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Protective immunity induced by a native *Toxoplasma gondii* antigen against *Toxoplasma* infection in Balb/c mice.

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Running title: Protective 44-kDa antigen against *T. gondii.*

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Abstract

The vaccine development towards *Toxoplasma gondii,* a parasitic protozoan is an elusive goal. Our objective was to investigate the immunogenic and protective effects of a native *T. gondii* antigen in BALB/c mice. Balb/c mice were immunized by injecting *T. gondii* native antigen subcutaneously three times, at one-week intervals between each injection. The serum levels of anti-*T. gondii* IgG, IgG subclass antibodies, IFN-γ, and IL-10 were quantified using ELISA. In a challenge, immunized mice with target antigen were given a lethal dose of RH strain of *T. gondii* tachyzoites; the number of surviving mice was counted. Western blotting identified the target antigen in tachyzoite antigenic extract at 44-kDa molecular mass. The 44-kDa antigen was isolated and partially characterized as a protein. The immunized mice exhibited significant ($p < 0.05$) elevated levels of specific anti-*T. gondii* IgG, IgG1 and IgG2a antibodies compared to control groups. Furthermore, the 44-kDa antigen significantly ($p < 0.001$) stimulates the synthesis of IFN-γ, as well as IL-10 indicating the native antigen might elicit immune responses of both Th1 and Th2 types. Furthermore, immunized BALB/c mice displayed prolonged survival time of up to 12 days against lethal challenge with *T. gondii* RH strain in comparison with non-immunized controls. In conclusion, immunization of BALB/c mice with 44-kDa native antigen generates immunoprotective responses against *T. gondii* infection and increases survival time. The 44-kDa antigen may have the potential as a promising candidate vaccine against *T. gondii* infection and further investigations based on recombinant target protein will be performed.

Keywords: *Toxoplasma gondii*, Vaccine, Native 44-KDa antigen, ELISA.

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1. Introduction

Toxoplasmosis is a parasite infection caused by a microbe called *Toxoplasma gondii*. It can be encountered in animals, birds, and soil. Infection with *T. gondii* causes significant clinical consequences in individuals associated with weakened immune systems and cases of hereditary transmission. *T. gondii* has three infectious forms of sporozoites that reside in the tissue as a cyst identified in oocysts, tachyzoites, and bradyzoites. Toxoplasmosis in humans can be spread by various means, including mother-to-child transmission, consuming raw meat containing dormant cysts, ingesting contaminated food, and exposure to water contaminated with mature oocysts (1). There is currently a lack of an efficient control approach to decrease toxoplasmosis in humans along with other global warm blood (2). Conventional treatment for toxoplasmosis involves the use of different drugs, such as pyrimethamine and sulfadiazine. However, many patients are unable to respond well to this medication combination due to its serious adverse reactions. Additionally, this treatment only targets the tachyzoite form of the parasite and is ineffective against the latent forms, specifically the slow-dividing bradyzoites found within tissue cysts (3). At the moment, vaccination is thought to be a very successful disease prevention method. Numerous studies have confirmed that vaccinations are effective in preventing and controlling a variety of viral diseases, including toxoplasmosis (4). As a result, several immunogens, such as DNA vaccines, recombinant antigens, native parasite antigens, and killed, and live-attenuated antigens, have been the basis of immunization strategies that only led to partial protection against *T. gondii* infection throughout the past 20 years (5,6). Notwithstanding testing various antigens from *T. gondii* micronemes, rhoptries, dense granules, organelles, and surface antigens, the researchers were unable to develop a vaccine that would effectively prevent *T. gondii* infection in people (7). Vaccines are a useful substitute for pharmaceutical treatments. Nevertheless, developing a toxoplasmosis vaccine that is safe, long-lasting, and efficacious has been challenging (8). Toxovax® is currently the only vaccination approved for use in sheep and goats. Some drawbacks of this live attenuated vaccine are its short shelf life, the possibility of infection for humans who handle the vaccine, and the potential for virulence reversion (9). This study aimed to assess the immunogenic and immunoprotective properties of the native Toxoplasma antigen isolated from tachyzoite. The specific antibody production levels and cytokines were assessed in immunized animals. The protective effect was assessed by examining the survival rate of *T. gondii*-challenged mice following immunization. This study investigated the protective capacity of the target toxoplasma antigen for the first time, to lay the fundamentals to produce protective approaches against toxoplasmosis.

2. Materials and Methods

2.1. Mice, parasites, and antigen preparation for Toxoplasma tachyzoites

Female BALB/c mice relatively susceptible to toxoplasmosis (age six to eight weeks) were maintained under standard pathogen-free circumstances. All experimental procedures were reviewed and approved by the Ethics Committee of Scientific Research, Faculty of Pharmacy, Minia University (Permit number: ES03/2021). The virulent RH strain of *T. gondii* was produced and maintained in vivo via repeated intraperitoneal passages in mice (every 2 to 3 days). Tachyzoite exudate was then combined with 10% DMSO in cryotubes and stored in cold ethanol at -20^{0}C for 24 hours before being frozen in liquid nitrogen at -196^0 C and used to challenge immunized animals.

2.2. Antigenic Preparation of Toxoplasma Tachyzoites and other parasites

The tachyzoites were homogenized through a process of freezing at a temperature of -196^{0}C for 15 minutes followed by thawing, which was repeated for 3 cycles. Afterward, the homogenized mixture

was centrifuged at a speed of 4,000 rpm for 15 minutes to eliminate any cell debris. Lowery *et al.* (10) evaluated the protein composition and stored the Toxoplasma tachyzoites antigen at a temperature of -20 ^{0}C till it was ready to be utilized. Attallah *et al.* (11) showed how to prepare an antigenic preparation of mature *Schistosoma mansoni*, *Fasciola gigantica,* and *Ascaris lumbricoides*. and determined the protein concentration of each antigen then stored at -20 ^{0}C until they were used.

2.3. SDS-PAGE and Isolation of the Target Toxoplasma Antigen

Utilizing vertical slabs of 12% polyacrylamide, *T. gondii* tachyzoites antigenic extract and pure Toxoplasma antigen were resolved in analytical SDS-PAGE (12) at 50-µg/lane. Standard molecular weights (BioRad, Hercules, CA 94547, USA) were run in parallel. Preparative slab gel electrophoresis running conditions were modified based on the prestained molecular weight marker (BioRad) to reduce protein smear and provide a considerable long migration distance between bands in the 44-kDa region of the serum sample. Coomassie blue staining and immunoblotting were used to identify the 44-kDa band in each run from a lane of the electrophoresed preparative gel. After cutting the neighboring band in the unstained preparative gel, the 44-kDa antigen was electroeluted from the polyacrylamide gel at 200 V for three hours in a dialysis bag (Sigma, USA). After dialysis, polyethylene glycol and 40% trichloroacetic acid (TCA) were used to concentrate the electroeluted antigen, which was subsequently centrifuged for 15 minutes at 6500 ×g. The precipitate was thrice washed with diethyl ether to remove any remaining TCA. The pellet was put back into PBS (pH 7.2). A sample of the electroeluted antigen was analyzed for protein concentration, and the remaining material was then kept at –20 ^{0}C. According to **Attallah *et al.*** (13), antisera was developed in White rabbits from New Zealand that were subcutaneously immunized with the purified 44-kDa antigen at three distinct injection sites used in Western technology to identify the reactive bands that react with rabbit antisera.

2.4. Western blot

In a protein transfer unit, resolved samples on SDS-PAGE were electrotransferred onto the NC membrane (0.45 µm pore size, Sigma). The NC membrane was blocked in 0.05 M Tris-buffered saline (TBS) containing 200 mM NaCl (pH 7.4), rinsed in TBS, and incubated with specific rabbit anti-Toxoplasma IgG antibody (ABC Diagnostics, New Damietta, Egypt) diluted in blocking buffer with constant shaking. An alkaline phosphatase conjugate of goat anti-rabbit IgG diluted 1:400, was incubated on the NC membrane for two hours following three TBS washes (30 minutes each) (The Binding Site, Birmingham, UK). The substrate, which consisted of premixed 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) in 0.1M Tris buffer, pH 9.6; ABC Diagnostics, was applied to the NC membrane after three further TBS washes (15 minutes each). The substrate reaction was halted by submerging the NC membrane in distilled water after the color response was observed in 5–10 minutes.

2.5. The developed antisera's reactivity and specificity with the native 44-KDa antigen using ELISA:

ELISA was used to assess the reactivity and specificity of IgG anti-44KDa antigen rabbit sera against purified Toxoplasma antigen, crude *T. gondii* Tachyzoites antigen, and crude antigens of *S. mansoni* (SWAP), *F. gigantica* (FWAP), and *A. lumbricoides*. Flat-bottomed, polystyrene, microtiter ELISA plates were coated overnight with the purified Toxoplasma antigen, and crude antigens (5-µg/mL Carbonate buffer, pH, 9.6). After blocking, each well received 50 µL of serum from a rabbit immunized with the target pure Toxoplasma antigen at a 1:750 dilution in PBS with 0.05% (v/v) Tween 20 (PBS-T20). The serum utilized as a negative control was taken from non-immunized rabbits. All samples were tested in duplicate. Anti-Rabbit IgG

alkaline phosphatase conjugate (The Binding Site, Birmingham, UK), diluted 1: 1500 in PBS-T20, was incubated on the plates for 2 hours at 37 degrees before being washed and incubated again for 1 hour. After washing, p-nitrophenyl phosphate substrate (1 mg per ml 0.1 M glycine buffer, pH 10.4) was added, and the plates were then incubated for 20 min at 37 ^{0}C. The color density was measured at a wavelength of 405 nm using a microplate reader (EZ Read 400, Biochrom Ltd, UK). The mean OD plus three standard deviations for the serum samples from normal rabbits was used to determine the cutoff OD for ELISA positive, which was set at 0.219.

2.6. Biochemical Characteristics of the native 44-KDa Toxoplasma antigen

Analytic SDS-PAGE was used to determine the purity of the native 44-kDa antigen (12). Protease or other chemical reagents were used to treat the antigen to analyze some of its native biochemical properties. The antigen was then tested in an ELISA using antisera against the 44-kDa antigen to see whether these treatments affected the antigen epitopes. The pure antigen was treated for one hour at a concentration of 1 mg/ml with 40% TCA (v/v) at 4 ºC and 0.2 M NaOH or 0.2 M HCl (v/v) at room temperature. Periodate oxidation with 20 mM sodium Meta-periodate was carried out at room temperature for an entire night. The reaction was then stopped by adding an equivalent volume of 130 mM glycerol. The antigen sample (at 200 mg/mL) was mixed with an equivalent volume of 20, 60, and 180 mM β-Mercaptoethanol. The purified antigen (1 mg/mL) was incubated with α-Chymotrypsin (1 mg/mL; Sigma) at 37 °C for 5, 10, 15, 30, and 45 minutes in the protease test. Crude tachyzoites and bovine serum albumin were analyzed in parallel as positive and negative controls.

2.7. Cellular and humoral immune responses

2.7.1. Immunization Schedule

Female BALB/c mice (6-8 weeks) were randomly divided into two groups (10 mice per group); **Group I** (control group)**:** Mice received sterilized phosphate-buffered saline (PBS, pH 7.4). **Group II** (immunized group): each mouse was subcutaneously immunized three times with 10 µg of Toxoplasma antigen with an equal volume of Freund's complete adjuvant (FCA) (volume 1:1) and with an equivalent volume of Freund's incomplete adjuvant (FIA) as an adjuvant protein booster, at one-week intervals (i.e. week 1, 2 and 3). The sera of all mice were collected from each group at week 1 after exposure to the target antigen to detect serum concentrations of IL-10 and INF-γ and to detect antibody levels, the serum was collected from each group before at week 0, and after immunization with pure toxoplasma antigen at weeks 2, 4, and 6.

2.7.2. Cytokine analysis

The mice sera were collected from all mice after one week of exposure to the target antigen to evaluate the cellular immune responses. The quantities of IL-10 and IFN-γ were determined using a highly sensitive and specific non-competitive "sandwich-type" ELISA (Biosensis, Thebarton, Australia) according to the manufacturer's instructions using EZ Read 400 (Biochrom Ltd). All assays were carried out in duplicate. The units of two cytokine concentrations were picograms per milliliter (pg/mL).

2.7.3. Detection of total IgG and IgG subclass antibody response in mouse sera

Collected mice sera at 0, 2, 4, and 6 weeks were used to quantify the levels of immunoglobulin. By using the enzyme-linked immunosorbent test (ELISA), antigen-specific IgG antibodies were examined. Microtiter plates were coated with pure Toxoplasma antigen and left overnight at 4 ^{0}C. Mouse sera (50 µL/well) were incubated at 1:750 dilutions after the plates were blocked with 10% bovine serum albumin (BSA) for an hour at 37 ^{0}C. Using p-Nitrophenyl phosphate as a substrate, immune complexes were revealed after bound antibodies were identified using ALP-conjugated anti-mouse IgG (The Binding Site, Birmingham, UK) diluted 1:1500 in PBS-T20. The EZ Read 400 microplate reader (Biochrom Ltd) was used to estimate the OD values at 405 nm. The

assay was performed twice for every serum sample. The mean OD plus three standard deviations for the serum from normal mice was used to determine the cutoff OD for ELISA positive, which was set at 0.219. The isotyping of mouse serum Immunoglobulin was determined using sandwich ELISA based on rat anti-mouse mAbs to IgG1, IgG2a, IgG2b, and IgG3 (The Binding Site, Birmingham, UK). The samples were tested in duplicate.

2.8. Protective effect of the 44-kDa Toxoplasma antigen

Female BALB/c mice (6-8 weeks old) were randomly divided into four groups (10 mice per group). In each group, the mice were monitored every three days for 21 days following the challenge to track the number of days survived and evaluate the rate of death every 3 days. **Group I:** mice were immunized with sterilized PBS, pH 7.4 formulated with an equal volume of Freund's complete adjuvant (FCA). **Group II:** mice were immunized subcutaneously with 10 µg of the purified Toxoplasma antigen mixed with FCA three times at 1-week intervals. An equal volume of Freund's incomplete adjuvant (FIA) was used as an adjuvant protein booster, the next time. **Group III:** mice were immunized with 10 µg of the Purified Toxoplasma Antigen as group II. Two weeks after the last immunization, each mouse was inoculated intraperitoneally with 6.45 $x10^3$ live tachyzoites in 200 µL PBS, pH 7.4. **Group IV:** mice were immunized with sterilized PBS and then inoculated with the 6.45 x 10^3 live tachyzoites in 200 µL PBS, 2 weeks after the last immunization. To confirm the findings, the aforementioned experiment was conducted once more.

2.9. Statistical analysis

SPSS 20.0 for Microsoft Windows, SPSS Inc., Chicago, IL, USA, was used for all statistical analyses. The data were summarized descriptively and provided as mean ±SD. Student's t-tests and ANOVA were used to analyze differences in continuous variables. All tests were two-tailed, and statistical significance was determined at a threshold of 0.05.

3. Results

3.1. Isolation and purification of the target Toxoplasma antigen

The 44-kDa antigen was extracted from Tachyzoites antigen via electroelution, enabling the production of monospecific anti-sera in rabbits that were immunized with the purified antigen, and the purity of the 44-kDa antigen was verified using 12% SDS-PAGE gel. The gel showed a single polypeptide band at the 44-kDa level, which was stained with Coomassie blue **(Figure 1A)**. The reactivity of the 44-KDa protein was confirmed using Western blot analysis **(Figure 1B)**.

3.2. Reactivity and specificity of rabbit IgG antibodies to 44-kDa Toxoplasma antigen

The rabbit anti-44-kDa antigen IgG antibodies exhibited strong reactivity towards both the Tachyzoites antigenic preparation and the purified 44-kDa antigen through ELISA. However, when tested against *Schistosoma mansoni* (SWAP), *Fasciola gigantica* (FWAP), and *Ascaris lumbericoides* (AWAP) antigens, the antibody yielded titers below the threshold for positivity. This confirms that the antibody is specific to Toxoplasma. as shown in **Figure 2.**

3.3. Biochemical Partial characterization of the 44-kDa Toxoplasma antigen

The reactivity of the purified 44-kDa antigen by rabbit-specific anti-Toxoplasma antibodies utilizing ELISA was unaffected by temperatures as high as 56 ^{0}C. Higher temperatures resulted in a reduction of antibody reactivity, demonstrating the conformational character of the discovered antigen **(Figure 3A)**. Furthermore, antibody reactivity to the purified 44-KDa antigen after treatment with acid or alkali was eliminated. However, after meta-periodate oxidization and reduction with β-Mercaptoethanol, the reactivity towards pure 44-

KDa antigen was preserved. The TCA precipitate fraction showed the same reactivity as the pure antigen, but the TCA supernatant fraction showed no reactivity, indicating that the detected antigen epitopes are protein-based **(Figure 3B)**. The reactivity was reduced and disappeared after 40 minutes of incubation with α-Chymotrypsin **(Figure 3C)**.

3.4. Immune responses against the 44-kDa Toxoplasma antigen in mice

3.4.1. Cellular immune responses

In mice immunized with 44-kDa Toxoplasma antigen, the present study revealed the production of a significant quantity of IFN-γ ($P < 0.001$) which is linked with Th1 cells and necessary for defense against intracellular infections. In addition, significant levels of IL-10 ($P < 0.001$) were determined, which is a cytokine linked with Th2 immune response (**Figure 4).** These results can be used as confirmation that immunization with 44-KDa activates both the Th1 and Th2 immune responses.

3.4.2 Specific IgG antibody and IgG Isotypes induced:

For detection of total specific IgG antibody response, besides the IgG isotypes (IgG1, IgG2a, and IgG2b, IgG3) serum samples from two groups were collected on day 0, 14, 28 post-immunization and 2 weeks later post-immunization (on day 42), and anti-*T. gondii* antibodies in mice from all groups were analyzed by ELISA. In the control group (G1), there was no significant difference in IgG antibody levels. Total IgG in mice of G2 increased moderately in the fourth week after immunization with continuous immunization and reached a peak in the sixth week after the last booster immunization as illustrated in **Figure 5A**. To assess the type of immune response (Th1 or Th2) elicited in immunized BALB/c mice, the 44-KDa antigen-stimulated high levels of both IgG1 and IgG2a in immunized mice versus a control group. There was no statistically significant difference ($P > 0.05$) between specific IgG1 and IgG2a levels suggesting that both Th1 and Th2 types of responses were elicited **(Fig. 5B).**

3.5. Immunization with 44-kDa antigen was effective against *T. gondii* infection:

To assess the protective efficacy of 44-kDa protein, intraperitoneal infection with 6.45 x 10^3 tachyzoites of the highly virulent *T. gondii* strain RH. **Figure 6A s**hows that the BALB/c mice of group IV (n=10) died within 6-12 days after the RH strain tachyzoite challenge. Notably, in the 44-kDa immunized group (G2), mice survival time was monitored with the highest survival time. Particularly, the survival time of G3 group mice was prolonged survival time with a 70 % survival rate compared with the control groups I and II ($P < 0.005$). The above experiment was repeated once, and similar results ($p > 0.05$) were obtained as shown in **Figure 6B.**

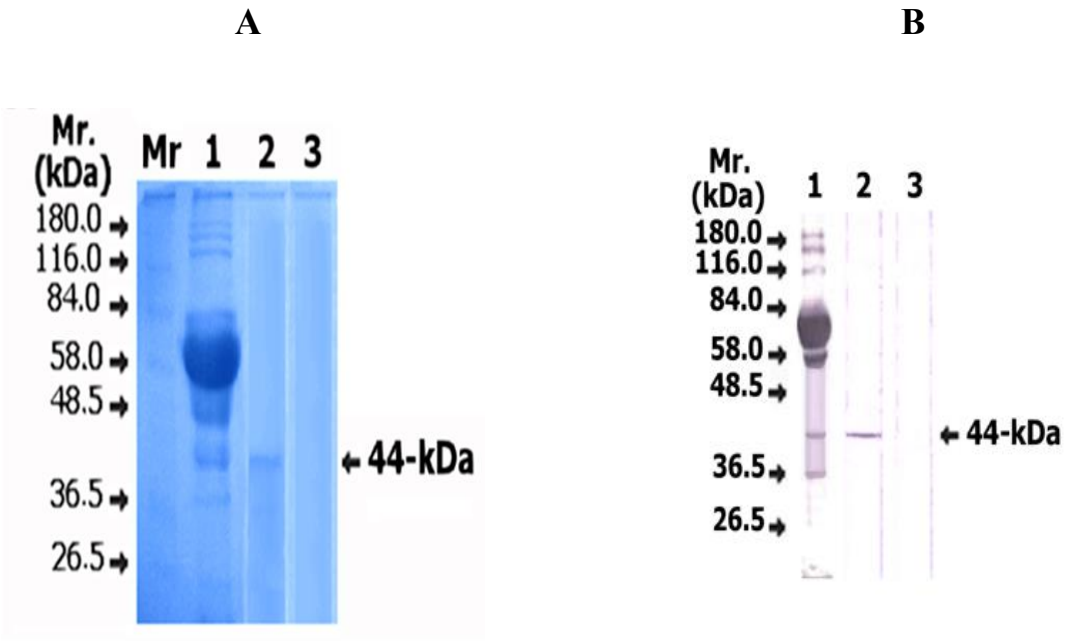


Figure 1. The 44-kDa Toxoplasma antigen was purified from the *T. gondii* tachyzoites antigen. A. The purified 44-kDa antigen was stained with Coomassie blue on SDS-PAGE. **B**. The reactivity of purified 44-KDa was confirmed using Western blot. Lane 1: Crude *T. gondii* tachyzoites antigen, lane 2: Purified 44-kDa fraction from Tachyzoites antigenic preparation, lane 3: Elution buffer. Molecular weight markers (Mr.) include α-2 macroglobulin (180-kDa), β-galactosidase (116-kDa), fructose-6-phosphate kinase (84-kDa), pyruvate kinase (58-kDa), Fumarase (48.5-kDa), lactic dehydrogenase (36.5-kDa), triose phosphate isomerase (26.5-kDa)

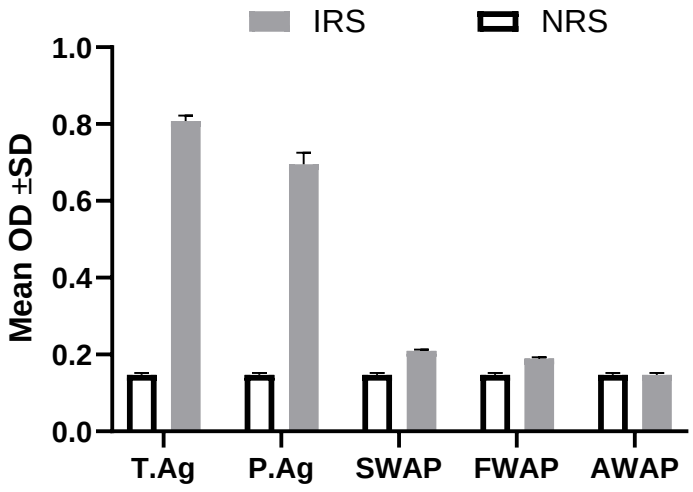
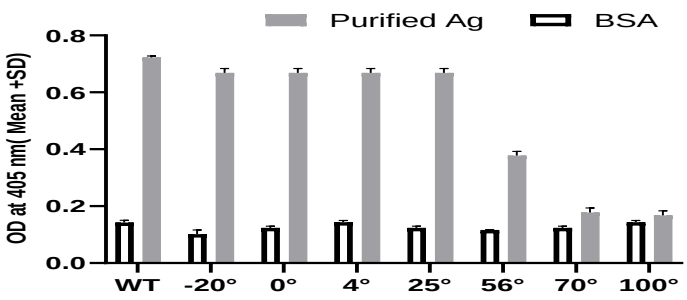


Figure 2. Immunoreactivity and specificity of the rabbit anti-44 kDa Toxoplasma purified antigen IgG antibody using ELISA. The immunized rabbit sera (IRS) showed high reactivity (OD > 0.219) towards the Tachyzoites antigen (T. Ag) and the Purified 44-kDa antigen (P. Ag) but showed no reactivity (OD < 0.219 i.e. below the threshold for positivity OD = 0.219) when tested against the antigens from *Schistosoma mansoni* (SWAP), *Fasciola gigantica* (FWAP), and *Ascaris lumbericoides* (AWAP). The normal rabbit sera (NRS) showed no reactivity towards all tested antigens.

A.



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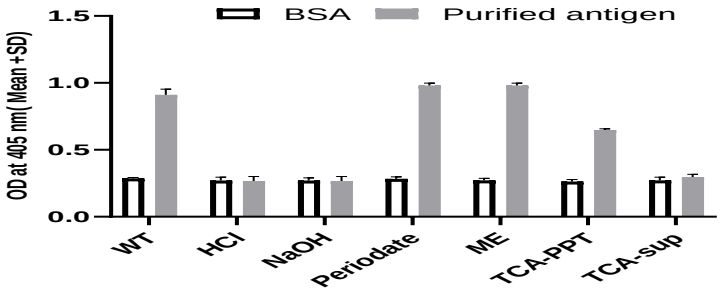

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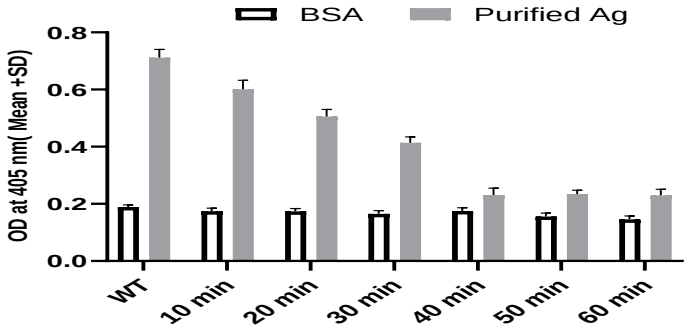
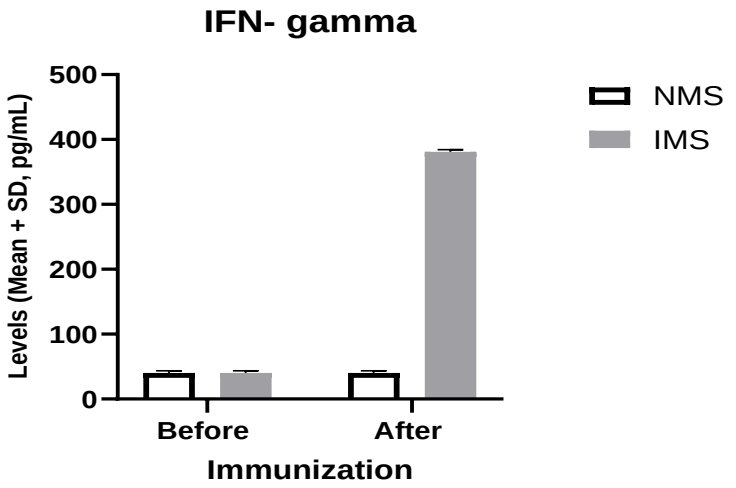


Figure 3. Partial biochemical characterization of native Toxoplasma antigen. A. Reactivity (mean OD value at 405 nm) of specific anti-44-kDa Toxoplasma antigen IgG antibody against the 44-kDa Toxoplasma purified antigen (without treatment; WT) using ELISA after incubation for 15 min at different temperatures. **B.** Reactivity of specific anti-Toxoplasma antibody against 44-kDa Toxoplasma purified antigen after α-Chymotrypsin treatment using ELISA. **C.** Immunoreactivity (expressed as mean OD value at 405 nm) of specific anti-44-kDa Toxoplasma antigen IgG antibody against the 44-kDa Toxoplasma purified antigen (without treatment; WT) after treatment with acid (HCl), base (NaOH), m-Periodate oxidation, β-Mercapto-Ethanol (ME) reduction and Tri-Chloro-Acetic acid precipitation (TCA-ppt) using ELISA.

A



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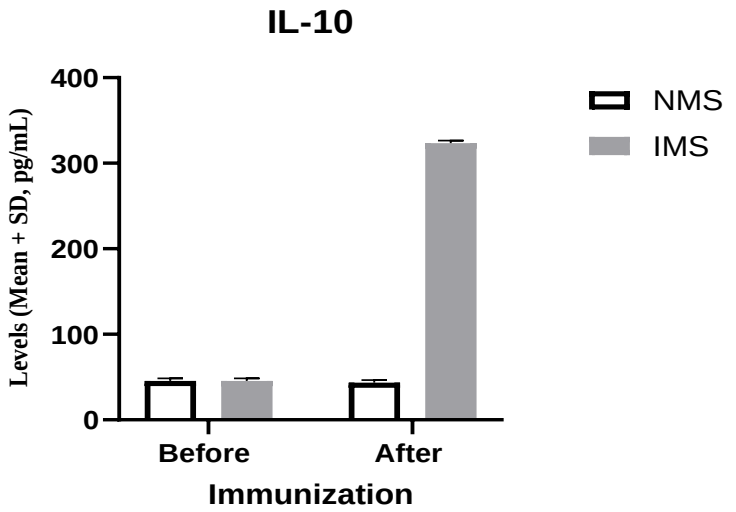
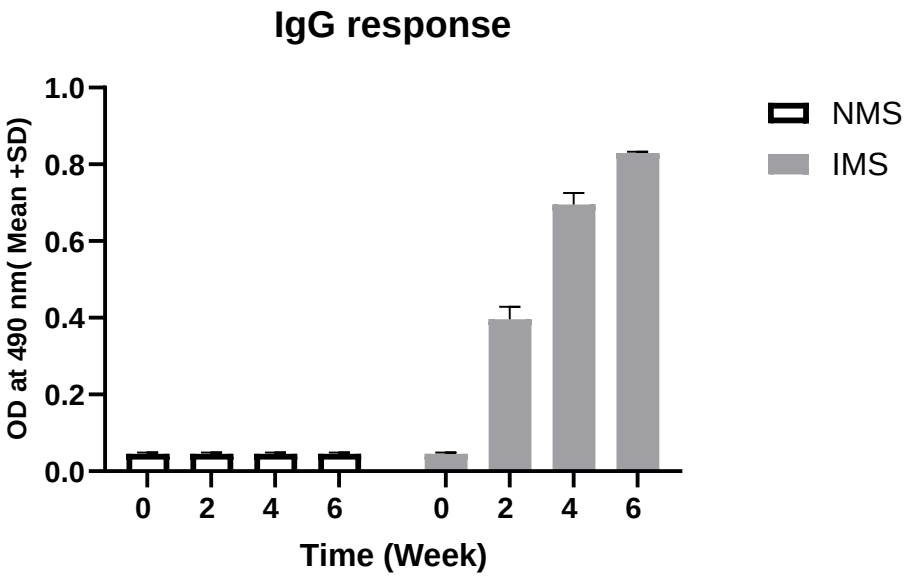


Figure 4. **Serum cytokine analysis of immunized mice stimulated with 44-kDa Toxoplasma Antigen. (A)** Significant levels ($p<0.001$) of Interferon-gamma INF-γ (Th1) were produced after one week. **(B)** Significant levels ($p<0.001$) of Interleukin 10 (IL-10) (Th2) were produced after one week from immunization in comparison with non-immunized sera (NMS).

A.



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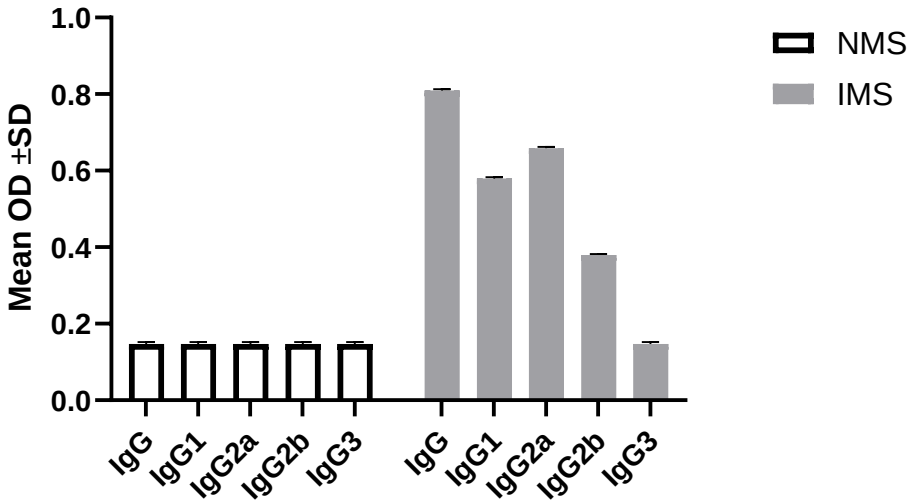
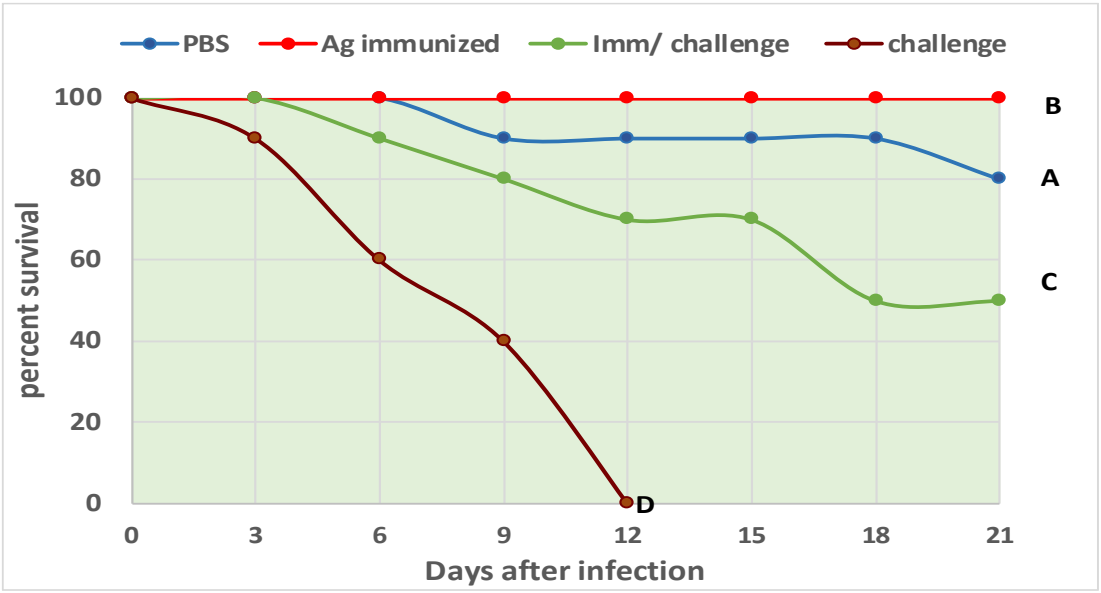


Figure 5. Humoral immune response in BALB/c mice elicited by 44-KDa immunization. **(A)** Reactivity of Sera from BALB/c Mice Immunized with the 44-kDa Purified Toxoplasma Antigen. **(B)** The specific IgG isotype profile was determined in BALB/c mouse serum. The data are provided as means ±SD, with a statistically significant difference ($P < 0.05$) compared to the control group.

A.



B

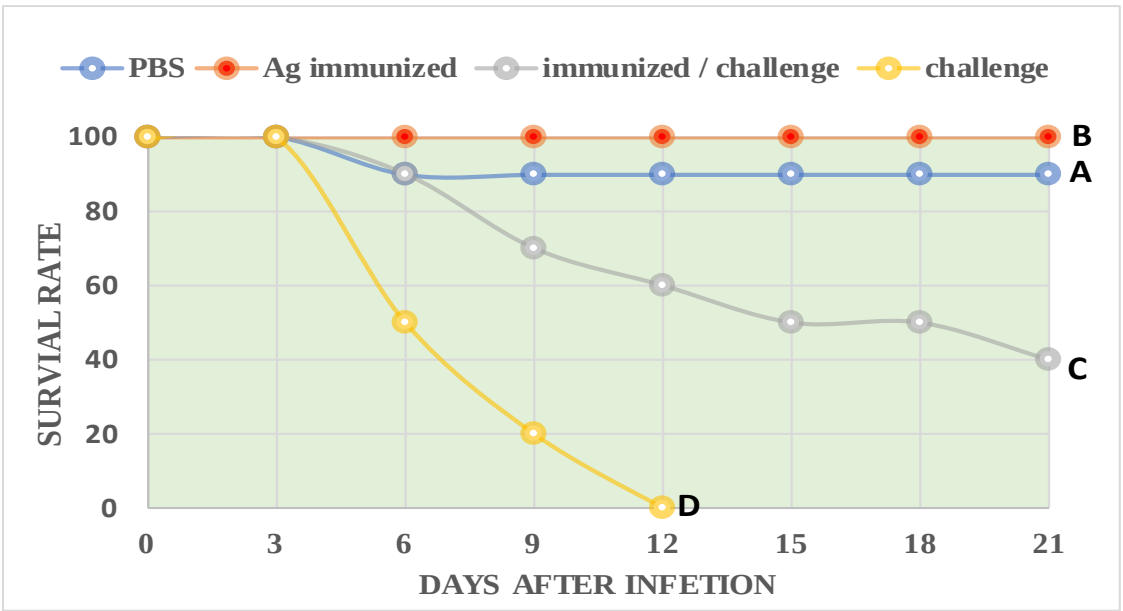


Figure 6. The Kaplan–Meier graph of the survival of immunized BALB/c mice with the 44-kDa *Toxoplasma gondii* antigen after challenge infection with live tachyzoites. (A) Each group consisted of ten mice, and survival times were recorded at three-day intervals for three weeks. Survival of mice in all groups was significantly higher than that of the G 4. **(B)** The experiment was performed once, yielding identical findings ($p > 0.05$).

4. Discussion

Toxoplasmosis is a severe disease that poses an immediate risk to human well-being and has the potential to affect human conduct, personality traits, and other observable characteristics besides psychiatric illnesses. *T. gondii* had a wide geographical range and a significant prevalence in numerous areas, impacting almost one-third of the global population in past periods (14). According to reports, *T. gondii* is an opportunistic pathogen that causes human infections ranging from 10% to 60% and up to 95% in some high-virulence regions (15,16). Numerous endeavors have been made to create a potent vaccination against *T. gondii*. However, owing to the intricate life cycle of this parasite, no efficacious vaccine has been formulated, although certain strategies show greater potential than others. The sole vaccine available for commercial use is Toxovax, which comprises primarily live modified tachyzoites derived from the S48 strain. This vaccine is authorized for use in Europe and is effective in avoiding abortion in sheep. However, it does not provide total protection against parasite infection, particularly in cases involving cyst-forming strains. However, there are several drawbacks to this vaccine, including poor storage conditions, high production costs, side effects, and the possibility of it returning to a pathogenic form (17). It is essential to develop a human vaccine to eliminate infection with toxoplasmosis and begins with vaccinating domestic cats can be a sensible and effective method to create a new veterinary vaccine that can stop the transmission of *T. gondii* from animals to humans and can reduce the occurrence of infection in both livestock and humans (18,19). The present study evaluated the prospect of employing novel virulence-related *T. gondii* 44-KDa protein as a candidate vaccine to prevent toxoplasmosis in the BALB/c mice model and how this protein could be used to prevent the disease. Attallah *et al.* (13) reported that several highly reactive bands in the *T. gondii* Tachyzoite antigenic preparation were found utilizing the western blot using the rabbit anti-*T. gondii* tachyzoites IgG antibodies. One highly reactive band was identified with a molecular mass at 44-kDa using a standard protein mixture. In the present study, the target 44-kDa antigen was isolated and purified from *T. gondii* tachyzoites antigen. The partial biochemical characterization of our target antigen confirmed its protein moiety. In ELISA. The rabbit anti-*T. gondii* tachyzoites IgG antibodies show high reactivity towards native 44-KDa but did not recognize any target epitopes in the other parasites such as Ascaris, Schistosoma, and Fasciola. These findings confirm the specificity of anti-44 KDa. The $CD8^+$ T-cytotoxic lymphocytes, which are crucial for cell-mediated immunity, as well as B cells, which are crucial for the humoral immune response, are required for protection against *T. gondii* infection (20,21). The antibodies made by B cells are significant immunological effectors triggered by vaccines. These antibodies can recognize and selectively bind to a pathogen or toxin (or to a part that resembles it). Antibody binding has two effects: it can prevent a pathogen from entering host cells or it can make it easier for other immune cells to take up and eradicate the infection. Another important immune effector is the cytotoxic CD8+ T cell, which can halt infectious diseases from spreading by releasing specific cytokines or by recognizing and destroying infected cells. CD4+ T helper (Th) cells provide growth factors and signals that aid in the development and maintenance of B and CD8+ T-cell responses (22). Mice immunized with 44-KDa showed markedly elevated levels of total IgG antibodies in the serum compared to mice treated with PBS and the concentration of 44-KDa-specific IgG antibodies in the blood of vaccinated mice showed a progressive increase, particularly between the fourth and sixth weeks of each booster immunization. Nevertheless, the IgG levels in the control mice did not exhibit any rise and remained consistently low throughout the entire experiment. Therefore, the 44-kDa vaccination successfully induced significant humoral and cellular immune

responses. Lakhrif *et al*. (23) illustrated that IgG antibodies had a significant role in protecting against *T. gondii* infection, limiting further infection, and reducing the reactivation of cysts during chronic infection **[23]**. Any effective vaccine can stimulate a balanced immune response comprising both Th1 and Th2 cells, with a little tendency towards the Th1-type response (24,25). Consequently, we identified the antibody isotypes induced by 44-KDa immunization through the assessment of IgG1 and IgG2a antibody subclasses and mice inoculated with native 44-KDa protein exhibited a balanced Th1/Th2 immune response that is characterized by comparable levels of IgG1 and IgG2a antibodies. Our results also are corroborated by the cytokine assay performed on serum, which revealed that the levels of Th1 cytokines (IFN-γ) and Th2 cytokines (IL-10) were significantly elevated in mice inoculated with pure 44-KDa compared to the control mice, and these results aligned with prior studies (2, 26-28). Our findings demonstrate that immunization with 44-KDa stimulates both cellular (Th1) and humoral (Th2) immune responses in BABL/c mice. The activities of T helper cells are significantly regulated by cytokines. They are primarily divided into Th1 type and Th2 type cytokines based on the distinctions in their functions (29). The amount of IFN-γ during a natural invasion by *T. gondii* dictates how the infection progresses. Immunity-related GTPases (IRGs), guanylate binding proteins (GBPs), inducible nitric oxide synthase (iNOS), and the inhibitory protein guan amine 2,3-dioxygenase (IDO) are just a few of the mechanisms by which IFN-γ can prevent *T. gondii* from replicating in infected cells. Tryptophan is depleted by IDO, and *T. gondii* cannot develop without it. To limit the reproduction of parasites, iNOS can produce the extremely toxic metabolite nitric oxide and consume arginine, which is also required for *T. gondii* growth. *T. gondii* is removed from the cytoplasm of the infected cells when the parasitophorous vacuole is destroyed, a process that can be disrupted by IRGs and GBPs (30). As part of the Th1 response, excessive doses of IFN-γ can cause immunopathological damage, which is why IL-10 plays a crucial role as a regulating cytokine. The 44-KDa immunized mice's Th1/Th2 cytokine balance was sufficient to prevent tachyzoites from spreading, but it wasn't high enough to cause appreciable inflammation (31). The most straightforward measure of a potential vaccine's efficacy is the percentage of vaccinated mice that can still be alive due to the *T. gondii* challenge. To test the mice's ability to survive, we administered intraperitoneal injections of tachyzoites from the extremely dangerous *T. gondii* RH strain (32). In our study, within 12 days of the RH tachyzoite test, every mouse in group IV perished. After contracting RH tachyzoites, the group of mice immunized with 44-KDa exhibited a more prolonged survival period that achieved (70%) survival rates. Therefore, mice vaccinated with 44-KDa had a considerable and efficient increase in their survival time, compared to those non-immunized mice, and our study was replicated and yielded consistent findings in which the mice that were inoculated with 44-KDa had an extended survival period when infected with RH tachyzoites, resulting in a survival rate of 60 %. This result agreed with **Şahar *et al.,*** (27) who observed that the mice challenged orally with *T. gondii* tachyzoites RH strain died within 13 days and the immunized mice achieved 70% survival rates. Chuang *et al.,* (33) also reported that 80% (8/10) of mice immunized with recombinant surface antigen 1 protein encapsulated in poly (lactide-co-glycolide) microparticles survived at least 28 days after a lethal subcutaneous tachyzoite challenge. According to Zheng *et al.* (28), there was a noteworthy rise in the survival rate among the groups that received rROP5 or rSAG1 vaccinations in isolation as opposed to the control group ($P < 0.05$ in both cases). In addition, mice immunized with rROP5 + rSAG1 had a longer survival period (12.1 ± 3.4 days; $P < 0.05$) than the groups that received single-antigen or control vaccinations. In conclusion, we have examined the effectiveness of a novel virulence-related *T. gondii*

tachyzoite, a native 44-KDa protein, and subcutaneously immunization of BABL/c mice with 44-kDa can elicit a protective immune response, involving both humoral and cellular components, and a balanced Th1 and Th2 response against *T. gondii* infection. Additionally, it can prolong the survival duration of the mice. Therefore, the antigen demonstrated a favorable ability to stimulate an immune response in the BALB/c mice models against *T. gondii* infection and could be considered as a candidate vaccine. Further research into the immunological alterations associated with purified antigen and their importance in avoiding infection will be recommended. Furthermore, the research suggests that it can be generated using recombinant DNA and be successful in reducing the risk of Toxoplasma infection as an initial move toward human immunization.

Authors contributions

All authors confirm contribution to the manuscript as follows: HI and AA Study conception and design, writing original draft. AS and MA Methodology and collection of data. AS, MA and HI Analysis and interpretation of results. All authors approved the final version of the manuscript.

Availability of data

The corresponding author can provide the original data upon request (Email: himosman@mu.edu.eg).

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Finding:

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