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Discovery of new *Toxoplasma gondii* antigenic proteins using a high throughput protein microarray approach screening sera of murine model infected orally with oocysts and tissue cysts

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Abstract

Background: *Toxoplasma gondii* is an obligate intracellular protozoan parasite that causes congenital toxoplasmosis, as well as other serious clinical presentations in immune compromised humans. The parasite has also been recently linked to behavioral diseases in humans and other mammalian hosts. New antigens are being evaluated to develop a diagnostic kit for the diagnosis of acute infection or a protective vaccine.

Methods: In this study, we have focused on the discovery of new antigenic proteins from *T. gondii* genomic data using a high throughput protein microarray screening. To date, microarrays containing > 2870 candidate exon products of *T. gondii* have been probed with sera collected from patients with toxoplasmosis. Here, the protein microarrays are probed with well-characterized serum samples from animal models administered orally with oocysts or tissue cysts. The aim was to discover the antigens that overlap in the mouse profile with human antibody profiles published previously. For this, a reactive antigen list of 240 antigens recognized by murine IgG and IgM was identified using pooled sera from orally infected mice.

Results: Analyses of screening data have identified plenty of antigens and showed strong immunogenicity in both mouse and human antibody profiles. Among them, ROP1, GRA2, GRA3, GRA4, GRA5, GRA6, GRA7, GRA8, GRA14, MIC1, MIC2 and MAG1 have shown strong immunogenicity and used as antigen in development of vaccines or serological diagnostic assays in previous studies.

Conclusion: In addition to the above findings, ROP6, MIC12, SRS29A and SRS13 have shown strong immunogenicity but have not been tested in development of a diagnostic assay or a vaccine model yet.

Keywords: *Toxoplasma gondii*, Oocysts, Sporozoite, Tissue cyst, Bradyzoite, Protein microarray, Antigen discovery, Vaccine, Diagnostics

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Background

Toxoplasma gondii is a protozoan parasite that has a widespread distribution worldwide among humans and animals [1]. The importance of this intracellular parasite comes from the clinical presentations formed in the fetus and in immune compromised patients. Infection of women early in pregnancy is associated with an increased risk of congenital toxoplasmosis *in utero* and may become lethal by dissemination to vital organs such as brain, heart or lungs. This may also occur in immunocompromised adults by therapeutic immunosuppression, such as in cancer chemotherapy or organ transplantation [1]. Recently, *T. gondii* has also been linked to behavioral syndromes such as schizophrenia or bipolar disorder [2].

The transmission of toxoplasmosis mainly occurs through contaminated water and foods [1]. Felidae cats are definitive hosts in which the production of oocysts occurs. Cats disseminate resistant oocysts in their feces that can be contagious up to 18 months in the environment. Humans, as well as other intermediate hosts, are often infected *via* domestic cats, but can also become infected by ingestion of tissue cysts in raw or under-cooked meat prepared from infected hosts.

Due to ease of transmission and resistance of oocysts in the environment, 1/3 of humans are estimated to be infected with *T. gondii* worldwide [3]. The rate of toxoplasmosis among pregnant women range between 37–58% in Europe [3], compared to 10.8% in the USA. [4]. In the USA, each year 400–4000 infants are estimated to be born with congenital toxoplasmosis, and up to 1.26 million cases of ocular toxoplasmosis are reported [3]. In addition, *T. gondii* oocysts are classified as a category B bioterrorism agents owing to a potential threat to water safety [5], as demonstrated by tainted water outbreaks that occurred in British Columbia, that affected 7718 people [6], Santa Isabel do Ivaí of Brazil that affected hundreds of people [7], Coimbatore, India that affected 178 people [8], and Izmir that affected 171 Air Force Academy recruits [9].

In addition to being the definitive host for *T. gondii*, abundance and close proximity of cats as a companion species in human populations plays an important role in transmission of infections to humans. According to the American Veterinary Medical Association (AVMA) and the European pet food industry Federation (FEDIAF) the total number of domestic cats in the USA is over 74 million, or approximately 1 cat per family of 4, and 74 million in the European Union [10, 11].

During acute toxoplasmosis in pregnant women, fetus can get infected by *T. gondii* and congenital

toxoplasmosis may occur. The first problem is misdiagnoses of acute toxoplasmosis in pregnant women during the first trimester due to the insufficiency of serological assays that cannot discriminate acute infection and chronic infection. Antigens specific for acute infection can be used in these serological assays for timely diagnosis of the disease. The other problem is that an efficient vaccine that can protect pregnant women against toxoplasmosis does not exist. Current diagnostic antigens that are used to develop diagnostic assays or vaccine candidate antigens are almost always selected randomly and/or based on biological properties such as being a surface protein, having a role in pathogenesis, or high immunogenicity [12, 13]. In this new era of advanced technology, methods to select antigens should specifically take advantage of recent advances such as *in silico* analysis and novel *in vitro* and *in vivo* immunoscreens for the generation of new antigens. The present study addresses this bottleneck in selection of antigenic proteins against toxoplasmosis using a high throughput protein microarray screening approach [14]. Initially, we have generated protein microarray chips containing 2870 candidate exon products of *T. gondii*. Then, we probed them with well grouped human sera [(i) acute (IgM- positive and low avidity IgG); (ii) chronic with IgM persistence (IgM-positive and high avidity IgG); (iii) chronic (IgM-negative and high avidity IgG); and toxoplasmosis negative] [15, 16]. There was a shortcoming in this approach that experimental infections are not possible in humans and we could not determine the antigens that were present at the beginning of the infection. This knowledge was important since these antigenic proteins can be very important to develop a diagnostic assay or a vaccine.

Following the two protein array studies in human toxoplasmosis, here we report a comprehensive *in vivo* study in a mouse model screened by protein microarrays. Mouse is an intermediate host with immune response similar to humans. For this purpose, two mouse groups were infected orally with oocysts and tissues cysts mimicking natural route of infection. Sera were collected prior to infection (Day 0) and at 1, 2, 3, 6, 10, 15, 40 and 120 days after infection. Then, protein microarray slides were probed with these sera to identify immunodominant exon products present in acute and chronic murine toxoplasmosis. Infecting the mouse groups with oocysts or tissue cysts also gave us the opportunity to determine the dominant antigens presented *via* the fecal-oral and ingestion routes of entry (i.e. mimicking natural route of infection).

Methods

Animals

Six to eight weeks-old female Swiss outbred mice were obtained from the Bornova Veterinary Control Institute Animal Production Facility. *Toxoplasma gondii* oocysts were obtained from a recently weaned kitten, approximately 3–4 months-old.

During *in vivo* studies, mice and cat were housed in different rooms under standard and suitable conditions. Specifically, rooms had ambient temperature and humidity, adequate light cycle, and diet was specific for each animal type. All animals were checked for humane endpoints every day such as rapid weight loss more than ~20% of gross body weight, inability to assess water or food, or loss of skin elasticity indicative of dehydration.

Obtaining tissue cysts and oocysts

A kitten was fed with *T. gondii* PRU strain tissue cysts obtained from mouse brain as described [17]. Initially, brains of previously infected mice were extracted under sterile conditions and homogenized in approximately 2 ml sterile 0.9% NaCl containing penicillin (10 U/ml), streptomycin (10 µg/ml) and gentamicin (2 µg/ml) using an injector with 20G 1" (0.9 × 25 mm) needle. The tissue cysts were counted by hemocytometer under phase contrast microscope (Nikon) and the remaining homogenate was used to passage the strain *in vivo*.

To feed the kitten, a part of the infected mouse brain was crushed between cover glass and slide and tissue cysts were examined under phase contrast microscope. The remaining brain with tissue cysts was fed to the cat. Before feeding the kitten, the presence of oocysts in kitten stool was examined using sucrose flotation technique as described [17].

After feeding, the feces of the kitten were collected daily and oocysts were purified as described [17–19]. Briefly, ~10 g of feces were softened by adding to a 50 ml tube, filled with tap water and incubated for 2 h at room temperature. The tap water was discarded and approximately 50 ml sucrose/phenol solution (53 g sucrose, 0.8 ml liquid phenol, 100 ml tap water) was added

to the softened feces, and slowly emulsified using a spatula. Next, the homogenate was filtered through two layers of sterile gauze and centrifuged at 400× g for 10 min. Then, 500 µl supernatant from the top of the tube was collected and mixed with 4.5 ml 2% H₂SO₄ to stimulate sporulation. The oocysts were then incubated at room temperature for 3–5 days and checked for sporulation by microscopy. As the oocysts sporulated, 3 ml of 1 N NaOH was added to the mixture to stop stimulation.

For purification of oocysts, 4 ml of 2.2 M sucrose solution was mixed with the sporulated oocysts and then 5 ml distilled H₂O was carefully overlaid and centrifuged at 1200× g for 20 min. Supernatant containing oocysts was collected without disturbing the sucrose solution, and 4 ml of 2.2 M sucrose solution was mixed with the supernatant again. Then, 5 ml distilled H₂O was carefully overlaid and centrifuged at 1200× g for 20 min. The supernatant containing oocysts was collected, resuspended in 50 ml of distilled water, and centrifuged at 2000× g for 10 min. About 1–2 ml of the supernatant collected from the top of the tube was mixed with an equal volume of sterile 0.9% NaCl. The resulting purified oocysts were quantified by hemocytometer under phase contrast microscopy and immediately used to infect mice.

Infection of mice and collection of sera

Two groups of Swiss outbred mice (*n* = 10) were administered orally with fresh 8–10 sporulated oocysts and 10–15 tissue cysts using a stainless steel curved feeding needle (Harvard Apparatus). Serum samples were collected by tail bleeding from each anesthetized mouse prior to inoculation (day 0) and on day 1, 2, 3, 6, 10, 15, 40 and 120 after infection. On day 120, mice were euthanized and brains were homogenized in approximately 2 ml sterile 0.9% NaCl containing penicillin (10 U/ml), streptomycin (10 µg/ml) and gentamicin (2 µg/ml) using a syringe with a 20G 1" (0.9 × 25 mm) needle. The presence of tissue cysts in each homogenate was confirmed

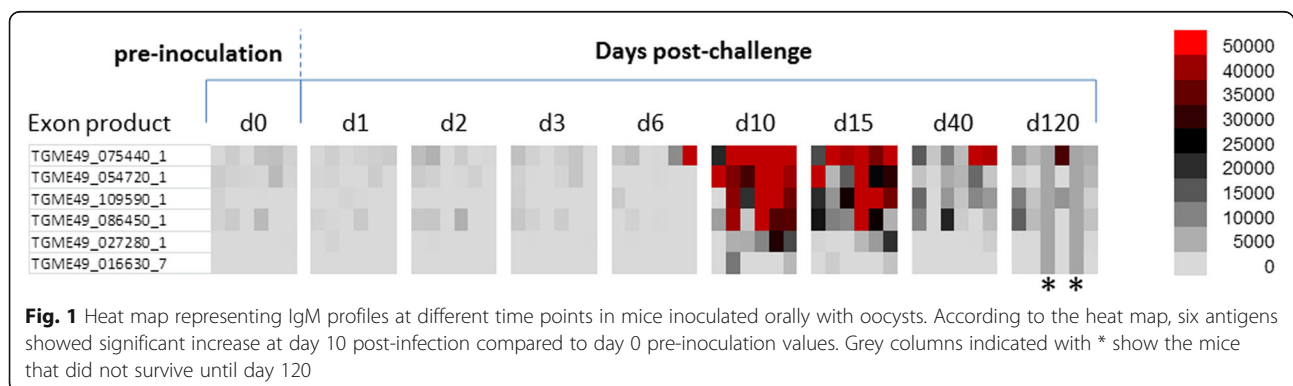


Table 1 Antigens that showed significant IgM response at day 10 in mice infected with oocysts

| Rank ^a | Gene ID_exon | Description | Benjamini-Hochberg corrected <i>P</i> -value (d0 vs d10) |
|-------------------|-----------------|-----------------------------------|--|
| 1 | TGME49_075440_1 | Granule antigen protein GRA6 | <i>P</i> < 0.0001 |
| 2 | TGME49_054720_1 | Dense granule protein GRA8 | <i>P</i> < 0.0001 |
| 3 | TGME49_109590_1 | Rhoptry protein 1 | <i>P</i> = 0.0007 |
| 4 | TGME49_086450_1 | Dense granule protein 5 precursor | <i>P</i> = 0.0131 |
| 5 | TGME49_027280_1 | Dense granule protein GRA3 | <i>P</i> = 0.0077 |
| 6 | TGME49_016630_7 | Trigger factor protein, putative | <i>P</i> = 0.0183 |

^aBased on average signal value, as per Figs. 1 and 2

using a phase contrast microscopy to determine if mice were infected with *T. gondii*.

Protein microarray fabrication to determine IgM and IgG kinetics

A two-step antigen discovery process was employed. In the first step, two large arrays (TG1 and TG2/3) comprising more than 2870 proteins were probed with a subset of the murine immune sera (sera from nine mice collected at day 10–15 and 40) to determine the IgG and IgM responsive predominant antigens. The first published array (TG1) comprised 1357 exon products from 615 genes [15] and the second array (TG2/3) contained 1513 exons (from 772 genes) [16]. These 2870 proteins were down-selected from > 8000 genes (> 43,000 exons) present in the *T. gondii* genome by a bioinformatic filtering process, as previously described [15, 16]. To isolate the exons, primers were designed from the genomic sequence of type II strain ME49 of *T. gondii* that was obtained from the *Toxoplasma* Genomics Resource (<http://toxodb.org/toxo/>). In the final screening, a completely new mouse array (TG4) was generated. Initially, a set of sera from day 40 for IgG and two sets of sera from day 10 and day 15 were probed against the full TG1 and TG2/3 arrays to determine the murine profile. Finally, a total of 240 exon

products reactive to the murine sera were identified on the basis of this screen. These 240 plasmids were expressed and printed using the standard methods to produce a mouse-appropriate, down-selected reactive antigen array, termed here “TG4” (see Additional file 1: Table S1 for the list of 240 prioritized proteins). The final TG4 array was generated and probed with all the mouse sera samples (from day 0 to day 120) as described previously [15, 16]. Then, the results of the array were compared to the human array profiles to determine the shared antigens both in humans and mice.

The nomenclature used in ToxoDB *Toxoplasma* Genomic Resource (<http://toxodb.org/toxo/>) is used throughout, with each gene ID appended with the exon number expressed.

Statistical analysis

The statistical analyses of arrays were handled as described previously [15, 16]. Briefly, raw spot and local background intensities, protein annotation, and sample phenotypes are imported and merged in the R statistical environment, where all subsequent procedures are performed unless specified otherwise. Microarray spot intensities are quantified using ScanArrayExpress software (Perkin Elmer, USA) utilizing automatic local background subtraction for each

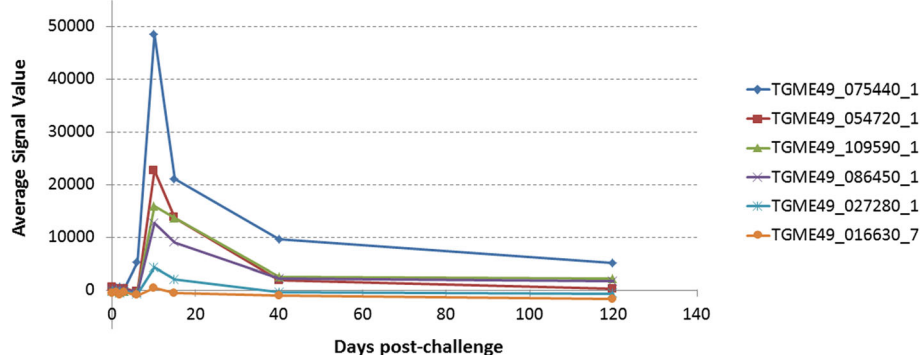


Fig. 2 Line chart of IgM kinetics at different time points in mice inoculated orally with oocysts. Line chart of six antigens, shown in Table 1 overlaid with Benjamini-Hochberg-corrected *P*-values comparing data for day 10 post-infection with pre-inoculation values. Signal values are average of adjusted array signals (raw *in vitro* transcription/translation [IVTT] signals subtracted of median of sample-specific control IMT spots) ± SD

spot. The generated signal intensity values are considered raw values. Next, all raw values are transformed using the base 2 logarithm (\log_2). Antibody magnitude is defined as signal intensity of reactive antigens with respect to NoDNA probes. To calculate the magnitude of antibody responses, the median normalized signal intensity (\log_2 scale) of the NoDNA probes per individual is subtracted from target probe normalized signal intensities for each individual.

All statistical analyses for microarray data were performed using the R statistical environment software (<http://www.r-project.org>). A Bayes regularized t-test is adapted from Cyber-T for protein arrays. To account for multiple testing conditions, the Benjamini & Hochberg [20] (BH) method is used to control the false discovery rate. After Benjamini-Hochberg correction, *P*-values smaller than 0.05 are considered significant, and the corresponding protein is considered differentially reactive.

Results

Serum samples were collected from two groups of mice, infected orally with viable oocysts and tissue cysts, before infection (Day 0) as well as 1, 2, 3, 6, 10, 15, 40 and

120 days after infection. Next, IgM and IgG antibody kinetics of these sera were analyzed by protein microarrays comprising 240 prioritized proteins.

IgM kinetics of mice infected with oocysts

During the screening in the group of mice infected with viable oocysts, the IgM response reached maximal intensity and breadth around day 10, as seen in the heat map in Fig. 1. Responses against 6 antigens increased significantly at day 10 compared to day 0 (*P*-values are listed in Table 1). Of the 5 antigens listed in Table 1, one of them is rhoptry protein and four are dense granule proteins which were all excreted secreted antigens (ESA) (Table 1). The IgM response decreased steadily after day 10 (Fig. 2).

IgG kinetics of mice infected with oocysts

During the screening, IgG response against 40 antigens reached maximal intensity and breadth around day 40 as seen in the heat map in Fig. 3. IgG responses against 40 antigens increased significantly at day 40 compared to day 0 (*P*-values are listed in

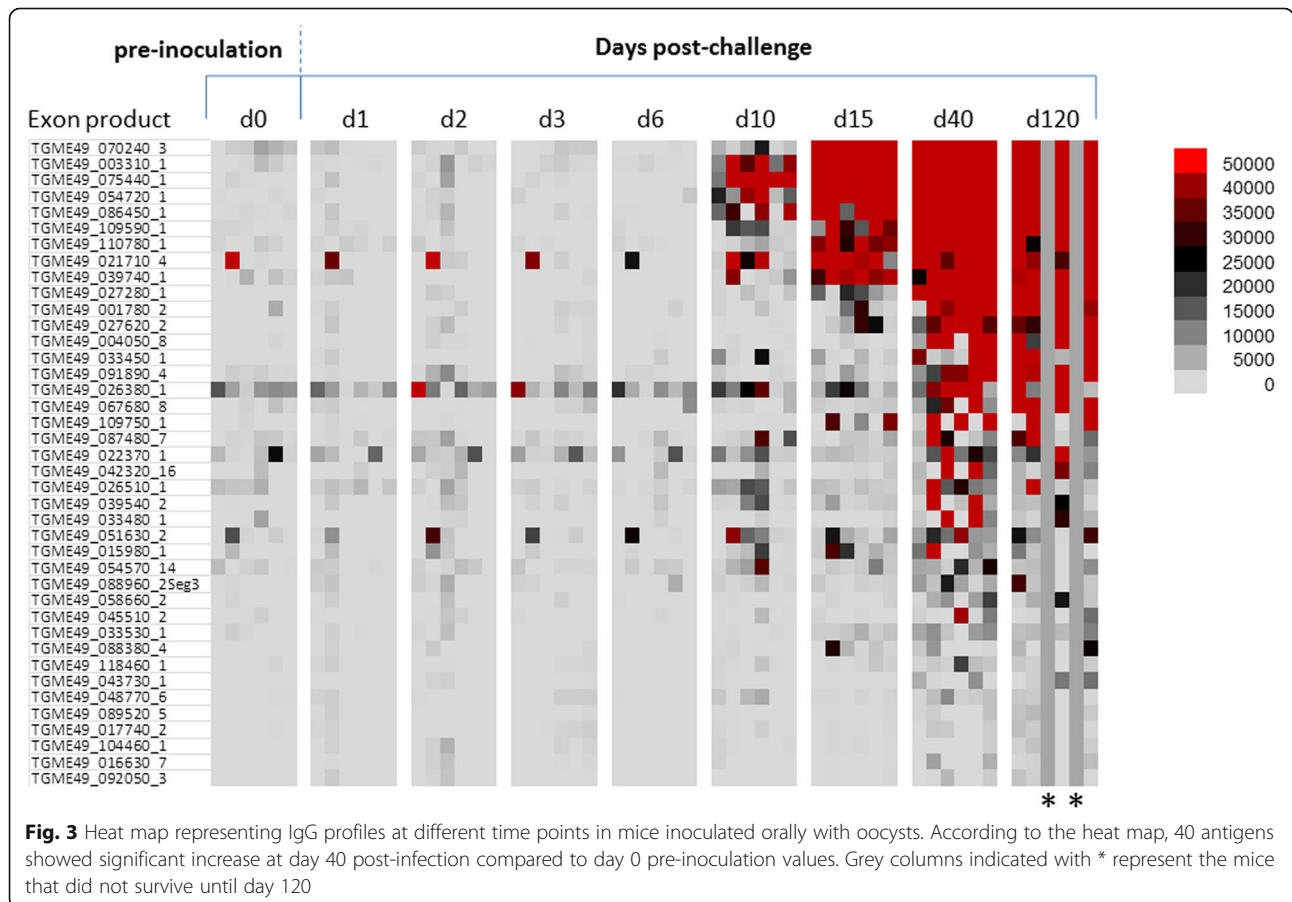


Fig. 3 Heat map representing IgG profiles at different time points in mice inoculated orally with oocysts. According to the heat map, 40 antigens showed significant increase at day 40 post-infection compared to day 0 pre-inoculation values. Grey columns indicated with * represent the mice that did not survive until day 120

Table 2 Antigens that showed significant IgG response at day 40 in mice infected with oocysts

| Rank ^a | Gene ID_exon | Description | Benjamini-Hochberg corrected <i>P</i> -value (d0 vs d40) |
|-------------------|---------------------|---|--|
| 1 | TGME49_070240_3 | Unspecified product (MAG1) | <i>P</i> < 0.0001 |
| 2 | TGME49_003310_1 | Dense granule protein GRA7 | <i>P</i> < 0.0001 |
| 3 | TGME49_075440_1 | Granule antigen protein GRA6 | <i>P</i> < 0.0001 |
| 4 | TGME49_054720_1 | Dense granule protein GRA8 | <i>P</i> < 0.0001 |
| 5 | TGME49_086450_1 | Dense granule protein 5 precursor | <i>P</i> < 0.0001 |
| 6 | TGME49_109590_1 | Rhoptry protein 1 | <i>P</i> < 0.0001 |
| 7 | TGME49_110780_1 | Dense granule protein GRA4 | <i>P</i> < 0.0001 |
| 8 | TGME49_021710_4 | TBC domain-containing protein | <i>P</i> = 0.0014 |
| 9 | TGME49_039740_1 | Dense granule protein GRA14 | <i>P</i> < 0.0001 |
| 10 | TGME49_027280_1 | Dense granule protein GRA3 | <i>P</i> < 0.0001 |
| 11 | TGME49_001780_2 | Microneme protein MIC2 | <i>P</i> = 0.0003 |
| 12 | TGME49_027620_2 | Dense granule protein GRA2 | <i>P</i> < 0.0001 |
| 13 | TGME49_004050_8 | Subtilisin SUB1 | <i>P</i> = 0.0007 |
| 14 | TGME49_033450_1 | SAG-related sequence SRS29A | <i>P</i> = 0.0151 |
| 15 | TGME49_091890_4 | Microneme protein MIC1 | <i>P</i> = 0.0003 |
| 16 | TGME49_026380_1 | Hypothetical protein | <i>P</i> = 0.0429 |
| 17 | TGME49_067680_8 | Microneme protein MIC12 | <i>P</i> = 0.0151 |
| 18 | TGME49_109750_1 | Succinate-Coenzyme A ligase, beta subunit | <i>P</i> = 0.0275 |
| 19 | TGME49_087480_7 | Hypothetical protein | <i>P</i> = 0.0483 |
| 20 | TGME49_022370_1 | SAG-related sequence SRS13 | <i>P</i> = 0.0445 |
| 21 | TGME49_042320_16 | B-box zinc finger domain-containing protein | <i>P</i> = 0.0190 |
| 22 | TGME49_026510_1 | Sec23/Sec24 trunk domain-containing protein | <i>P</i> = 0.0359 |
| 23 | TGME49_039540_2 | LEM3 (ligand-effect modulator 3) family | <i>P</i> = 0.0280 |
| 24 | TGME49_033480_1 | SAG-related sequence SRS29C | <i>P</i> = 0.0358 |
| 25 | TGME49_051630_2 | Slc30a2 protein | <i>P</i> = 0.0361 |
| 26 | TGME49_015980_1 | Hypothetical protein | <i>P</i> = 0.0358 |
| 27 | TGME49_054570_14 | Hypothetical protein | <i>P</i> = 0.0483 |
| 28 | TGME49_088960_2Seg3 | Hypothetical protein | <i>P</i> = 0.0358 |
| 29 | TGME49_058660_2 | Rhoptry protein ROP6 | <i>P</i> = 0.0358 |
| 30 | TGME49_045510_2 | Phospholipid-translocating P-type ATPase | <i>P</i> = 0.0483 |
| 31 | TGME49_033530_1 | Hypothetical protein | <i>P</i> = 0.0364 |
| 32 | TGME49_088380_4 | Heat shock protein HSP90 | <i>P</i> = 0.0430 |
| 33 | TGME49_118460_1 | P-type ATPase | <i>P</i> = 0.0430 |
| 34 | TGME49_043730_1 | Rhoptry protein ROP9 | <i>P</i> = 0.0364 |
| 35 | TGME49_048770_6 | Hypothetical protein | <i>P</i> = 0.0430 |
| 36 | TGME49_089520_5 | Hypothetical protein | <i>P</i> = 0.0498 |
| 37 | TGME49_017740_2 | 3-ketoacyl-(acyl-carrier-protein) reductase | <i>P</i> = 0.0483 |
| 38 | TGME49_104460_1 | Zinc finger, C3HC4 type (RING finger) domain-containing protein | <i>P</i> = 0.0483 |
| 39 | TGME49_016630_7 | Trigger factor protein, putative | <i>P</i> = 0.0498 |
| 40 | TGME49_092050_3 | Calcium dependent protein kinase CDPK8 | <i>P</i> = 0.0483 |

^aBased on average signal value, as per Figs. 3 and 4

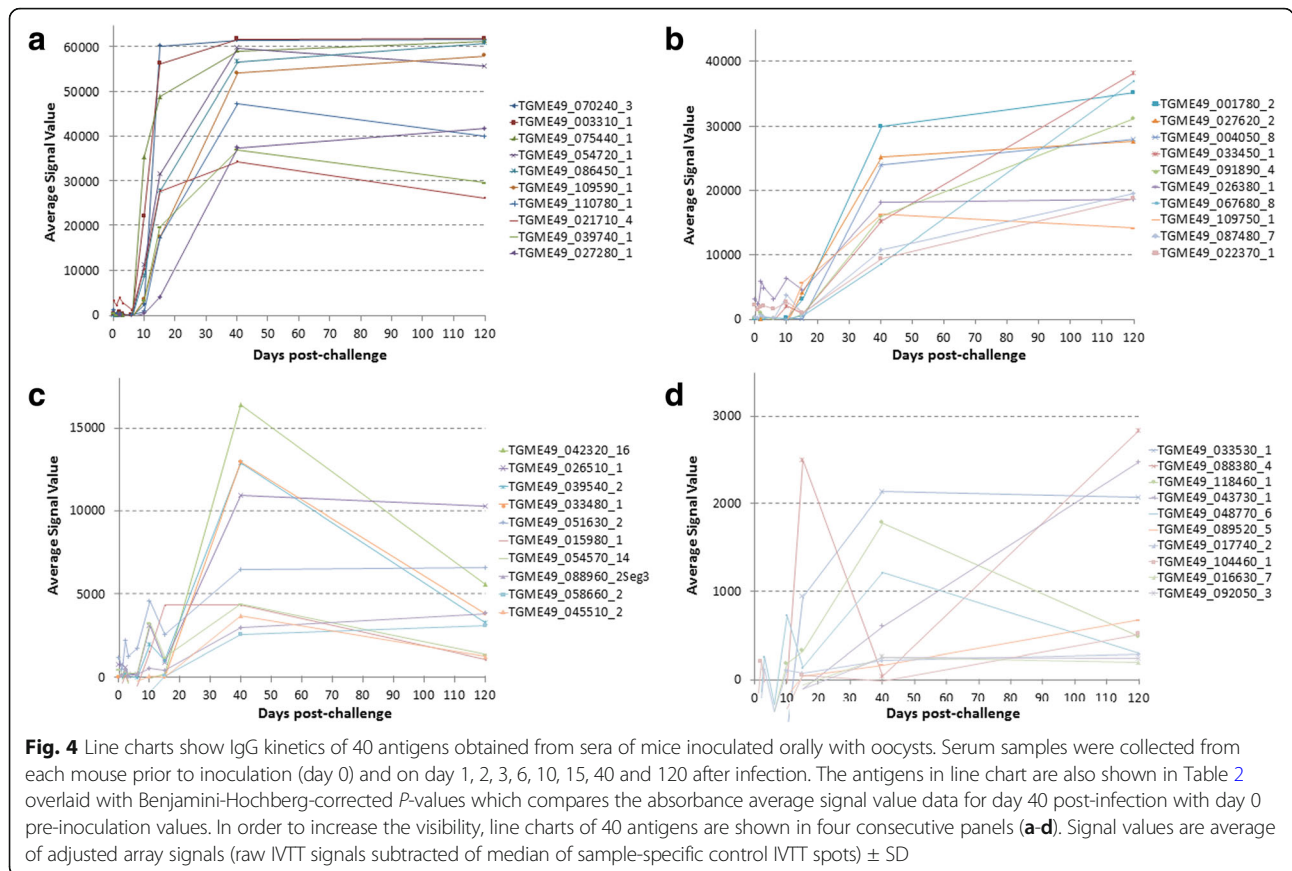


Table 2). The IgG response mostly first peaked at day 10 or afterwards (Fig. 4a-d). Among the first five highest signal giving proteins, four of them are dense granule proteins which were ESA (Table 2).

IgM kinetics of mice infected with tissue cysts

During the screening in the group of mice infected with viable tissue cysts, the IgM response reached maximal intensity and breadth around day 10, as seen in the heat map in Fig. 5. IgM responses against 6 antigens increased significantly at day 10 compared to day 0 (*P*-values are listed in Table 3). Of the 5 antigens listed in Table 1, one of them is rhoptry protein and two of them are dense granule proteins which all ESA. The IgM response decreased steadily after day 15 (Fig. 6).

IgG kinetics of mice infected with tissue cysts

During the screening, IgG response against 40 antigens reached maximal intensity and breadth around day 40 as seen in the heat map in Fig. 7. IgG responses against 40 antigens increased significantly at day 40 compared to day 0 (*P*-values are listed in Table 4). The IgG response mostly gave first peaks at day 10 or afterwards (Fig. 8a-d). Among the first five highest signal giving proteins, one of them is rhoptry protein and three of them are dense granule proteins which were ESA (Table 4).

Discussion

The realization of the importance of antigen discovery to develop diagnostic assays or a protective vaccine has

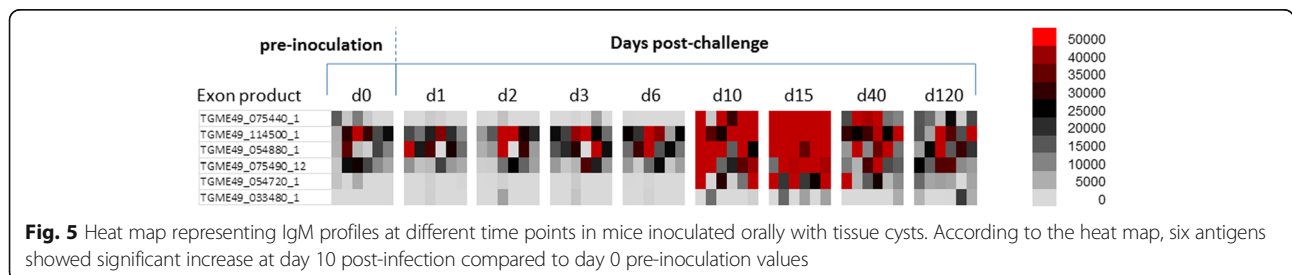


Table 3 Antigens that showed significant IgM response at day 10 in mice infected with tissue cysts

| Rank ^a | Gene ID_exon | Description | Benjamini-Hochberg corrected P-value (d0 vs d10) |
|-------------------|------------------|------------------------------|--|
| 1 | TGME49_075440_1 | Granule antigen protein GRA6 | $P = 0.0493$ |
| 2 | TGME49_114500_1 | Subtilisin SUB2 | $P = 0.0497$ |
| 3 | TGME49_054880_1 | Alpha-galactosidase | $P = 0.0493$ |
| 4 | TGME49_075490_12 | Hypothetical protein | $P = 0.0493$ |
| 5 | TGME49_054720_1 | Dense granule protein GRA8 | $P = 0.0493$ |
| 6 | TGME49_033480_1 | SAG-related sequence SRS29C | $P = 0.0493$ |

^aBased on average signal value, as per Figs. 5 and 6

increased in the last decade. During the development of a diagnostic serological assay to detect acute toxoplasmosis, antigens expressed during the acute toxoplasmosis can be much preferred. Plenty of antigens such as SAG1, SAG2, SAG2A, ROP1, ROP2, GRA1, GRA2, GRA3, GRA4, GRA5, GRA6, GRA7, GRA8, MIC1, MIC2, MIC3, MIC4, M2AP, AMA1, HSP20, BAG1 (HSP30), MAG1, NTPase, P25, P35, P68 and multi-epitope peptide containing epitopes of SAG1, SAG2 and SAG3 have been tested by ELISA methods with well categorized sera obtained from human or animal models so far to diagnose acute toxoplasmosis [13].

In the case of development of a protective vaccine against toxoplasmosis, vaccine formulations should contain antigens from each stage of the parasite to sequentially block the parasite during the invasion of host cells. More than 60 proteins have been tested as vaccine candidate antigens up to date [12], but none of them conferred the desired efficacy to be able to proceed to clinical trial. Randomly chosen vaccine candidate antigens, such as structural proteins or metabolic enzymes, have delayed the progress in vaccine development pipeline for *T. gondii*.

In the life-cycle of *T. gondii*, tissue cysts (containing bradyzoites) and oocysts (containing sporozoites) are the main forms of the parasite that transmit the disease in

nature. As they enter the host cell, bradyzoites and sporozoites both convert to motile tachyzoites which invade the tissues. Just as the immune response starts to respond to the parasite, tachyzoites change into slowly dividing bradyzoites and remain latent in a tissue cyst [21]. Thus, a successful vaccine against *T. gondii* is likely to contain antigens from all forms of the parasite and selected using a rational approach.

To address these issues, we screened the serum samples from mice orally infected with tissue cysts and oocysts. Sera were collected prior to administration (Day 0) as well as 1, 2, 3, 6, 10, 15, 40 and 120 days after infection from these mice to characterize the immune response during the first dates and thereafter. By screening these mice sera, we identified the antigens that give prominent immune response during infection initiated with oocysts or tissue cysts. In addition, antibody kinetics of each antigen was displayed.

In the mice infected with oocysts, 6 IgM and 40 IgG immune-reactive antigens were discovered that showed significant increment in response compared to baseline (Figs. 1, 2, 3 and 4). In the group infected with tissue cysts, 6 IgM and 40 IgG immune-reactive antigens were identified (Figs. 5, 6, 7 and 8). Oocyst and tissue cyst fed mice showed significant IgM response at day 10. Thereafter, a decline was observed in IgM response after day 10 in oocyst-infected mice but in the group of mice

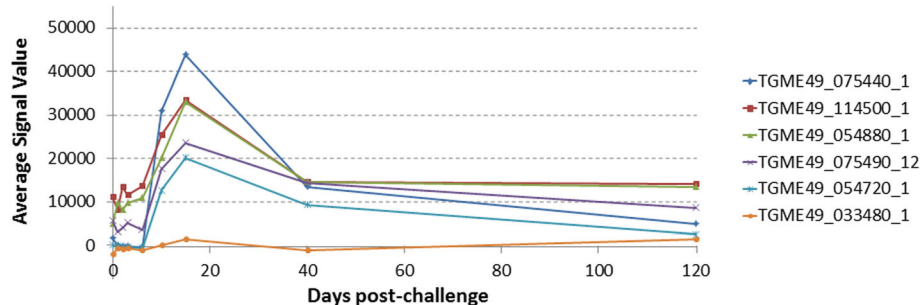
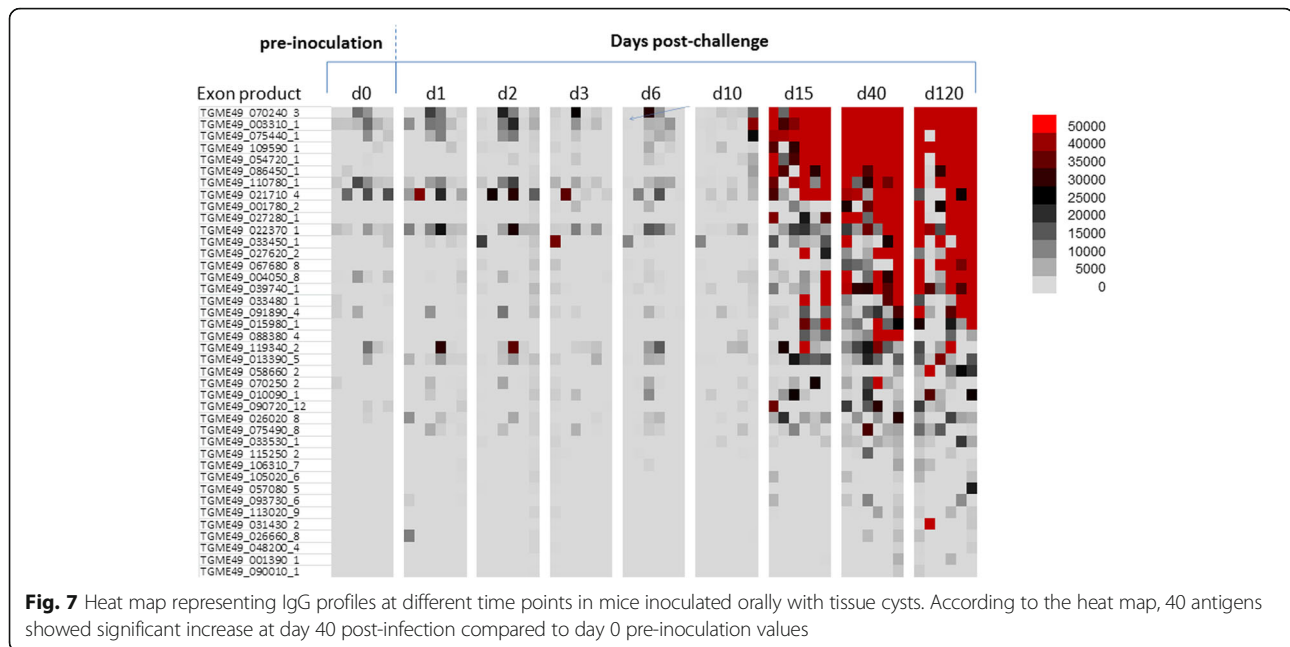


Fig. 6 Line chart represent IgM kinetics specific for six antigens probed with sera collected from mice at different time points inoculated orally with tissue cysts. These six antigens are also shown in Table 3 overlaid with Benjamini-Hochberg-corrected P-values comparing the absorbance average signal value data for day 10 post-infection with day 0 pre-inoculation values. Signal values are average of adjusted array signals (raw IVTT signals subtracted of median of sample-specific control IVTT spots) \pm SD



infected with tissue cyst, the increment in IgM response continued until day 15 and then declined. This is consistent with tissue cyst bradyzoites requiring more time to invade the host cells. The IgG response first appeared mostly around at day 15, peaked at day 40 and remained elevated or increased through day 120. Overall, there are 12 antigens discovered during IgM screening and 80 proteins during IgG screening.

If we look from the point of view of selecting an antigen(s) to develop a serological diagnostic assay or vaccine, we have to focus on the antigens that play significant roles in pathogenesis. During the invasion, MIC proteins are secreted from small apical organelles called micronemes. MICs are adhesion proteins that help the parasite to attach to the host plasma membrane. ROP proteins are secreted from *unique* elongated organelles called rhoptries. ROPs are secreted from the apical end into the cytoplasm of host cells and help the formation of parasitophorous vacuole (PV) derived from host cell membrane. Once the parasite gets inside PV, GRAs which are dense granule proteins are released into the PV that modifies the vacuole membrane [22]. Thus, antigens to be used in diagnostic serological assays or vaccine formulations are needed to: (i) actively induce strong immune response at the very beginning of the infection (i.e. strong IgM response during the first 10–15 days of infection); (ii) induce long-lasting immunity (i.e. strong IgG response until day 120); (iii) be antigenic in both forms of the parasite because transmission can occur through oocysts or tissue cysts.

From the accumulated screening data, two antigens GRA6 (TGME49_075440) and GRA8 (TGME49_054

720) have all of the above-mentioned properties. In addition, GRA3 (TGME49_027280), GRA5 (TGME49_086450), ROP1 (TGME49_109590) and SAG-related sequence SRS29C (TGME49_033480) have similar properties excluding antigenicity in IgM response in oocyst infected mice. Besides, there are 11 antigens that induce long-lasting IgG response in both oocyst and tissue cyst infected mice which are the cyst matrix protein MAG1 (TGME49_070240); dense granule proteins GRA2 (TGME49_027620), GRA4 (TGME49_110780), GRA7 (TGME49_003310) and GRA14 (TGME49_039740); microneme proteins MIC1 (TGME49_091890), MIC2 (TGME49_001780) and MIC12 (TGME49_067680); surface proteins SAG-related sequence SRS13 (TGME49_022370) and SRS29A (TGME49_033450); rhoptry protein ROP6 (TGME49_058660).

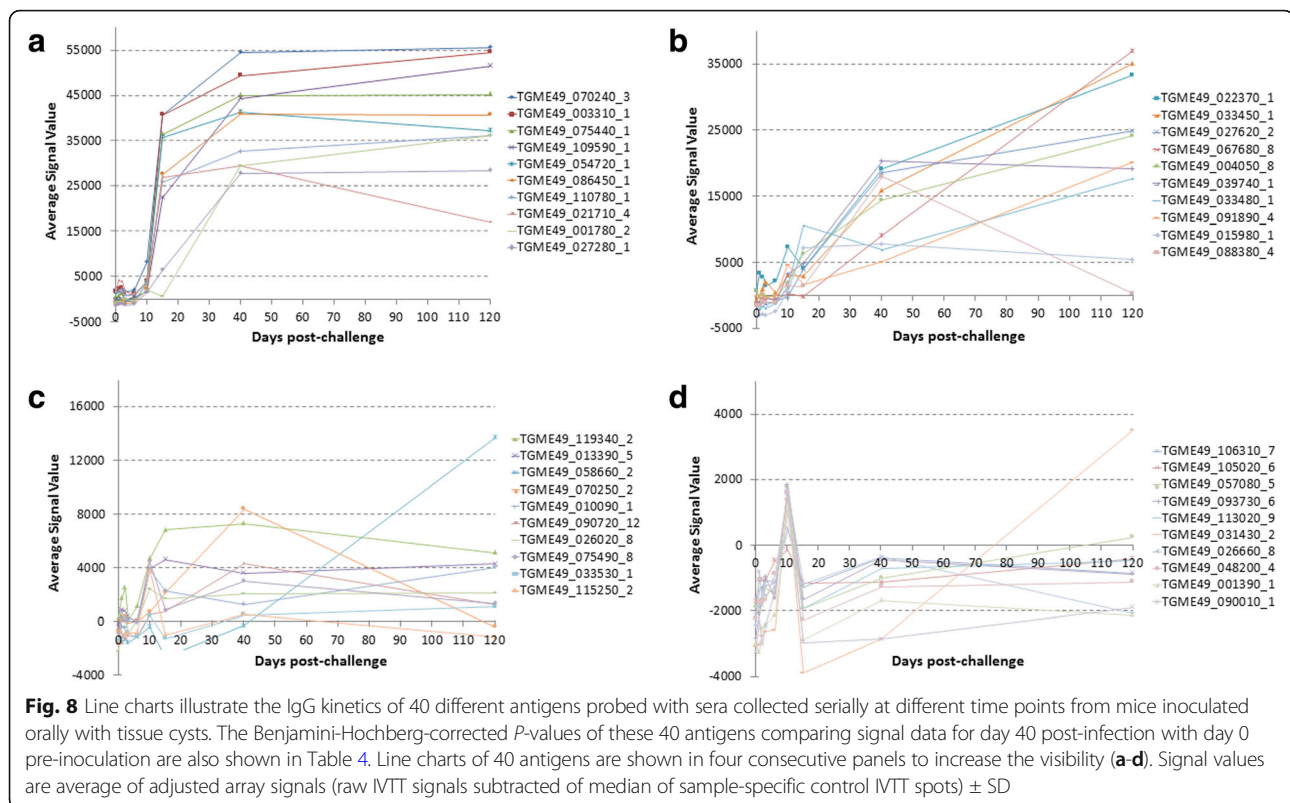
When we match the human screening data from our previous studies [15, 16] with these mouse response antigens, 16 of these antigens showed a strong response in human sera samples except SAG-related sequence SRS29C (TGME49_033480).

Two studies about transcriptomic and proteomic analyses of *T. gondii* confirmed that GRA2, GRA3, GRA4, GRA5, GRA6, GRA7, GRA8, GRA14, MIC1, MIC2, MIC12, ROP1, ROP6 and SRS29C, expression levels are elevated in sporozoites at days 4 and 10 of infection relative to day 0 of infection, suggesting an important role in host cell invasion [23, 24]. Among the above-mentioned antigens, ROP1, GRA2, GRA4, GRA5, GRA6, GRA7, GRA14, MIC1, MIC2 and MAG1 have been used as vaccine candidate antigens in previous studies and showed strong immunogenicity [12, 25–44]. GRA3 and GRA8

Table 4 Antigens that showed significant IgG response at day 40 in mice infected with tissue cysts

| Rank ^a | Gene ID_exon | Description | Benjamini-Hochberg corrected <i>P</i> -value (d0 vs d40) |
|-------------------|------------------|--|--|
| 1 | TGME49_070240_3 | Unspecified product (MAG1) | <i>P</i> = 0.0006 |
| 2 | TGME49_003310_1 | Dense granule protein GRA7 | <i>P</i> = 0.0017 |
| 3 | TGME49_075440_1 | Granule antigen protein GRA6 | <i>P</i> = 0.0006 |
| 4 | TGME49_109590_1 | Rhoptry protein 1 | <i>P</i> = 0.0006 |
| 5 | TGME49_054720_1 | Dense granule protein GRA8 | <i>P</i> = 0.0006 |
| 6 | TGME49_086450_1 | Dense granule protein 5 precursor | <i>P</i> = 0.0006 |
| 7 | TGME49_110780_1 | Dense granule protein GRA4 | <i>P</i> = 0.0092 |
| 8 | TGME49_021710_4 | TBC domain-containing protein | <i>P</i> = 0.0180 |
| 9 | TGME49_001780_2 | Microneme protein MIC2 | <i>P</i> = 0.0037 |
| 10 | TGME49_027280_1 | Dense granule protein GRA3 | <i>P</i> = 0.0006 |
| 11 | TGME49_022370_1 | SAG-related sequence SRS13 | <i>P</i> = 0.0187 |
| 12 | TGME49_033450_1 | SAG-related sequence SRS29A | <i>P</i> = 0.0475 |
| 13 | TGME49_027620_2 | Dense granule protein GRA2 | <i>P</i> = 0.0092 |
| 14 | TGME49_067680_8 | Microneme protein MIC12 | <i>P</i> = 0.0187 |
| 15 | TGME49_004050_8 | Subtilisin SUB1 | <i>P</i> = 0.0317 |
| 16 | TGME49_039740_1 | Dense granule protein GRA14 | <i>P</i> = 0.0006 |
| 17 | TGME49_033480_1 | SAG-related sequence SRS29C | <i>P</i> = 0.0477 |
| 18 | TGME49_091890_4 | Microneme protein MIC1 | <i>P</i> = 0.0354 |
| 19 | TGME49_015980_1 | Hypothetical protein | <i>P</i> = 0.0010 |
| 20 | TGME49_088380_4 | Heat shock protein HSP90 | <i>P</i> = 0.0012 |
| 21 | TGME49_119340_2 | Hypothetical protein | <i>P</i> = 0.0280 |
| 22 | TGME49_013390_5 | Plectin, putative | <i>P</i> = 0.0370 |
| 23 | TGME49_058660_2 | Rhoptry protein ROP6 | <i>P</i> = 0.0355 |
| 24 | TGME49_070250_2 | Dense granule protein GRA1 | <i>P</i> = 0.0388 |
| 25 | TGME49_010090_1 | Protein kinase domain-containing protein | <i>P</i> = 0.0354 |
| 26 | TGME49_090720_12 | Vacuolar proton translocating ATPase subunit, putative | <i>P</i> = 0.0297 |
| 27 | TGME49_026020_8 | Transporter, major facilitator family protein | <i>P</i> = 0.0485 |
| 28 | TGME49_075490_8 | Hypothetical protein | <i>P</i> = 0.0388 |
| 29 | TGME49_033530_1 | Hypothetical protein | <i>P</i> = 0.0099 |
| 30 | TGME49_115250_2 | GAMM1 protein, putative | <i>P</i> = 0.0485 |
| 31 | TGME49_106310_7 | RecF/RecN/SMC N terminal domain-containing protein | <i>P</i> = 0.0187 |
| 32 | TGME49_105020_6 | Hypothetical protein | <i>P</i> = 0.0387 |
| 33 | TGME49_057080_5 | 3'5'-cyclic nucleotide phosphodiesterase domain-containing protein | <i>P</i> = 0.0408 |
| 34 | TGME49_093730_6 | DHHC zinc finger domain-containing protein | <i>P</i> = 0.0108 |
| 35 | TGME49_113020_9 | STAS domain-containing protein | <i>P</i> = 0.0134 |
| 36 | TGME49_031430_2 | Oligosaccharyl transferase stt3 protein, putative | <i>P</i> = 0.0365 |
| 37 | TGME49_026660_8 | Hypothetical protein | <i>P</i> = 0.0187 |
| 38 | TGME49_048200_4 | Ribosomal RNA- dimethyltransferase, putative | <i>P</i> = 0.0488 |
| 39 | TGME49_001390_1 | Hypothetical protein | <i>P</i> = 0.0180 |
| 40 | TGME49_090010_1 | Hypothetical protein | <i>P</i> = 0.0485 |

^aBased on average signal value, as per Figs. 7 and 8



have been evaluated with ELISA and showed some immunity with human samples [45–48]. SRS29C have been identified as an important negative regulator of acute virulence in murine models [49].

Conclusion

Our approach has revealed that ROP6, MIC12, SRS29A and SRS13 have shown strong immunogenicity and at the same time not tested in development of a diagnostic assay or a vaccine model yet. In addition, it seems that GRA3 and GRA8 can be a good vaccine candidate that has not been tested in an animal model yet. The protective efficiency and immunity of SRS29C can be analyzed in a comprehensive vaccine study. In development of a diagnostic assay to detect acute toxoplasmosis, the evaluation of ROP6, MIC12, SRS29A and SRS13 IgM/IgG kinetics with well categorized human sera can give some clue about acute toxoplasmosis. Multivalent vaccine formulations or ELISAs using several antigens of *T. gondii* have been found more successful in previous studies in terms of protection against toxoplasmosis and diagnosing acute toxoplasmosis. Thus, blending the novel antigens discovered in this study with previously immunogenic antigens in diagnostic assays or vaccine formulations may give better results. We must also bear in mind that the protection against toxoplasmosis is

conferred mainly by cellular immune response and through humoral immune response [22]. Screening of cellular immunity of humans and animals against toxoplasmosis will definitely give more clues about protective antigens but a robust high-throughput wet lab approach does not exist currently. On the other hand, the antigens discovered in this study are reactive with IgM and IgG antibodies and showed strong immunogenicity and could give researchers some clue to develop a vaccine against toxoplasmosis or diagnostic serological kit.

Additional file

Additional file 1: Table S1. 240 exon products reactive with murine sera comprising the mouse array TG4. (XLSX 20 kb)

Abbreviations

ROP: rhopty protein; GRA: dense granule protein; MIC: microneme protein; MAG: cyst matrix protein; SRS: SAG-related sequence; AVMA: American Veterinary Medical Association; FEDIAF: The European Pet Food Industry Federation; TG1, TG2/3, TG4: microarrays developed to detect *T. gondii* antigens; ToxoDB: *Toxoplasma* Genomic Resource; TGME49: *T. gondii* ME49 strain; IVTT: *in vitro* transcription/translation; SD: Standard deviation

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the Figshare repository, <https://figshare.com/s/2ecdc4a875cfcfb3>.

Authors' contributions

MD, LL, AJ, HC, SGI, PLP, ADD, DHD and AYG conceived the study. AYG and DHD obtained funding. MD, DHD, LL and PLP analyzed the data. DHD, MD, PLP and AYG prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All *in vivo* studies were performed according to Guide for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committee (IACUC) of Ege University (Turkey) for animal ethical norms (Permit number: 2009–155).

Competing interests

MD, AYG, DHD and PLF are inventors on patent applications pertaining to this work. DHD and PLF own stock in a company (Antigen Discovery Inc.) that has licensed the technology. The terms of this arrangement have been approved by the UCI Conflict of Interest Committee. LL, AJ, HC, SGI, ADD declare that they have no competing interests.

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