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Original Article

Bioinformatic Prediction and Production of Four Recombinant Proteins from Different Developmental Stages of *Trichinella spiralis* and Testing of Their Diagnostic Sensitivity in Mice

Cheng-Cheng ZHAI [1,2], Xiao-Lei LIU [3], Xue-BAI [3], Ze-Jun JIA [2], Shao-Hong CHEN [1], Li-Guang TIAN [1], Lin AI [1], Bin TANG [3], Ming-Yuan LIU [3], *Xiu-Ping WU [1], *Jia-Xu CHEN [1]

1. *National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, WHO Collaborating Center for Tropical Diseases, Key Laboratory of Parasite and Vector Biology, National Health and Family Planning Commission, Shanghai 200025, China*
2. *Editorial Department of Chinese Journal of Clinical Medicine, Department of Research, Zhongshan Hospital, Fudan University, Shanghai 200032, China*
3. *Key Laboratory for Zoonoses Research, Ministry of Education, Institute of Zoonoses, Jilin University, Changchun 130062, China*

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***Correspondence**
Email:
wuxp@nipd.chinacdc.cn

Abstract

Background: Trichinellosis is a serious food-borne parasitic zoonosis, thus finding high quality antigens is the key to serodiagnosis of trichinosis. This article reports the characterization and sensitivity of four recombinant proteins expressed by four genes (*Wn10*, *Zh68*, *T668*, and *Wm5*) from different developmental stages of *Trichinella spiralis* for the diagnosis of trichinellosis in mice.

Methods: This study was conducted in Jilin University and National Institute of Parasitic Diseases of Chinese Center for Disease Control and Prevention in 2017-2018. The structures and functions of the proteins encoded by four genes were predicted by bioinformatics analysis. The four genes were cloned and expressed, and the recombinant proteins were purified. Anti-*Trichinella* IgM and IgG antibodies in the sera of mice infected with *T. spiralis* from 1-45 d post-infection (dpi) were evaluated by ELISA.

Results: The optimal antigen epitopes of four proteins (P1, P2, P3, and P4) encoded by the four genes from T- and B-cells were predicted, and four purified recombinant proteins (r-P1, r-P2, r-P3, and r-P4) were successfully produced. For IgM, the antibody levels detected by the four recombinant antigens were approximately equal to the cut-off value. Anti-*Trichinella* IgG antibodies were first detected by r-P1 at 8 dpi, followed by r-P2, r-P3, and r-P4 at 10 dpi, 14 dpi, and 16 dpi, respectively, and the antibody levels remained high until 45 dpi.

Conclusion: The recombinant antigens r-P1, r-P2, r-P3, and r-P4 could be antigens that react with antibodies, they showed high sensitivity in the detection of anti-*Trichinella* IgG antibodies in mice. Among these proteins, r-P1 may be a candidate antigen for the detection of anti-*Trichinella* IgG antibodies in the early infection phase and exhibited the best sensitivity among the antigens.

Introduction

Trichinellosis, a serious food-borne parasitic zoonosis, is caused by ingestion of raw or inadequately cooked meat that contains infectious larvae of the nematode of the genus *Trichinella* distributed worldwide (1,2). From 1986 through 2009, 65,818 cases and 42 deaths were reported in 41 countries (3,4). Thus, trichinellosis is a serious threat to the health of both animals and humans and public health concern in many countries (5). However, non-specific clinical symptoms make this disease very difficult to diagnose, particularly at the early stages (6). Molecular assays, such as PCR (7) and loop-mediated isothermal amplification (LAMP) (8) may be useful for meat inspection in slaughterhouses for quality control by food authorities, and in medical laboratories. Serological tests, such as western blot analysis (9) and ELISAs for the detection of anti-*Trichinella* antibodies are valuable approaches for the diagnosis of human trichinellosis (10). The sensitivity and specificity of the assays used are determined by the quality of the selected antigens (11).

The most commonly used antigen for the diagnosis of trichinellosis is muscle larvae (ML) excretory-secretory (ES) antigens, but cross-reactions often occur (12,13). Besides, a variety of diverse antigens are expressed at different developmental stages (14,15), which may explain why ML ES is not recognized by antibodies induced by *Trichinella* in the intestinal stage and the false negative results observed during the early stages of infection (16). ES antigens from four developmental stages of *Trichinella spiralis* were used to detect the *Trichinella*-specific IgM and IgG antibody in our previous study, however, the results were not satisfactory (17).

In our previous study, several antigenic genes were detected at different developmental stages of *T. spiralis,* including the cystatin-like gene *Wn10* (screened from intestinal ML at 6 h post-infection), serine protease gene *Zh68* (screened from three-day-old adult worms), serine protease gene *T668* (screened from newborn larvae), and serpin gene *Wm5* (screened from ML) (18). Cystatin (19) and serine proteases (20) play significant roles in the life cycle of *T. spiralis*. In the present study, some of the structures and functions of proteins encoded by these four genes were predicted by bioinformatic analysis. Subsequently, the four genes were cloned and expressed in an *E. coli* expression system, and the four recombinant proteins (r-P1, r-P2, r-P3, and r-P4) were purified. Anti-*Trichinella* IgM and IgG antibodies derived from the sera of mice infected with different doses of *Trichinella* from 1-45 dpi were analysed by ELISA, and the sensitivity of the four recombinant antigens were compared and evaluated. The data obtained in this study will provide basis for future studies.

Materials and Methods

Parasites

T. spiralis (T1, ISS534 isolates) was maintained by serial passage in BALB/c mice at the Institute of Zoonoses, Jilin University (Jilin, China). Larvae were collected by artificial digestion using a standard protocol (21). The animals were treated strictly according to the National Institutes of Health guidelines (publication no. 85–23, revised 1996).

The animal protocols were approved by the Ethical Committee of Jilin University, affiliated with the Provincial Animal Health Committee, Jilin Province, China (Ethical Clearance number IZ-2009-08). This study was conducted in Jilin University and National Institute of Parasitic Diseases of Chinese Center for Disease Control and Prevention in 2017-2018.

Serum samples

BALB/c mice have orally infected with *T. spiralis* larvae. Sixty mice were randomly divided into four groups (fifteen mice per group): group-1 mice were infected with 30 larvae, group-2 mice with 100 larvae, group-3 mice with 200 larvae, and group-4 mice with 300 larvae. Tail blood was collected at 1-45 dpi from the four groups of infected mice, and serum samples were prepared. Serum samples obtained from mice before infection served as negative controls. The serum samples were stored at -80 °C until use.

Bioinformatic analysis of the encoded proteins

Bioinformatic methods were used to predict the structures and functions of the proteins (P1, P2, P3, P4) encoded by *Wn10*, *Zh68*, *T668* and *Wm5*. The GenBank accession numbers of P1, P2, P3, P4 were ABY60755, ABY60762, AAR36900, ABI32311, respectively. The hydrophilic and hydrophobic domains analysis was performed using the expert protein analysis systems ExPASy-ProtScale (K-D methods) and ExPASy-ProtParam. TMHMM is a method for prediction of transmembrane helices, based on the hidden Markov model. Prediction of structural domains was conducted by using the SMART tool. The secondary structures were predicted by ExPASy-SOMPA. Homology modelling by SWISS-MODEL was used to predict the tertiary structures. By combining the prediction results obtained from the two websites BceP-red and ABCpred and by comparing with the results predicted by Protean, the optimal candidate region for the linear epitopes of the B lymphocyte antigen of the four proteins was predicted. Simultaneously, four websites, namely, SYFPEITHI, BIMAS, IEDB and NetMHC, were used to predict the T lymphocyte antigen epitopes.

Preparation of recombinant proteins

After isolating *T. spiralis* intestinal ML at 6h post-infection stage, three-day-old adult worms stage, newborn larvae stage and ML stage, four types of RNAs were extracted using RNA extraction kits (Takara, Japan). Then, the cDNAs were used as templates to amplify the target genes by PCR. Primer sequences were designed based on the gene sequences in NCBI Gene (Table 1). Four genes were cloned into the expression vector pET-28a (+), digested by *Eco*R Ⅰ and *Xho* Ⅰ restriction enzymes and the four recombinant plasmids containing the four genes were transformed into *E. coli* BL21. Then, the four recombinant proteins were expressed as insoluble inclusion bodies by induction with 1 mM IPTG, and urea-denatured proteins were purified by Ni-affinity chromatography and an AKTA Purifier system (General Electric, Schenectady, NY, USA) (22). Four recombinant proteins were named r-P1, r-P2, r-P3 and r-P4. These antigen concentrations were determined by spectrophotometer (NanoDrop 2000, Thermo, America) and were stored at -20 °C until use.

ELISA for the detection of anti-Trichinella antibodies

The optimal dilutions of various reagents were determined using checkerboard titration. An indirect ELISA method was utilized to detect anti-*Trichinella* IgM and IgG antibodies. In brief, 96-well microtitre plates (NUNC, Denmark) were coated with r-P1, r-P2, r-P3, and r-P4 at 100 μl/well, and the corresponding ES antigens were diluted to 10 μg/mL in the coating buffer (bicarbonate buffer, pH 9.6). Coating was performed for 2 h at 37 ℃ and then overnight at 4 ℃. The corresponding ES proteins were used as positive controls with the sera of infected mice at 26 dpi. Serum obtained from normal mice before infection was used as a negative control. After blocking with PBS-0.5% Tween 20 (PBST) containing 5% skimmed milk at 37 °C for 2 h, mouse serum diluted 1:50 in blocking buffer and horseradish peroxidase (HRP)-conjugated anti-mouse IgM and IgG (Abcam, UK) diluted 1:5000 and 1:10,000, respectively, were sequentially added,

and the samples were incubated at 37 °C for 1 h. Then, 3,3',5,5'-tetramethylbenzidine (TMB) solution was added at 100 µl/well, and the reaction was stopped by the addition of 2 M H_2SO_4 at 100 µl/well. The optical density (OD) at 450 nm was measured using a micro-plate reader (Thermo, USA). Each sample was measured in duplicate wells, and the values were averaged. The cut-off values for the ELISAs were set to 2.1-fold higher than the average OD value of the negative control samples. Sample ratios <2.1 were considered negative.

Table 1: Parameters of primer pairs for gene *Wh10*, *Zh68*, *T668* and *Wm5*

NCBI-Gene	***Gene***	***Primer sequence***	***Molecular weight***	***Translated protein***
EU263325	*Wn10*	F:5'---CGCGGATCCGAATTCATGAGTTTTATGCACTGTATTTTC---3' R:5'---GTGGTGGTGCTCGAGTTAACATTCAACAGTTGACTTGGT---3'	49.51 kDa	Cystatin-like protein
EU263327	*Zh68*	F:5'---CGCGGATCCGAATTCATGATCCTTTTCAAGTGCTTATTT---3' R:5'---GTGGTGGTGCTCGAGTTAACGGAAAAAAGTGAATGATGG---3'	51.15 kDa	Serine protea
AY491941	*T668*	F:5'---CGCGGATCCGAATTCATGATTAGACGTCTTTTTCAATAT---3' R:5'---GTGGTGGTGCTCGAGTTACTTAGAAAAGTGATAATATGT---3'	50.45 kDa	Serine proteases
DQ864973	*Wm5*	F:5'---CGCGGATCCGAATTCATGGAAACAGAAATTGCAAAACC---3' R:5'---GTGGTGGTGCTCGAGTTAATTACCAGAAAAACGTCCAAT---3'	46.09 kDa	Serine protease inhibitor

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 to identify changes in the levels of IgM and IgG antibodies. Statistical analyses were performed using SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). Mauchly's test of sphericity and repeated measures analysis of variance (ANOVA) were used to determine the differences between antibody levels from various antigens, differences between different infection-dose groups, and differences in the serum antibody levels at different time points within a group. $P<0.05$ was considered statistically significant.

Results

Bioinformatic analysis of the encoded proteins

Analysis of hydrophilic and hydrophobic domains

As shown in Fig. 1, the higher the positive value, the stronger the hydrophobicity; the lower the negative value, the stronger the hydrophilicity. P1, P2, and P4 did not have distinct hydrophobic regions (Fig.1a,b,d). The hydrophilic balance coefficients of P1, P2 and P4 were -0.4, -0.36, and -0.171, respectively, and all the proteins were hydrophilic. For P3 (Fig. 1c), there was a clear hydrophilic region after amino acid 280. The hydrophilic equilibrium coefficient was -0.503, which is also indicative of a hydrophilic protein.

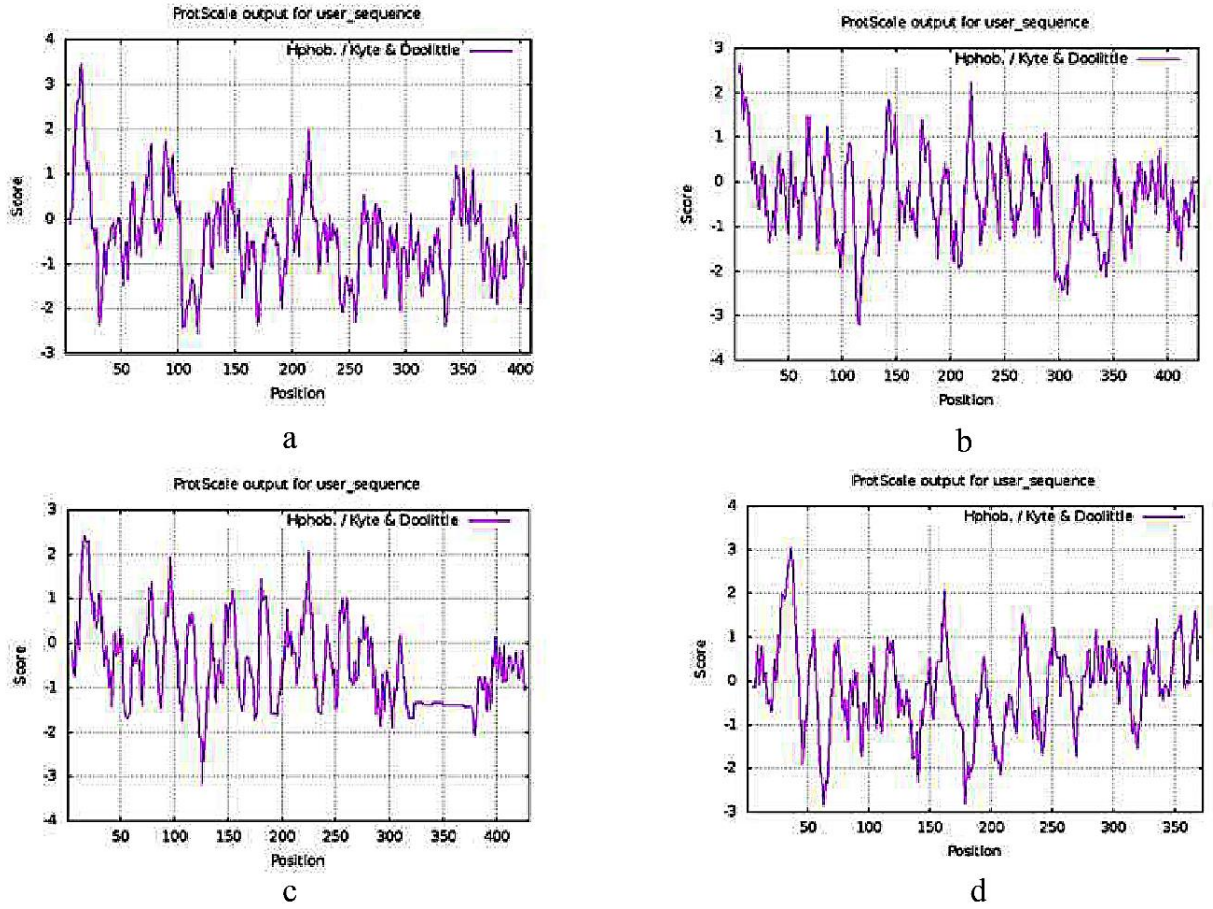

Fig. 1: Hydrophobicity and hydrophilicity of four proteins of *T. spiralis* analyzed by ExPASy-ProtScale (K-D methods).
a: P1; b: P2; c: P3; d: P4

Analysis of transmembrane domains

The protein sequence contains transmembrane region, which suggests that it may act as a membrane receptor or an anchor protein, or ion channel protein located on the membrane. All the amino acids of P1, P2, and P4 were located on the surface of the cell membrane, none of these three proteins were membrane proteins (Fig. 2a,b,d). P3 showed a typical transmembrane region between amino acids 13-30, thus might be a membrane protein (Fig. 2c).

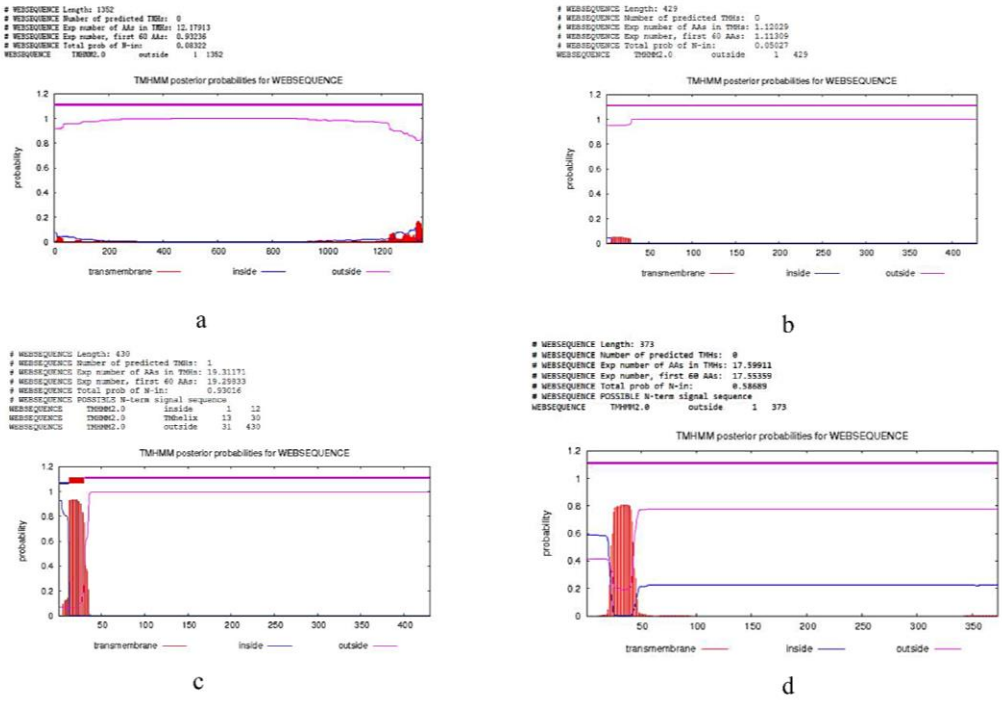

Fig. 2: Transmembrane region of four proteins of *T. spiralis* analyzed by TMHMM.
a: P1; b: P2; c: P3; d: P4

Analysis of structural domains

Domains are the functional, structural and evolutionary units of proteins, which play an important role in the classification and prediction of protein structures. P2 and P3 may have highly conserved trypsin-like serine protease domain (Fig. 3a.b), P3 exhibited a transmembrane region between the amino acids 13 and 30. Amino acids 14-373 of P4 represent a serine protease inhibitor domain (Fig. 3c). No typical structural domain was identified in P1.

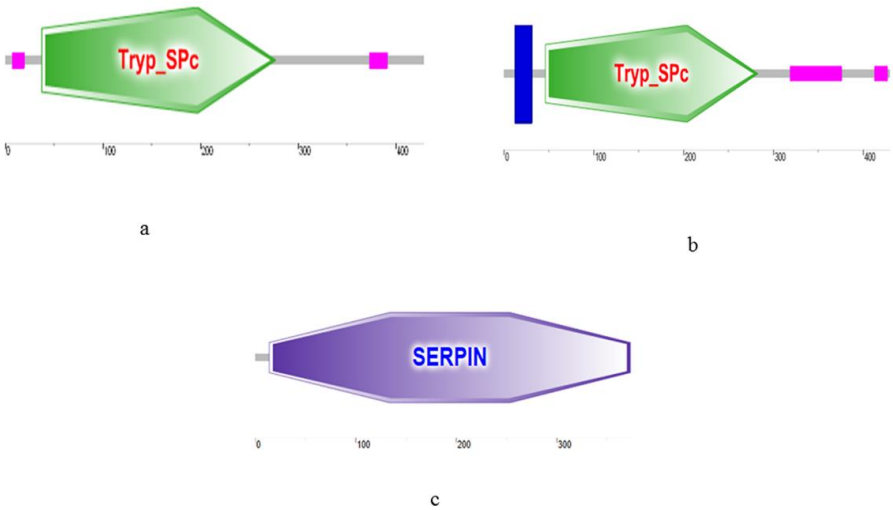

Fig. 3: Structure domain of P2, P3 and P4 of *T.* spiralis analyzed by SMART.
a: P2; b: P3; c: P4.

Secondary structure prediction

As shown in Fig.4, the α-helix accounted for 44.39% of P1, β-turn accounted for 5.12%, and random coil accounted for 32.44% (Fig. 4a). For P2, the proportions for α-helix, β-fold, β-turn and random coil were 17.25%, 23.08%, 8.86%, and 50.82%, respectively (Fig. 4b). For P3, they were 13.26%, 24.88%, 12.09%, and 49.77%, respectively (Fig. 4c); while for P4, the were 48.26%, 0, 10.99%, and 21.45%, respectively (Fig. 4d).

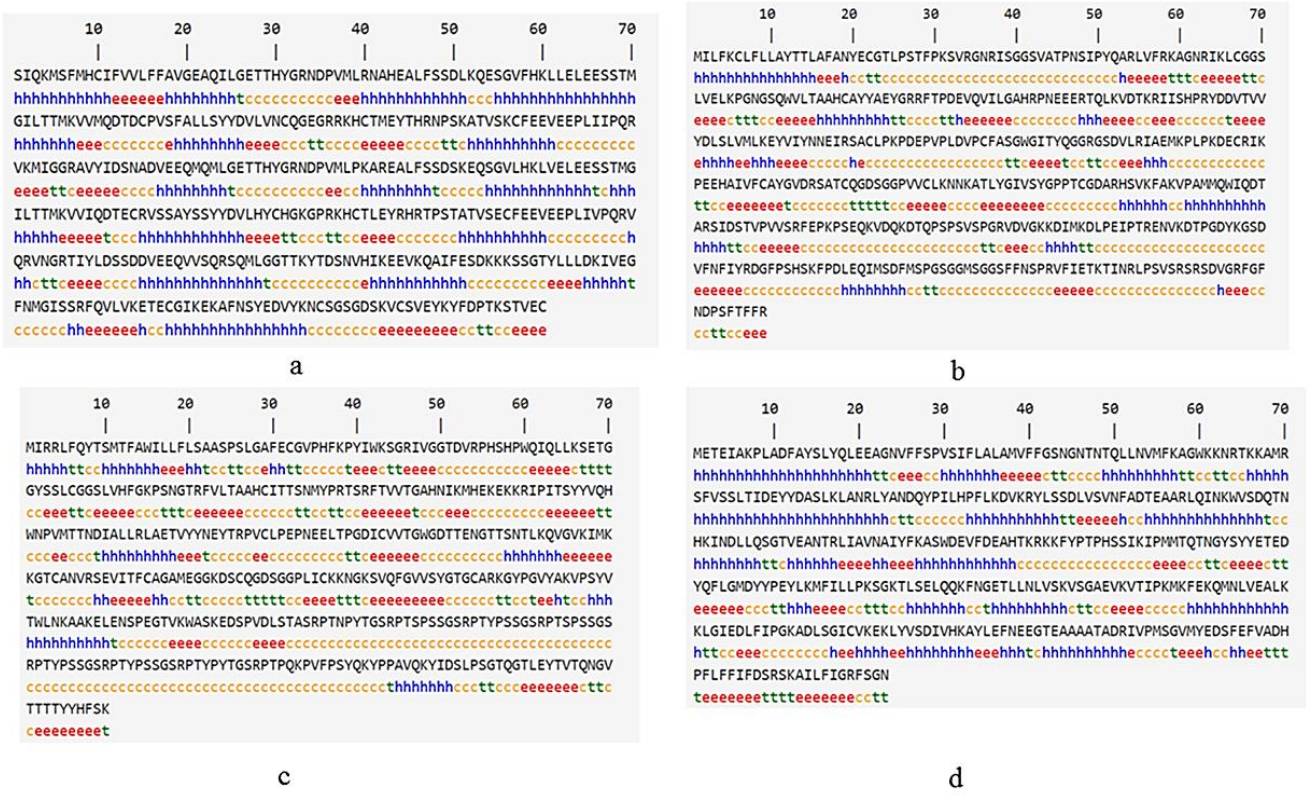

Fig. 4: Secondary structure of four proteins of *T. spiralis* predicted by ExPASy-SOMPA.
a: P1; b: P2; c: P3; d: P4

Tertiary structure prediction

The biological function of protein depends on its spatial structure heavily. Tertiary structure is the ultimate goal of protein structure prediction. α-helix, β-fold, β-turn and random coil can be clearly shown in tertiary structure. The optimal tertiary structures of four proteins are shown in Fig. 5.

B-cell epitope prediction

The optimal candidate region for the linear epitope of the B lymphocyte antigen of P1 was YSSYYDVLHYCHGKGP (amino acids 230-245); for P2, the region was HRPNEEERTQ (amino acids 111-120); for P3, the region was HNIKMHEKEKKRIPIT (amino acids 119-134) and for P4, the region was GVMYEDSFEFVADHPF (amino acids 337-352).

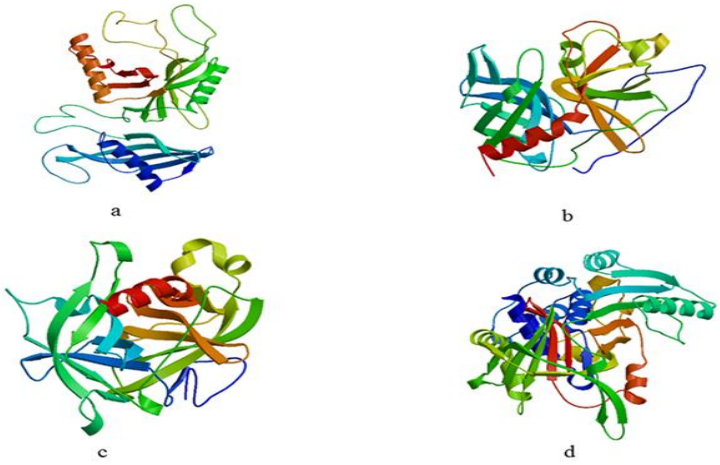

Fig. 5: Tertiary structure of four proteins of *T.* spiralis predicted by the SWISS-MODEL.
a: P1; b: P2; c: P3; d: P4

T-cell epitope forecasting

The optimal antigen epitope of MHC I of P1 was GVLHKLVEL (amino acids 195-203); for P2, the epitope was ILFKCLFLL (amino acids 2-10); for P3, the epitope was FLSAASPSL (amino acids 19-27); for P4, the epitope was YLSSDLVSV (amino acids 111-119). As for MHC Ⅱ, the optimal antigen epitopes of P1 was VLFFAVGEAQILGET (amino acids 14-28); for P2, the epitope was KCLFLLAYTTLAFAN (amino acids 5-19); for P3, the epitope was ILLFLSAASPSLGAF (amino acids 16-30); for P4, the epitope was YLKMFILLPKSGKTL (amino acids 222-236).

Total RNA extraction and gene amplification results

The four genes were successfully cloned (Fig. 6). Gene sequencing results were verified to be correct by Shanghai Shenggong Biological Co., Ltd. (Shanghai, China).

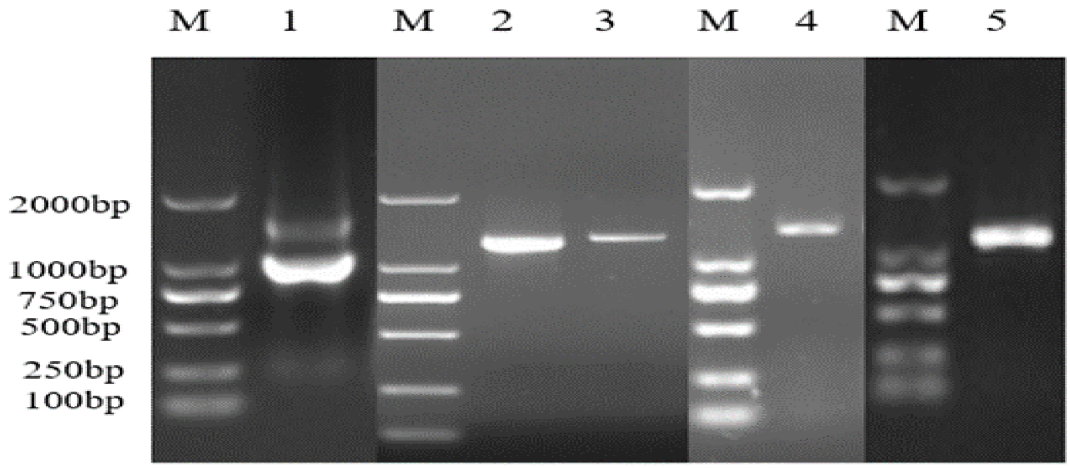

Fig. 6: Total RNA and gene amplification
M: DL2000 Maker; 1: Total RNA of *Trichinella spiralis*; 2: *Wn10* gene amplification; 3: *Zb68* gene amplification; 4: *T668* gene amplification; 5: *Wm5* gene amplification

Production of recombinant proteins

E. coli BL21 cells harbouring pET-28a (+) or the recombinant expression plasmids were induced by IPTG for 4 h, followed by SDS-PAGE. Each gel showed an additional band, indicating the presence of the recombinant protein at the correct molecular weight. After purification to greater than 95%, the recombinant proteins could be used for the immunological test; four single bands were observed. Using this approach, four recombinant proteins were obtained. The concentrations of r-P1, r-P2, r-P3, and r-P4 were 150 μg/mL, 300 μg/mL, 200 μg/mL, and 100 μg/mL, respectively. The SDS-PAGE analysis of the expression and purification of the recombinant proteins are presented in Fig. 7.

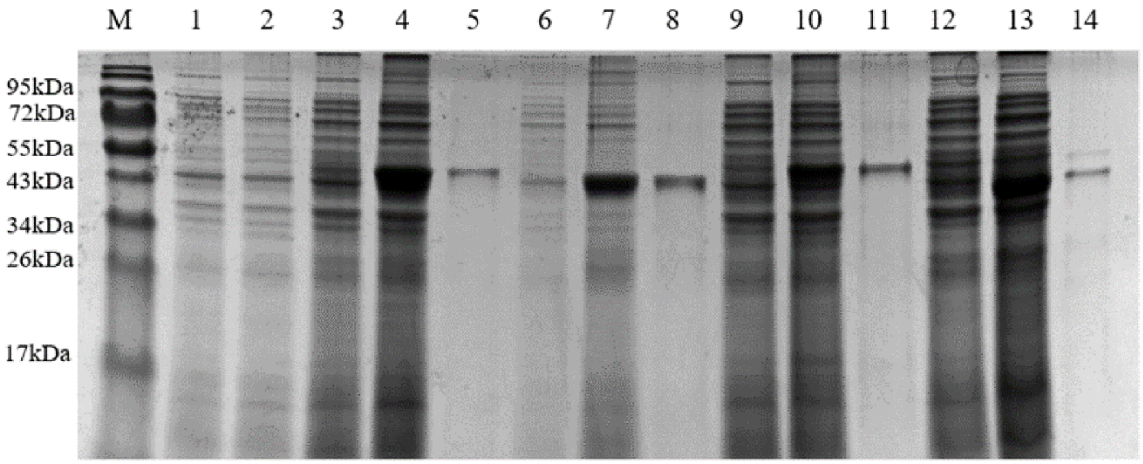

Fig. 7: SDS-PAGE analysis of r-P1, r-P2, r-P3 and r-P4 of *T. spiralis*
M: Marker; 1: pET-28a without IPTG induction; 2: pET-28a induction with IPTG for 4h; 3: pET-*Wn10* without IPTG induction; 4: pET-*Wn10* induction with IPTG for 4h; 5: r-P1 after purification; 6: pET-*Zh68* without IPTG induction; 7: pET-*Zh68* induction with IPTG for 4h; 8: r-P2 after purification; 9: pET-*T668* without IPTG induction; 10: pET-*T668* with IPTG induction for 4h; 11: r-P3 after purification; 12: pET-*Wm5* without IPTG induction; 13: pET-*Wm5* with IPTG induction for 4h; 14: r-P4 after purification

ELISA results for the detection of anti-Trichinella antibodies

Levels of the anti-Trichinella IgM antibody detected using recombinant proteins

All the antibody levels fluctuated around the cut-off value, and the OD values fluctuated from 0 to 0.6. When r-P1 was used, only the levels in mice infected with 300 larvae/mouse were higher than the cut-off value after 19 dpi, and the levels in the other three infection groups were approximately equal to the cut-off value. Moreover, when r-P2, r-P3, or r-P4 was used as the antigen, anti-*Trichinella* IgM antibodies could be detected at higher than the cut-off value for a sustained period of time only when the infection dose was 30 larvae/mouse (Fig. 8).

Levels of the anti-Trichinella IgG antibody detected with recombinant proteins

The OD values fluctuated between 0 and 2.0, which were much higher than the values for the anti-*Trichinella* IgM antibodies. When r-P1 was used, the levels of the anti-*Trichinella* IgG antibodies rapidly increased at 8 dpi or 10 dpi and remained high until 45 dpi.

In mice infected with the low dose (30 larvae/mouse), the levels of IgG were higher than those of antibodies in the high-dose group.

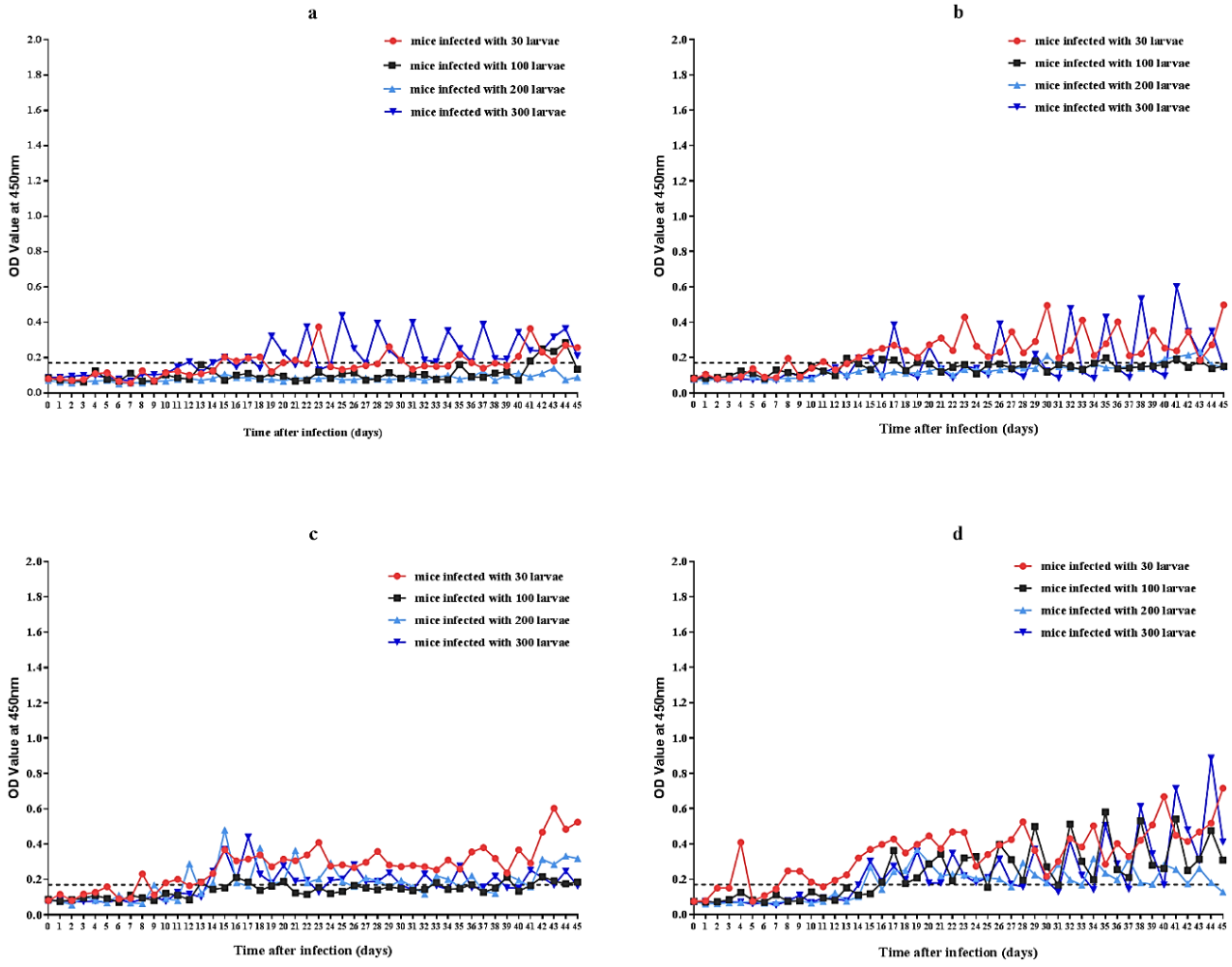

Fig. 8: The levels of anti-*Trichinella* IgM antibodies in the sera of mice infected with four doses of muscle larvae
a: r-P1; b: r-P2; c: r-P3; d: r-P4

When r-P2 was used, IgG antibodies were detected from 19 dpi to 45 dpi when 30 larvae were administered per mouse, and IgG antibodies could be observed as of 10 dpi with the other three infection doses. The sera of the infected mice were stably positive from 14 dpi to 16 dpi at all the infection doses when r-P3 and r-P4 were used as antigens. Besides, r-P4 showed the highest IgG levels from 21 dpi to 45 dpi (Fig. 9).

Statistical analysis results

The data did not satisfy the symmetry of the spherical test, but the method was corrected. There were significant differences in anti-*Trichinella* IgM and IgG antibody levels in the serum of infected mice with the four different dose groups ($P<0.05$). Differences in antibody levels at different time points after infection were statistically significant ($P<0.05$).

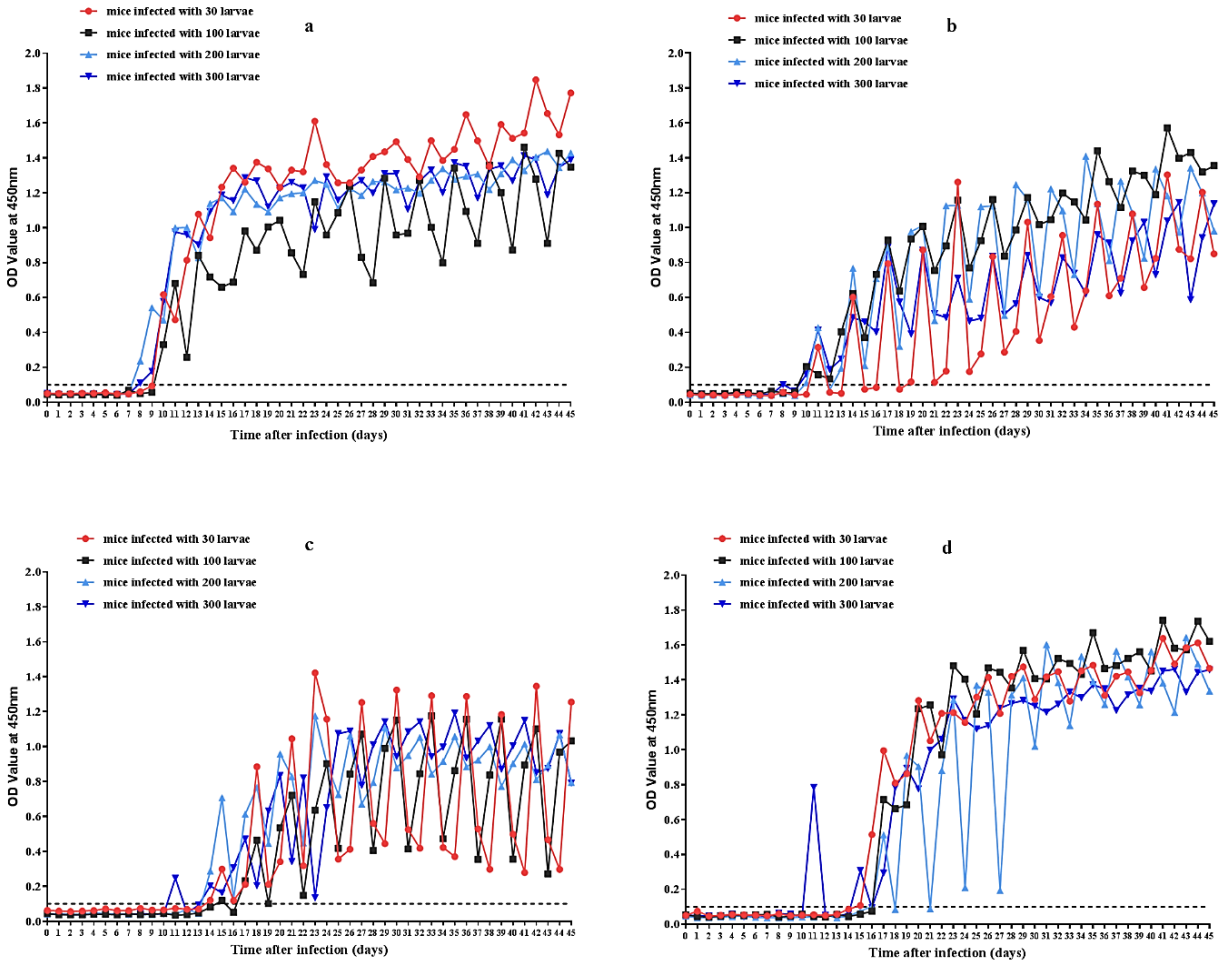

Fig. 9: The levels of anti-*Trichinella* IgG antibodies in the sera of mice infected with four doses of muscle larvae
a: r-P1; b: r-P2; c: r-P3; d: r-P4

Discussion

The biological function of a protein is largely dependent on the spatial structure of the protein. To investigate the biological characteristics and functions of P1, P2, P3 and P4 of *T. spiralis,* the structures were predicted. In this study, P1, P2, P3, and P4 represented hydrophilic proteins. P3 may represent a membrane protein. Based on prediction of their tertiary structures and antigen epitopes, the proteins were found to contain sufficient antigen epitopes to bind to antibodies, thus the four recombinant antigens may have immunodiagnostic value and react with antibodies. The results of diagnostic sensitivity of four gene recombinant protein confirms their antigenicity, however, the biological function and application potential need to be further elucidated.

ES antigen is the most widely used diagnostic antigen of trichinellosis, but due to its multitudinous limitations, more and more components of ES are being studied. Different antigens can be expressed during different developmental stages of *T. spiralis*. In general, 4691 proteins have been identified in the Ad, NBL, and ML stages, 1067 of differentially expressed (23), including serine protease,

DNase Ⅱ (24), trypsin enzyme Ⅱ family protein and paramyosin (22). They are expected to become early diagnostic antigens and are potential trichinellosis vaccine candidate molecules (25). Moreover, it may be more useful for diagnosis to find the common components among all the life stages of *T. spiralis* since these antigens are relevant regardless of the infection phase (26). The four recombinant proteins included a cystatin-like protein, two serine proteases, and a serine protease inhibitor. They may represent components of ES proteins of *T. spiralis*.

Several studies have investigated the diagnostic effect of ES antigens from various developmental stages by ELISA. For example, in mice infected with 100 ML, anti-*Trichinella* IgG antibodies were detected as early as 10 dpi by intestinal infective larval ES antigens; however, as early as 12 dpi by ML ES antigens (27). Similarly, in mice infected with 100 ML, anti-*Trichinella* IgG antibodies were first detected using adult worm ES antigens, crude antigens, and ML ES antigens at 8 dpi, 12 dpi, and 12 dpi, respectively. In mice infected with 500 ML, IgG was first detected at 10 dpi, 8 dpi, and 10 dpi, respectively (28).

In this present study, the sensitivity results demonstrated that the levels of IgM antibodies were low, possibly because the four genes were screened by using pig serum at 26 dpi with an IgG antibody. With anti-*Trichinella* IgG antibodies, r-P1 can be detected as early as 8 dpi, allowing diagnosis of a blind spot (14-19 dpi), which was superior to the results for the adult ES antigen, intestinal 6-h larval ES antigen, and ML ES antigen (17), and the antibody levels remained high. This finding indicated that r-P1 could be used to diagnose trichinellosis in mice from 8 dpi to 45 dpi by ELISA. Furthermore, r-P4 may be able to diagnose trichinellosis from at least 16 dpi to 45 dpi. Besides, in the low-dose group (30 larvae/mouse), the levels of IgG antibodies detected by r-P1 were higher than those detected in the other three high-dose groups, which was consistent with previous studies (28). Thus, high-dose infection did not generate high levels of IgG antibodies, as this effect might induce immunosuppression in the host.

There are few studies on the sensitivity of recombinant proteins. A recombinant protein named rTsEla showed that anti-*Trichinella* IgM, IgG and IgE in infected mice were first detected by ELISA at 6, 10, 12 dpi, and reached 100% at 8, 14 and 14 dpi, respectively, and showed higher sensitivity and specificity than ES antigen (97.37% vs 89.74%, $P>0.05$; 99.10% vs 98.20%, $P>0.05$) (29). However, using an L20h-Ts3 fusion protein (30), which is a 31-kDa recombinant ML ES protein, the results by ELISA showed that both the sensitivity and specificity were lower than those for the ES antigen (31).

Conclusion

The recombinant antigens r-P1, r-P2, r-P3, and r-P4 are antigens that can react with antibodies in *Trichinella* infected mice, and the optimal antigen epitopes from T- and B-cells were predicted. The protein r-P1 may be the best potential candidate antigen for the detection of anti-*Trichinella* IgG antibodies during the early stages of infection. The protein r-P4 could be used for diagnosis of mid- or late-stage infection. This study laid the foundation for further studies on the early diagnosis of trichinellosis in pigs and humans and provided scientific information regarding the application of ELISA for screening early diagnostic antigens of *T. spiralis*.

Acknowledgements

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Conflict of interest

We have no conflict of interest related to this study.

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