigen gene polymorphism and the histocompatibility laboratory. *Journal of Molecular Diagnostics* 3: 98–104.
- YUAN, Z. G., X. ZHANG, R. Q. LIN, E. PETERSEN, S. HE, M. YU, X. H. HE, D. H. ZHOU, Y. HE, H. X. LI, ET AL. 2011. Protective

- effect against toxoplasmosis in mice induced by DNA immunization with gene encoding *Toxoplasma gondii* ROP18. *Vaccine* 29: 6614–6619.
- ZHANG, J., Z. SHANG, X. ZHANG, AND Y. ZHANG. 2011. Modeling and analysis of *Schistosoma* Argonaute protein molecular spatial conformation. *Asian Pacific Journal of Tropical Biomedicine* 1: 275–278.
- ZHANG, N. Z., J. CHEN, M. WANG, E. PETERSEN, AND X. Q. ZHU. 2013. Vaccines against *Toxoplasma gondii*: New developments and perspectives. *Expert Review of Vaccines* 12: 1287–1299.