

# The Immunogenicity and Immunoprotection Identification of Adhesion Protein 65 (AP65) of *Trichomonas Vaginalis*

**Zhenchao Zhang**

Xinxiang Medical University <https://orcid.org/0000-0002-3386-9947>

**Xiaoxiao Song**

Xinxiang Medical University

**Zhengbo Zhang**

Xinxiang Medical University

**Haoran Li**

Xinxiang Medical University

**Yujuan Duan**

Xinxiang Medical University

**Hao Zhang**

Xinxiang Medical University

**Haoran Lu**

Xinxiang Medical University

**Chengyang Luo**

Xinxiang Medical University

**Shuai Wang**

Xinxiang Medical University

**Mingyong Wang** (✉ [wmy118@126.com](mailto:wmy118@126.com))

Xinxiang Medical University

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## Research article

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# Abstract

**Background:** Adhering to the epithelial lining along the urogenital track of the host is the prerequisite for *Trichomonas vaginalis* (*T. vaginalis*) to inflict its parasitism and pathogenicity, causing urogenital infection. The AP65 of *T. vaginalis* (TvAP65) involves in the process of adhesion. So, the present study was aimed at investigating the molecular characterization and vaccine candidacy of TvAP65 for protecting the host from the onset of Trichomoniasis.

**Methods:** The open reading frame (ORF) of TvAP65 was amplified and then inserted into pET-32a (+) to clone recombinant TvAP65 (rTvAP65). The immunoblotting determined the immunogenicity and molecular size of TvAP65, while immunofluorescence staining visualized and the precise localization of TvAP65 in *T. vaginalis* trophozoites. The animal challenged with the enzyme-linked immunosorbent assay (ELISA) test was used to evaluate the immunoprotection and the types of the immune response of TvAP65.

**Results:** By the sequence analysis, TvAP65 encoded a 63.13 kDa protein that aligned 567 amino acid residues together with a high antigenic index. The western blotting then revealed that rTvAP65 and native TvAP65 could interact with the antibodies in the rat serums post hoc rTvAP65 immunization and the serums from the mice that were experimentally infected with *T. vaginalis*, respectively. Immunofluorescence stained TvAP65 on the surface of *T. vaginalis* trophozoites. Moreover, following emulsification with Freund's adjuvant, rTvAP65 was subsequently administered to BALB/c mice three times at 0, 2, and 4 weeks and the results from this animal challenge experiments showed significant increases in immunoglobulins of IgG2a, IgG1, and IgG, and proinflammatory factors of IFN- $\gamma$ , and IL-2, and 10. Lastly, rTvAP65 vaccinated animals had a prolonged survival time ( $26.80 \pm 4.05$ ) after challenged by *T. vaginalis*.

**Conclusions:** TvAP65 mediated the adhesion of *T. vaginalis* to the host epithelia for the pathogenesis of the parasite and can be considered as a candidate protein for designing a functional vaccine that induces cell-mediated and humoral immunity against the *T. vaginalis* infection.

## Background

Trichomoniasis caused by *T. vaginalis* has been one of the most prevalent sexually transmitted diseases. The WHO reported that *T. vaginalis* infected approximately 276 million people worldwide in 2008 [1, 2], which showed rise to an increase of 11% in comparison with the number in 2005 [3]. Moreover, the United States had claimed approximately 5 million populaces getting *T. vaginalis* infection annually, and Japan also published its rate of *T. vaginalis* infection as 24.3% in females. Also, the prevalence of *T. vaginalis* infection in the underdeveloped nations and areas such as rural Uganda and South Africa had been projected to be 23.8% and 18.0%, respectively [4]. Clinically *T. vaginalis* infection in females can be complicated by trichomonas vaginitis, cervicitis and atypical pelvic inflammation [5]. In pregnant women, *T. vaginalis* may cause premature membrane rupture, premature delivery, and abortion. In recent years,

studies have shown that *T. vaginalis* infection may predispose females to cervical neoplasm and infertility [6]. and males can also be the victims of *T. vaginalis* for the development of urinary tract disease including prostate cancer. As one of the pathogens of sexually transmitted diseases, *T. vaginalis* can raise the risk of coinfection with other sexually transmitted diseases including human immunodeficiency virus (HIV) infection [7, 8].

At present, trichomoniasis can be effectively therapeutized by Metronidazole in clinical treatment [9, 10]. However, more and more clinical studies confirm the generation of Metronidazole-resistant *T. vaginalis* strains and shortcomings of other surrogate drugs against *T. vaginalis* [11]. Therefore, considerable studies have been undergoing for developing novel therapeutic agents to treat *T. vaginalis* infection. Besides, many researchers have proposed that either DNA vaccines or recombinant antigens could effectively stimulate immune responses against *T. vaginalis* [12], thus, the vaccine inoculation could be an optimal approach to eradicate infectious diseases like trichomoniasis. However, to date, the commercialized anti-trichomoniasis vaccine has not been available in current clinical settings [13, 14]. The discovery of vaccines depends on the identification of candidate antigens in *T. vaginalis*.

Adhesion to the epithelia of the urogenital tract by *T. vaginalis* as an early but critical step for developing infection depended on the adhesion proteins including AP120, 65, 51, 33, and 23 [15–17]. Among these proteins, AP65 is a dominant functional protein, which is not only a part of hydrogenosomes but also mediates binding to the cells of its host [18, 19]. Garcia et al. found that TvAP65 was a more crucial adherent protein of trichomonads than other adhesins, and functioned as the hydrogenosomal NAD-dependent decarboxylating malic enzyme [20]. *T. vaginalis* can bind to erythrocytes to obtain lipids and iron, and iron acquisition from hemoglobin in this process is achieved by the action of AP51 and AP65 [17]. Besides, many previous studies showed that the amount of AP65 secreted by *T. vaginalis* increased upon contact with host cells, and this protein was expressed and transcriptionally modulated by iron [20].

Anti-AP65 serum IgG antibodies could inhibit the adherence of live *T. vaginalis* to the epithelia of the host, and the antibody-binding epitopes of AP65 were localized to the N-terminal sequence of this protein [20]. In addition, the receptor-binding epitope of AP65 was also located at the amino terminus. Further studies showed that the adhesion of TvAP65 to the host cell surface was determined by the polypeptide formed by the N-terminal 1–25 amino acid in TvAP65, which may constitute the epitope binding to the surface receptor on host cells [21]. Analysis of the AP65 protein sequence revealed that AP65 contained malic enzyme and oxidized coenzyme I binding sites [22]. AP65 in the secreted protein preparation was successfully internalized by vaginal epithelial cells, resulting in induced signaling in vaginal epithelial cells for the expression of genes of IL-8 and COX-2 [21]. The TvCyP1 cyclophilin can inhibit and activate the expression of the AP65 gene by regulating Myb-like transcription factor 1 (Myb1) and Myb3, respectively [23]. Moreover, AP65 was identified to be immunogenic and prevalent throughout *T. vaginalis* by immunoscreening and immunofluorescence assays (IFA) [24].

To date, the immunogenicity of AP65 remains undefined, so this study was aimed to characterize its immunogenicity through in vitro and in vivo experiments.

## Methods

### The ethics statement for this study

All animal experiments were conducted under the ethical guideline and regulations issued by the Animal Ethics Committee of Xinxiang Medical University (Reference No. 2015016), which guided the researchers to make all efforts to alleviate various sufferings of the animals during experiments. In this study, the infected mice were euthanized at the humane endpoints when the mice appeared moribund. Euthanasia was executed by confining the animals in a closed space with 60–70% CO<sub>2</sub> for five minutes.

### Preparation of experimental animals and *T. vaginalis*

Six weeks old BALB/c female mice and SD rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and bred under a specific pathogen-free (SPF) environment.

The strain of *T. vaginalis* used in this study was isolated from the vaginal discharges of the patients with the diagnosis of trichomoniasis, placed in TYM medium containing 50 mg/mL ciprofloxacin, 100 mg/mL ceftriaxone, 2.5 mg/mL amphotericin B and 10% calf serum, and cultured in a humidified chamber containing 5% CO<sub>2</sub> with the temperature of 37 °C. For further experiments, 2×10<sup>6</sup> parasites were harvested at their stationary phase, which was confirmed as the actin genotype E strain by PCR-restriction fragment length polymorphism (PCR-RFLP).

### Preparation of soluble proteins from *T. vaginalis* trophozoites

Approximate 5×10<sup>7</sup> *T. vaginalis* trophozoites were harvested and washed by the centrifugation three times with the speed of 2500 rpm for ten minutes with 0.1 M PBS (pH 7.2). After the pellet of trophozoites was re-suspended in 2 mL PBS, the mixed resuspend underwent repeated freeze-thaw cycles three times at the temperatures of -20 and 4 °C to disintegrate the parasite plasma membranes. For purifying the parasite protein, the lysed parasitic mixture was first sonicated on the ice at a speed of 60 W/s, then centrifuged at 12000 rpm for 30 min at 4 °C, and thereafter, the concentration of the protein in the supernatant was measured with the Bradford method, and finally, the soluble proteins from *T. vaginalis* were aliquoted and stored at -70 °C for future uses.

### Total RNA extraction from *T. vaginalis*

The E.Z.N.A.<sup>TM</sup> Total RNA Kit I (OMEGA, Zhengzhou, China) was used to extract total RNA from *T. vaginalis* trophozoites, and the extracted RNA was re-suspended with the DEPC-treated water, which was further treated with ribonuclease inhibitor (TaKaRa, Dalian, China), and RNase-free DNase I (TaKaRa) to

rid contamination of the genomic DNA for conducting reverse transcription. Purified RNA with the ratio of OD260/OD280 in between 1.9 and 2.0 was considered to reach the required purity.

## Cloning TvAP65

Trophozoite cDNA was obtained by RT-PCR, and then the open reading frame (ORF) of TvAP65 (GenBank accession no. U35243.1) was amplified from the cDNA with a *Bam*HI-anchored forward primer (5'-CGCGGATCCATGCTCGCATCT TCAGTCGC-3') and *Xho*I-anchored reverse primer (5'-CCGCTCGAGTTAGTA GAGTTGCTCGTATTCAGCC-3'), and cloned into the pMD19-T vector (TaKaRa). Afterward, the cloned recombinant pMD19-T-TvAP65 was sequenced and then transformed in *E. coli* (DH5a) competent cells purchased from Yi Fei Xue Biotechnology (Nanjing, China) for the future amplification of TvAP65. The online sequence check (<http://www.ncbi.nlm.nih.gov/BLAST/>) was blasted to verify the sequence homolog between the fragment of rTvAP65 and the sequence in the GenBank.

## Bioinformatics analysis of sequences

The homology between the cloned TvAP65 and AP65 in the Genebank was completed through BLASTX and BLASTP (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the amino acid sequence of TvAP65 underwent the alignment analysis with CLUSTALW1.8, and the putative motifs, possible secondary structures, and potential signal peptides were predicted by applying the online analysis tools and programs as previously reported [25].

## Protein purification of TvAP65 and pET-32a

TvAP65 fragment was successfully sub-cloned into pET-32a (+) expression vector system (Novagen, USA) from the recombinant plasmid pMD19-T-TvAP65, and subsequently confirmed to be inserted in the right place of the recombined plasmid. Afterward, sequencing confirmed recombinant plasmid pET-32a-TvAP65 was introduced into competent *E. coli* BL21 (DE3), and when OD 600 of the culture reached 0.6 at 37°C, isopropyl-b-D-thiogalactopyranoside (IPTG; Sigma–Aldrich, USA) was added into the bacterial growth culture media to induce the recombinant protein expression. Then, following five more hours incubation with IPTG at 37°C, the bacteria were harvested and further lysed with 10 mg/mL lysozyme (Sigma–Aldrich, USA). Lastly, the extracts from lysed bacteria were run through 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The recombined TvAP65 protein was then purified through a Ni<sup>2+</sup>-nitrilotriacetic acid (Ni-NTA) column (GE Healthcare, USA), and post-purification purity was determined by a 12% SDS-PAGE gel [26]. After measuring the concentration, the rTvAP65 protein was stored at -20°C for future experiments. Additionally, through the same process, the pET-32a proteins with 109 amino acid residues composed of 6 histidines and Trx•Tag<sup>TM</sup> thioredoxin protein were obtained for future uses.

## Production of Anti- rTvAP65 serums

SD rats were inoculated by subcutaneously injecting a combo of Freund's complete adjuvant with 0.3 mg purified rTvAP65 protein at the ratio of 1:1 to different sites on the rats for producing antigen-specific polyclonal antibodies. 14 days later, the rats were boosted then 4 rounds of injections with the combo of Freund's incomplete adjuvant with 0.3 mg purified rTvAP65 protein as above at a 7-day interval. After completing the immunization, the polyclonal serums were made and stored for the use in the next experiments. The serums used as negative control were made before the first injection above [27]. Moreover, for generating antiserum against *T. vaginalis*, the parasites were injected into mice, and serums were collected ten days post-infection.

## Immunoblot analysis of TvAP65

SDS-PAGE separated the proteins containing recombinant TvAP65 as well as soluble trophozoite proteins of *T. vaginalis*, and the separated proteins were then transferred to the nitrocellulose membrane (Millipore, Shanghai, China). Following the transfer, the membranes were incubated with either mouse (recombinant TvAP65) or rodent antisera (soluble trophozoite proteins of *T. vaginalis*) as primary antibodies at the concentration of 1:100 or 1:200 respectively for one hour; afterward, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or anti-rat IgG (Sigma, Shanghai, China) as the secondary antibodies were added in the incubation accordingly for another 1 hour. All the incubations were carried out at 37°C environment. The proteins were detected with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen (Boster Bio-Technology, Wuhan, China).

## Localization of TvAP65 in trophozoites of *T. vaginalis*

Harvested *T. vaginalis* trophozoite cells were smeared on a poly-L-lysine coated glass slides for 15 minutes drying. The mounted slides underwent the 10-minute fixation with 4% paraformaldehyde PBS at room temperature, after that, the parasites on the slides were permeabilized with 1% Triton X-100 solution for 10 minutes. Finally, the slides were blocked at 37 °C with PBST containing 4% (w/v) BSA for one hour. For the immunofluorescence study, sequentially the slides with the parasites were first incubated either with the rat anti-TvAP65 serum or the control serums at the dilution ratio of 1:100 for overnight at 4 °C; further with goat anti-rat IgG antibody labeled with Cy3 (Beyotime, Shanghai, China) at dilution ratio 1:1,000 in the dark for 40 minutes; then with DAPI (Beyotime) to stain the nuclei for 5 min; and lastly with fluorescent mounting medium (Beyotime). During the staining procedure, PBS was continuously used to wash the slides. Finally, the processed slides were visualized under laser confocal microscopy (Nikon, Beijing, China).

## Immunization and challenge infection

80 BALB/c mice with the age of 6 weeks old were randomized into 4 groups of 20 each, and then immunization combos were made including the rTvAP65 mixture of 100 µg of rTvAP65 with Freund adjuvant at the ratio of 1: 1, the pET-32a mixture of 100 µg of pET-32a protein with Freund adjuvant at the ratio of 1:1, and the adjuvant mixture of Freund adjuvant alone. For testing the immunogenicity of rTvAP65, the mice in the first three groups were injected subcutaneously either with the rTvAP65 mixture, or pET-32a mixture, or adjuvant mixture, while the fourth group of mice was designated as the blank control without the inoculation of any kind. The procedure was carried out as previously reported [25]. Ten days after the last vaccination, the mice all were given the intraperitoneal injection of  $1 \times 10^7$  trophozoites of *T. vaginalis*, and then under surveillance on the infectious and survival status throughout the entire period after challenge. If any animal presented with the infectious symptoms caused by *T. vaginalis*, they were euthanized by CO<sub>2</sub>.

Thirty days after the challenge with *T. vaginalis*, the survival rate of mice was calculated with the following formula: the number of survived mice after immunization / the total number of mice before immunization  $\times 100\%$ .

## Determination of antibody levels in serums

The blood samples from mice in each group ( $n = 5$ ) were collected at 0, 2, 4, and 6 weeks. Serum was isolated from the blood sample and stored at  $-20^\circ\text{C}$  for evaluating antibodies and measuring cytokines. IgG isotypes and anti-TvAP65 antibodies in the serums were quantitatively studied with indirect ELISA [12]. Briefly, the wells of microtiter plates (Costar, New York, NY, United States) were first coated by rTvAP65 (2.5 µg/mL, 100 µL/well) in the carbonate buffer with a pH value of 9.6 at  $4^\circ\text{C}$  for overnight, and then blocked with 4% BSA at  $37^\circ\text{C}$  for 2 hours. Mice serums were prepared by dilution with the addition of PBS at a ratio of 1 to 10. Afterward, the diluted mice serums were added into the plates for 2 hour incubation at  $37^\circ\text{C}$ , thereafter, the plates were rinsed with PBST three times and sequentially treated with the HRP-conjugated secondary antibodies goat anti-mouse IgG2a, IgG1, and IgG, (SouthernBiotech, Birmingham, AL, United States). For conducting ELISA, 100 µL of 3, 3', 5, 5'-tetramethylbenzidine was pipetted into each well of the plates, the reaction was terminated with 100 µL (2 M) sulfuric acid. The 450 nm absorption was used to read the plates by an automatic ELISA reader (MULTISKANFC, Thermo Scientific, Waltham, MA, United States), and all plates were read in triplicate.

## Measurement of the secretory levels of various cytokines

The secretory levels of pro-inflammatory cytokine were determined in the serum from all experimental rodents. Interferon-gamma (IFN- $\gamma$ ), and interleukin-2, 4, 10, 17 were measured using commercially available ELISA kits (Boster, Wuhan, China) with the recombinant IFN- $\gamma$ , IL-17, 10, 4, and 2 as the corresponding controls for quantification. The data obtained from three individual experiments were further analyzed.

# Statistical analyses

One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to analyze the differences among different experimental groups. The survival-related data were analyzed using the Kaplan–Meier method. SPSS for Windows 16 (SPSS Inc., Chicago, IL) was used for all the statistical analyses, and  $P < 0.05$  indicated statistical significance.

## Results

### Cloning and sequence analysis of TvAP65

The ORF of rTvAP65 was 1704 bp (Fig.1A), which encodes a protein of 567 amino acids with a molecular weight of 63.13 kDa. Through sequence analysis, the ORF of TvAP65 was decoded as a protein with 70 basic, 67 acidic, 205 hydrophobic, and 122 polar amino acids with a theoretical Isoelectric point (pI) of 7.94. When comparing with the known proteins and DNA sequences in the NCBI gene bank (<http://www.blast.ncbi.nlm.nih.gov/blast.cgi/>), the TvAP65 nucleotide sequence was 96% identical to the *T. vaginalis* malate dehydrogenase (XM\_001579690.1) and hydrogenosomal malic enzyme subunit A proprotein (U16836.1) genes. The TvAP65 protein sequence showed 96% homology with the malate dehydrogenase of *T. vaginalis* (XP\_001579740.1) and 94% homology with the hydrogenosomal malic enzyme subunit C protein of *T. vaginalis* (AAA92716.1) in NCBI. No GPI anchors, transmembrane domains, or signal peptides were found in the sequence of TvAP65, but three O-glycosylation sites, and thirty-five phosphorylation sites were contained in the protein. As shown in Fig. 1B, the sequence of TvAP65 had nine hydrophilic regions, 28—73, 84—155, 192—234, 256—281, 297—306, 332—372, 420—466, 500—520 and 540—567, nine highly antigenic consecutive regions, 28—77, 87—108, 124—173, 194—234, 269—282, 299—207, 328—459, 479—522 and 533—567, and this flexible regions accounted for most of the TvAP65 sequence. Interestingly, the protein also composes of one malate dehydrogenase region, one NAD(P) binding domain of the malic enzyme site, and three malic enzyme domains.

### Expressing and purifying recombinant TvAP65

The supernatant of bacterial sonication was run on the SDS–PAGE. Ensuing to Ni-NTA chromatography, rTvAP65 was isolated at the size of 82 kDa by SDS–PAGE gel (Fig. 2A), which should be 63.13 kDa after deducing 18 kDa fused protein.

### Analysis of the recombinant and native TvAP65 by immunoblot

The immunoblot showed that rTvAP65 could interact with the serums from the mice with the experimentally induced *T. vaginalis* infection, but not with the serums from the controls (Fig. 2B). The western blotting with the rat anti-TvAP65 serum further confirmed that 70 kDa band was in accordance with the native TvAP65 protein of *T. vaginalis* trophozoites (Fig. 2C) but slightly larger than the predicted molecule weight.

## Location of TvAP65 in *T. vaginalis* trophozoites

The location of TvAP65 in *T. vaginalis* was unveiled by immunofluorescence staining with anti-rTvAP65 (Fig. 3). In comparison with the negative control, the TvAP65 was visualized to be mainly localized on the surface of trophozoites after expressing TvAP65.

## Protective effect of rTvAP65 inoculation to the experimental rodents

BALB/c mice were grouped and subjected to three sequential immunizations with the recombinant TvAP65 as an antigenic vaccine against *T. vaginalis* (Table 1) and then injected with  $1 \times 10^7$  of *T. vaginalis* trophozoites into the peritoneal cavities of the mice. The survival rate was obtained after the challenges with *T. vaginalis* (Fig.4), finding that the survival rate in the rTvAP65 immunized group was significantly higher than that of the mice either treated with Freund adjuvant only or the adjuvant mixed with pET-32a protein, within which 75–80% of mice died from the infection in eighteen days. Moreover, the mice with the rTvAP65 injection had significantly longer survival time than the ones injected with the adjuvant alone or adjuvant and pET-32a protein in response to *T. vaginalis* infections ( $26.80 \pm 4.05$ ,  $P < 0.05$ ).

Table 1  
Immunization protocol in experimental and control groups

Groups	1st(0 Day)	2nd (14 Day)	3rd (28 Day)
Blank control	No immunity	No immunity	No immunity
Adjuvant control (μl)	200	200	200
pET-32a protein control (μg)	100	100	100
Recombinant TvAP65 protein (μg)	100	100	100

## Humoral immunity

In order to evaluate the immune response to the three sequential vaccinations, the serum levels of IgG and its subclasses were measured every time following vaccine giving. In comparison, the IgG serum

levels in the mice with the injections of rTvAP65 were significantly elevated more than those in the control ( $P < 0.001$ ). Additionally, the OD value of IgG kept increasing in response to rTvAP65 injection, and the IgG titers peaked after the third vaccination. The controls could not be found to have significant differences in IgG levels (Fig. 5A). Besides, the levels of IgG2a and IgG1 in mice with the subjection of TvAP65 made the highest levels ( $P < 0.001$ ; Fig. 5B and C), but the serum concentrations of IgG2a were lower than that of IgG1, indicating Th2-type mediated cell immunity might be induced by rTvAP65.

## Measurement of cytokine concentration in serums of rTvAP65 immunized mice

The serum samples were obtained from the vaccinated mice with the injections of TvAP65, pET-32a protein, adjuvant alone at weeks 0, 2, 4, and 6 for determining the concentrations of serum IFN- $\gamma$ , and IL-2, 4, 10, 17. The results indicated that rTvAP65 induced significant raises in the concentration of serum IFN- $\gamma$ , IL-2, and IL-10 (Fig. 6A, B, and D) in comparison with those in the control animals at weeks 0, 2, 4, and 6 after the vaccination by rTvAP65 ( $P < 0.001$ ), and following the third immunization, IFN- $\gamma$ , and IL-2 and 10 reached their peak levels in response to the injections of TvAP65. However, the IL-4 and 17 levels in the TvAP65 immunized mice were not found to be significantly different from the levels in the controls (Fig. 6C and E).

## Discussion

Trichomoniasis is a very prevalent sexually transmitted diseases has not been completely controlled with the current management regime due to refractoriness. Since vaccination can block pathogens with the maximal cost-effectiveness [28, 29], the vaccine against *T. vaginalis* has been suggested as the alternative method to possibly eradicated infections and complications caused by *T. vaginalis*. However, the very first challenging step in developing vaccines is to identify a potential candidate antigen for being a putative targeting site for the vaccine. Based up underlying pathogenesis of the infection by *T. vaginalis*, colonization of *T. vaginalis* in the epithelium of urogenital tract is the triggering step for the development of trichomoniasis [30]. The previous studies reported that the colonization depended on several adhesins including adhesin protein 120, 65, 51, 33, and 23 [31, 32], all of which were found to be located on the surface of *T. vaginalis* with the correlation between the quantification of adhesins and the affinity of adherence. Among all the adhesins, TvAP65 has got our attention. On one hand, as the prominent trichomonad adhesin, TvAP65 is a hydrogenosomal NAD-dependent decarboxylating malic enzyme [20]. On the other hand, after secreted from the parasite, TvAP65 can be internalized into host cells to subsequently induce the gene expression of IL-8 and COX-2 in host cells [21]. Thus, TvAP65 displayed a dual role in parasitism as well as regulation of host cells. Besides, the localization research in the present study verified the location of TvAP65 is at the surface of trophozoites, therefore, this adhesion protein possesses the potentiation as the targeting site for a vaccine.

In the current study, we focused on the molecular properties of TvAP65 and the immune protective effect of TvAP65 against *T. vaginalis*. As shown in the experiments, the TvAP65 DNA was sequenced to be 1804 bp, which includes a 1704 bp ORF that can encode a protein with 567 amino acid and the predictive molecular weight of 63.13 kDa. The DNASTAR analysis on the protein sequence predicted that TvAP65 possesses the extensively distributed hydrophilic and flexible regions, which contribute to high surface probability and antigenic index, indicating antigenicity of TvAP65.

Furthermore, the cloned TvAP65 had been further blasted with the sequences in NCBI databases, showing that rTvAP65 possesses 96% homology to those of malate dehydrogenase of *T. vaginalis*. In addition, further identifying that the TvAP65 sequence contains a malate dehydrogenase region, an NAD (P) binding domain of malic enzyme site, and three malic enzymatic domains. Malate dehydrogenases catalyze the interconversion of malate to oxaloacetate, while malic enzymes catalyze the conversion of pyruvic acid to malic acid [33]. Thus, the regulatory function of TvAP65 on malic acid metabolism in the pathogenicity of *T. vaginalis* needs further investigation.

Sequence analysis further found a lack of similarity in the epitope sequences of TvAP65 to its human homolog such that potentially catastrophic adverse reactions with autoimmunity could be circumvented for its future applications. Besides, TvAP65 also functioned through the stages of the life cycle of *T. vaginalis*, and was expressed in all isolates of *T. vaginalis* [34, 35]. Taken all together, TvAP65 was qualified to be the ideal candidate for vaccine development.

Moreover, a band representing the 70 kDa protein in the extract from trophozoites was western blotted out by the anti-rTvAP65 serums, suggesting that post-translational modifications on the native TvAP65 may cause larger molecular weight than its the predicted weight of 63.13 kDa. Based on the sequence analyses, the TvAP65 protein might undergo the processes of phosphorylation and glycosylation. Also, serum samples from the *T. vaginalis* trophozoites infected mice could specifically bind to the recombinant TvAP65 in the western blot, demonstrating that TvAP65 induces humoral immunity against TvAP65.

Pathogen-specific antibodies not only regulates the immune reaction, but also can block the pathogens from binding to its specific receptors on the cell surfaces [36], and recruit macrophage to phagocytose the cells with intracellular parasites [37]. In this study, the IgG serum levels were significantly raised in the *T. vaginalis* infected mice immunized with rTvAP65 in comparison with that in mice with the control vaccine. Further measurement on IgG subclasses found a higher level of IgG1 than IgG2a, indicating that TvAP65 may induce a Th2-mediated immune response against *T. vaginalis*.

At present, several studies focused on the development of effective vaccines against *T. vaginalis* [29, 38]. Intraperitoneal administration of *T. vaginalis* was used to establish an infectious mouse model for studying the vaccine candidate against *T. vaginalis*. Several studies evidenced that the initial efficiency of the mimicry with intraperitoneal inoculation of *T. vaginalis* was higher than that of vaginal infection [39,

40]. Thus in the present study, the protective nature of rTvAP65 against *T. vaginalis* infection was evaluated by injection of  $1 \times 10^7$  trophozoites into the peritoneal cavity of the mice.

Furthermore, *in vivo* protective capacity is the crucial criterion for assessing the efficacy of a vaccine candidate [28, 41]. The survival time and rate of the vaccinated animals following the challenge with living parasites are the most acceptable approach for investigating the protective effect of a vaccine. In our study, rTvAP65 possesses a higher protective effect (55%) than that of the controls. Moreover, the results from the survival assay revealed longer survival time ( $26.80 \pm 4.05$  days) after rTvAP65 vaccination. Taken together, rTvAP65 could induce specific immune responses in BALB/c mice against *T. vaginalis* infections, however, whether the immune responses induced by rTvAP65 can prevent the mice from trichomoniasis and subsequent diminish the related mortality still needs long term outpatient follow-up.

Cytokines can activate Th1/Th2 cells [42], such as interferon-gamma (IFN- $\gamma$ ) that activates Th1 cells against infections [43], and IL-2 that fight off infections through expediting T cells to differentiate into effector and memory T cells when being stimulated by an antigen [44]. Moreover, the B cell proliferation, differentiation, and maturation, as well as the differentiation of CD4<sup>+</sup> T cells to Th2 cells are activated and regulated by IL-4, a cytokine marker of Th2 cells [45]. Our study found that rTvAP65 could induce the secretion of IL-2, IFN- $\gamma$ , and IL-4, suggesting that as a vaccine candidate rTvAP65 might trigger a Th1 as well as Th2 immune responses.

IL-10 enhances the B cells through downregulating the production of Th1 cytokines and expression of MHC class II antigen and costimulatory molecules [46] through its inhibitory regulation on the activity of NF- $\kappa$ B [47], and the JAK-STAT signaling pathway [48]. Xie et al. generated the recombined  $\alpha$ -actinin subunit of *T. vaginalis* as a vaccine candidate, which significantly boosted IL-10 secretion [39]. However, in our study, rTvAP65 did not significantly induce the expression of IL-10 in mice primed with rTvAP65, even though it did augment the IL-4 expression. Moreover, IL-17 is secreted from Th17 cells and induces numerous immune signaling molecules [49, 50] to regulate pro-inflammatory responses [51], and allergic responses. Although Th17 cells belong to a subset of CD4<sup>+</sup> cells [52], rTvAP65 was not capable of regulating IL-17 levels. Thus, the reason that TvAP65 could not stimulate the expressions of IL-10 and IL-17 needs to be further investigated.

## Conclusions

As a surface protein of *T. vaginalis* trophozoites, TvAP65 possesses immunogenicity, which could trigger an immune response against *T. vaginalis*. Thus, the present study established TvAP65 as a novel antigen for being used as a protein vaccine against *T. vaginalis* infection, although the underlying mechanism of adhesion to the epithelia of urogenital tracts exerted by TvAP65 still needs to be further defined.

## Abbreviations

TV: *Trichomonas vaginalis* (*T. vaginalis*); AP65: adhesion protein 65; WHO: World Health Organization; HIV: human immunodeficiency virus; TYM: Trypticase Yeast extract Maltose; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; ORF: open reading frame; HRP: horseradish peroxidase; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; IPTG: isopropyl- $\beta$ -D-thiogalactopyranoside; BSA: bovine serum albumin; Ni-NTA: Ni<sup>2+</sup>-nitrilotriacetic acid; DAPI: 4',6-diamidino-2-phenylindole; Cy3: cyanine3; DAB: 3,3'-diaminobenzidine tetrahydrochloride; ELISA: enzyme-linked immunosorbent assay; IFN- $\gamma$ : interferon gamma; IL-2: interleukin-2; IL-4: interleukin-4; IL-10: interleukin-10; IL-17: interleukin-17.

## Declarations

## Ethics approval and consent to participate

The study was reviewed and approved by the Ethics Review Committee of Xinxiang Medical University (Reference No. 2015016).

## Consent for publication

Not applicable.

## Availability of data and materials

All of the data in the present research are contained in the article.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

Data curation, SW; Formal analysis, XXS; Investigation, ZCZ; Methodology, XXS, YJD, HZ, HRL and CYL; Project administration, MYW; Software, HRL; Visualization, ZCZ; Writing – original draft, ZCZ; Writing – review & editing, MYW and SW. All authors read and approved the final manuscript.

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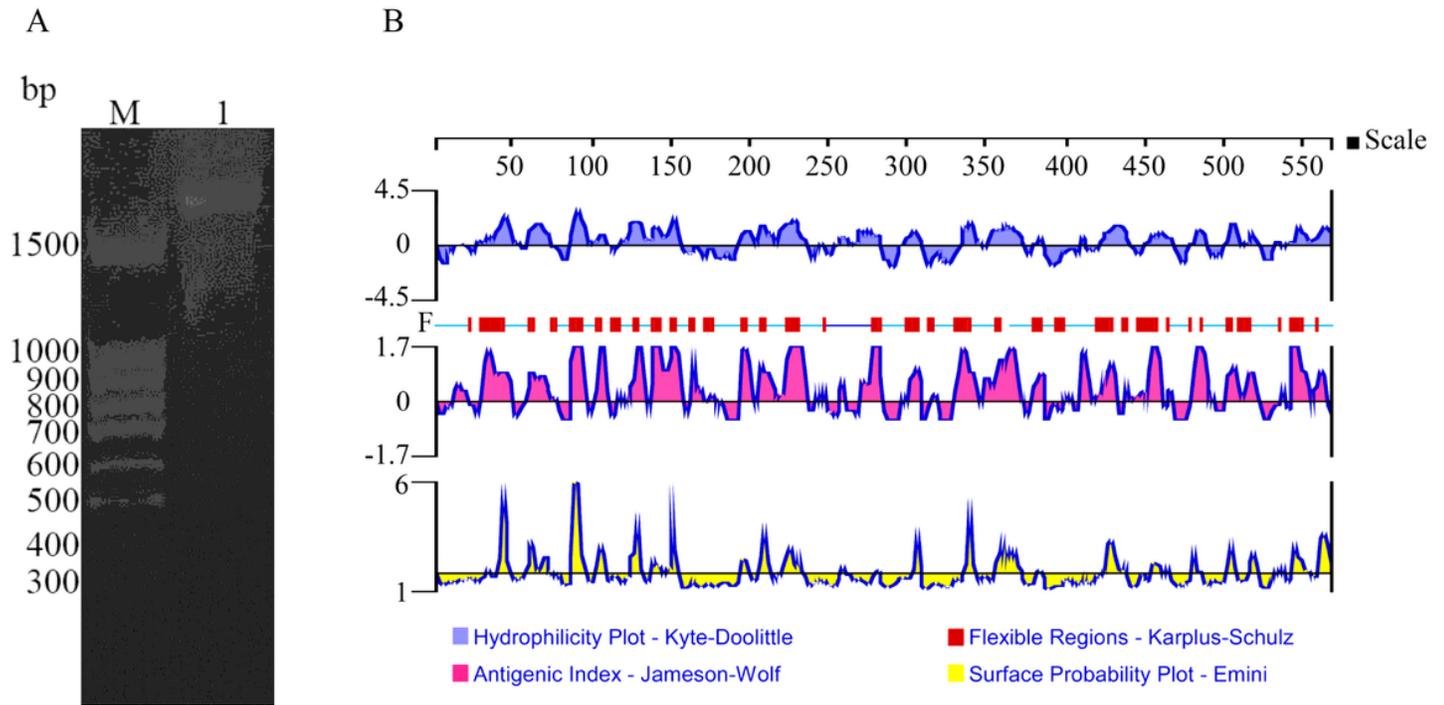
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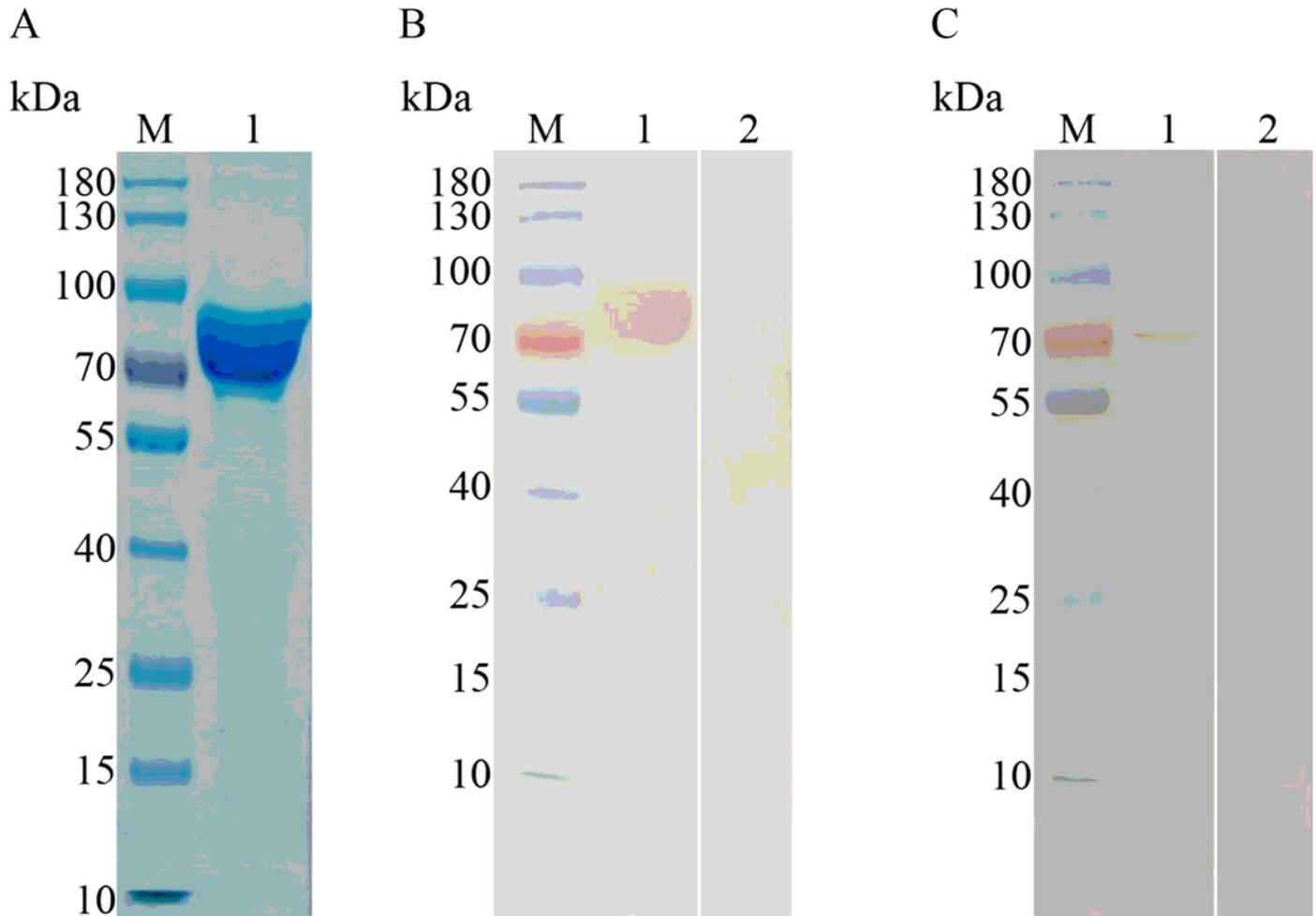
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## Figures



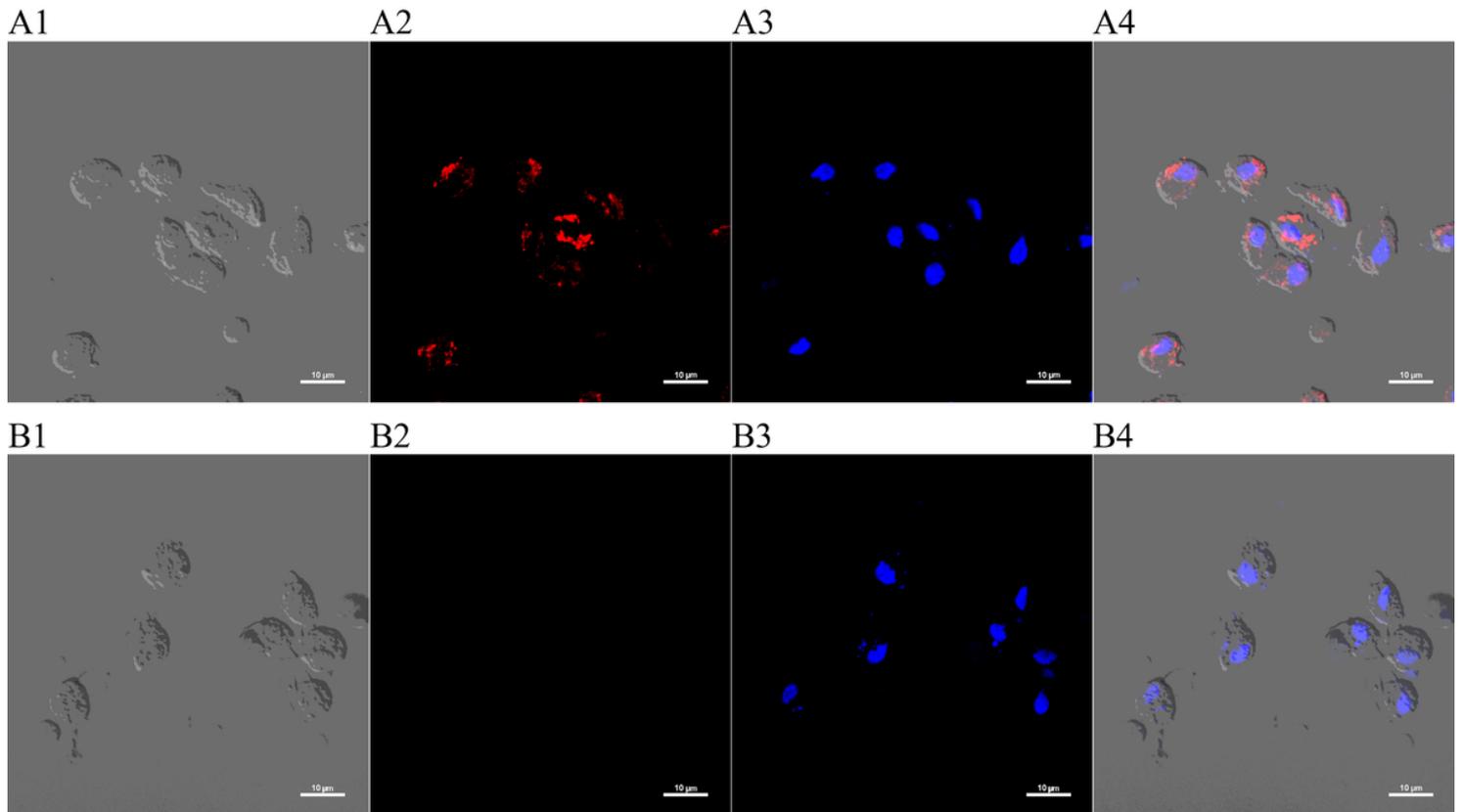
**Figure 1**

Amplification and bioinformatics analysis of the TvAP65 gene. A: The TvAP65 ORF displays in an Agarose gel after electrophoresis. (Lane 1: The rTvAP65 ORF; Lane M: Molecular weight marker DL for 1500bp); B: The linear B cell epitopes prediction of TvAP65 by DNASTAR based on hydrophilicity plot, flexible regions, antigenic index, and surface probability rules.



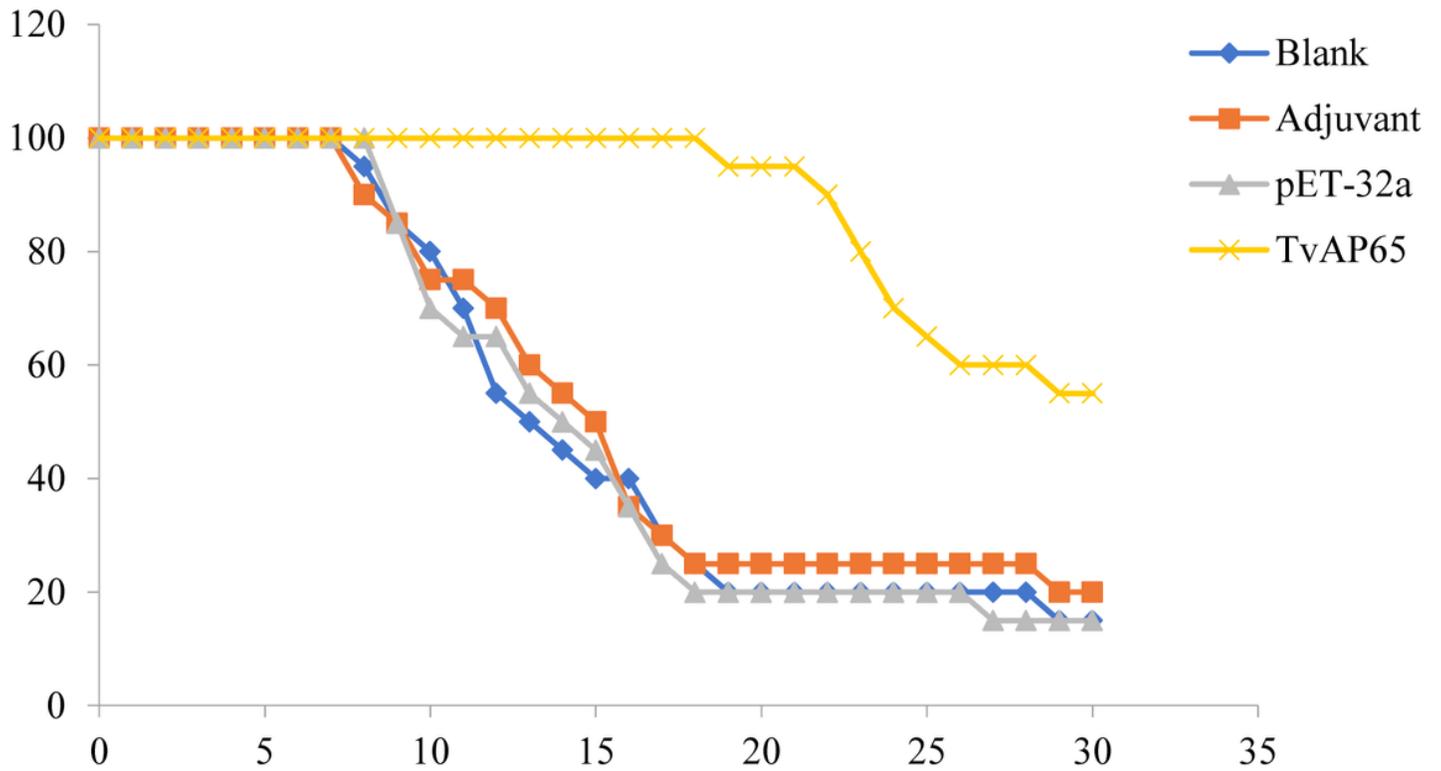
**Figure 2**

A: Purified rTvAP65 protein on SDS-PAGE. (Lane M: Molecular weight standard in kDa; Lane 1: The recombinant TvAP65). B: Immunoblot for rTvAP65. (Lane 1: The TvAP65 protein was blotted with the serums from the *T. vaginalis* infected mice; Lane 2: The rTvAP65 protein was blotted with the serums of normal mice). C: Immunoblot of crude somatic extracts from *T. vaginalis* trophozoites. (Lane 1: The crude somatic extracts from *T. vaginalis* trophozoites was blotted with the serums from the rTvAP65 immunized rats; 2: the crude somatic extracts from *T. vaginalis* trophozoites was blotted with the serums from non-immunized rats).



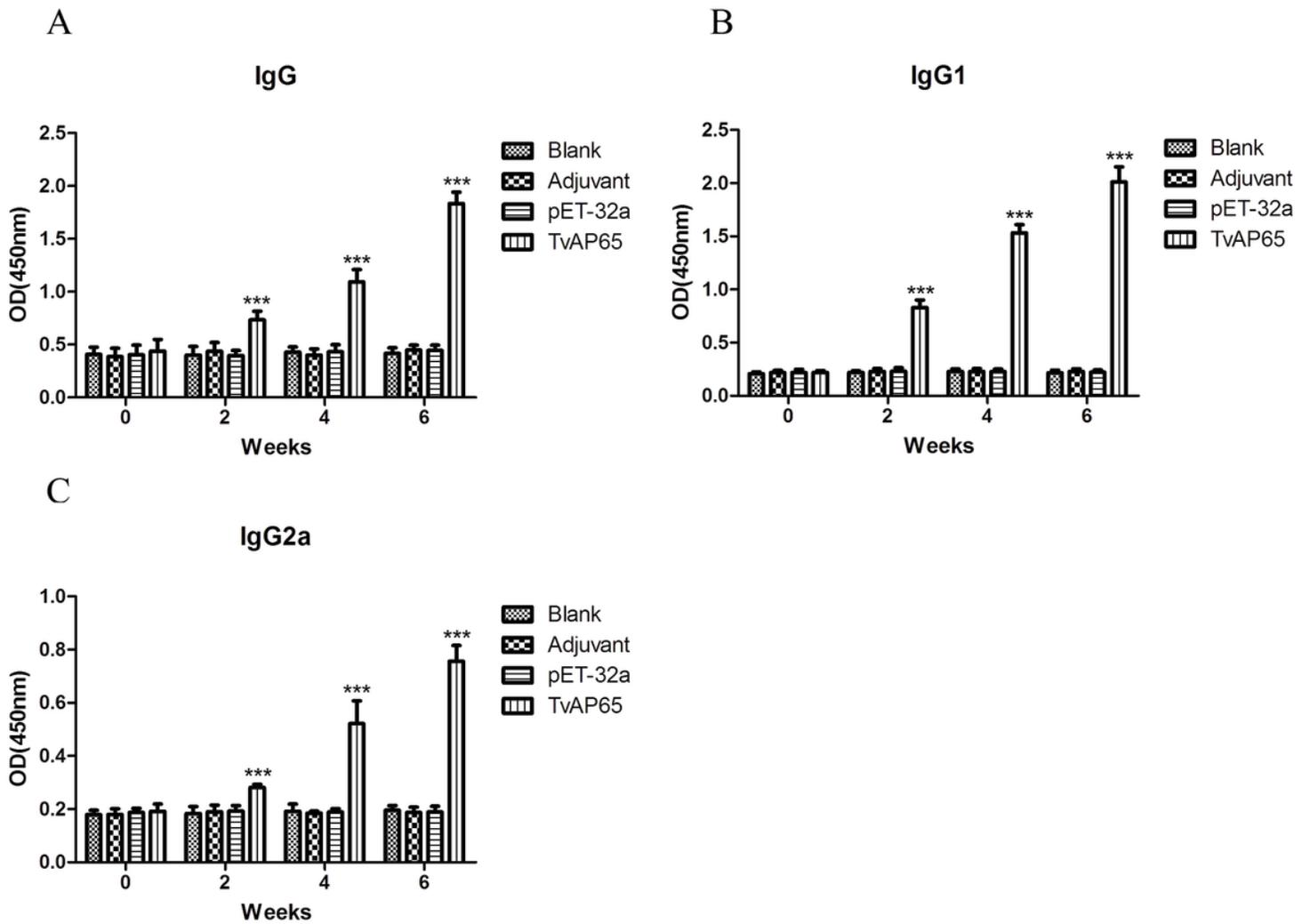
**Figure 3**

Expression and localization of TvAP65 protein in *T. vaginalis* trophozoites by immunofluorescence assay ( $\times 40$  magnification). Panel A: *T. vaginalis* trophozoites were probed with serum from rats immunized with TvAP65 protein. (A1) Differential interference contrast (DIC). (A2) Immunofluorescence localization using Cy3. (A3) Nuclei were stained with DAPI. (A4) DIC, Cy3 and DAPI merged. Panel B: Negative control, trophozoites were probed with serum from normal rats without immunization as the primary antibody. (B1) DIC. (B2) Cy3. (B3) DAPI. (B4) Merged.



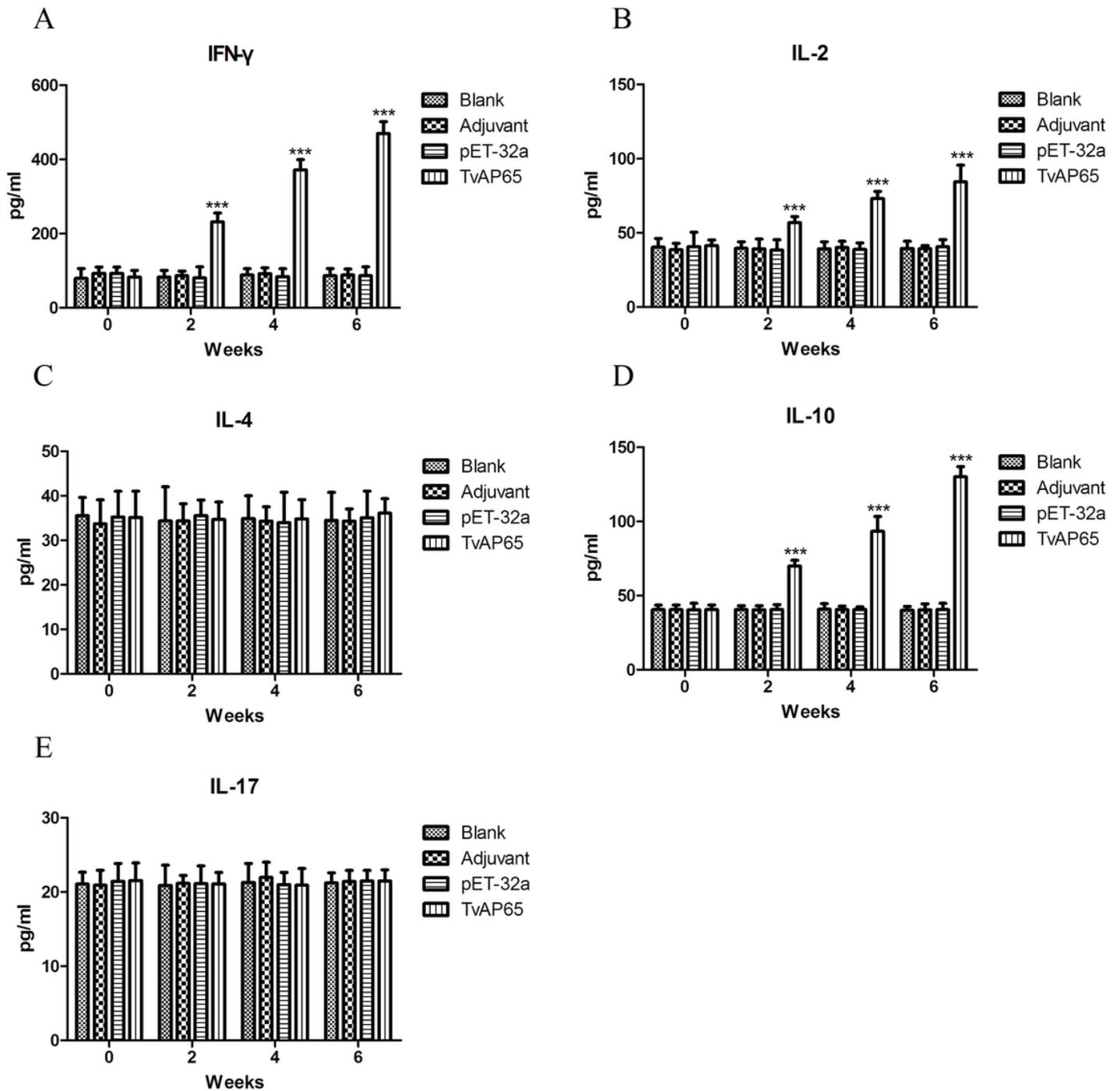
**Figure 4**

Survival curve of the mice following intraperitoneal injection of 107 *T. vaginalis* trophozoite actin genotype E strain ten days after the last vaccination.



**Figure 5**

The dynamic humoral immune reaction of BALB/c mice in response to the vaccination with rTvAP65 protein. 20 BALB/c mice were evenly divided into 4 groups in the randomized fashion. The mice in three experimental groups were immunized with a mixture of Freund adjuvant with either the rTvAP65 (1:1), or pET-32a protein (1:1), or the adjuvant alone, while the fourth group was taken as a negative control. The serum titers of IgG as well as its subclasses IgG1 and IgG2a were determined by reading the absorption at 450 nm at weeks 0, 2, 4, and 6. The expression levels were presented as the mean  $\pm$  SD. For designating the significant differences between groups at identical time points, (\*) refers to  $P < 0.05$ , (\*\*) to  $P < 0.01$  and (\*\*\*) to  $P < 0.001$ . A: IgG. B: IgG1. C: IgG2a.



**Figure 6**

Measurement of cytokine secretion. 20 BALB/c mice were randomized into four groups evenly, th which were vaccinated by the mixture (1:1) of Freund adjuvant with either rTvAP65 or pET-32a protein or Freund adjuvant alone or nothing. Then the concentrations of cytokines were quantitatively measured by ELISA assays at weeks 0, 2, 4, and 6, and the results are presented as the mean  $\pm$  SD with the unit of pg/mL.  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$  represent statistical significances and was labelled as (\*), (\*\*), and (\*\*\*), respectively. A: IFN- $\gamma$ . B: IL-2. C: IL-4. D: IL-10. E: IL-17.

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