

## Identification of early diagnostic antigens in soluble proteins of *Trichinella spiralis* adult worms by Western blot

Xu, D.M.<sup>1,2†</sup>, Wen, H.<sup>1†</sup>, Wang, L.A.<sup>1</sup>, Hu, C.X.<sup>1</sup>, Qi, X.<sup>1</sup>, Sun, G.G.<sup>1</sup>, Liu, R.D.<sup>1</sup>, Wang, Z.Q.<sup>1\*</sup> and Cui, J.<sup>1\*</sup>

<sup>1</sup>Department of Parasitology, Medical College

<sup>2</sup>The Third Affiliated Hospital, Zhengzhou University, 40 Daxue Road, Zhengzhou 450052, People's Republic of China

<sup>†</sup>Xu, D.M. and Wen, H. contributed equally to this article.

\*Corresponding author e-mail: wangzq@zzu.edu.cn, wangzq2015@126.com or Jing Cui; cuij@zzu.edu.cn

Received 7 July 2016; received in revised form 14 September 2016; accepted 20 September 2016

**Abstract.** Previous studies showed that crude antigens from *Trichinella spiralis* adult worms (AW) can be recognized by mouse infection sera at 8 days post infection. The aim of this study was to identify the early diagnostic antigenic bands in soluble proteins from *T. spiralis* AW by Western blot using early infection sera. The affecting factors of adult recovery were firstly observed in this study, and the results showed that the maximum number of adults was collected from small intestine when the female BALB/c mice were orally infected with 4000 ML and sacrificed at 3 days post infection. The results of Western blot analysis showed that seven protein bands (31, 35.1, 39, 40.6, 41.9, 47 and 50.6 kDa) could be recognized by early infection sera as early as at 8–10 days post infection, and were strongly reacted with mouse infection sera at 11–12 days post infection. Our results suggested that the seven protein bands of *T. spiralis* AW soluble proteins might be the early expressed antigens during the intestinal stage of *Trichinella* infection and therefore have potential value for the early diagnosis of trichinellosis.

### INTRODUCTION

The clinical diagnosis of human trichinellosis is rather difficult because its clinical manifestations are nonspecific (Dupouy-Camet *et al.*, 2002; Gottstein *et al.*, 2009). Currently, the serodiagnostic antigens most commonly used for trichinellosis are the excretory-secretory (ES) antigens of *Trichinella spiralis* muscle larvae (ML), as they are recommended by the International Commission on Trichinellosis (ICT) (Gamble *et al.*, 2004). However, the main disadvantage of detection of anti-*Trichinella* IgG antibodies against ML ES antigens is the occurrence of false negative results during the early stage of infection (Liu *et al.*, 2013; Cui *et al.*, 2015). Previous studies have shown that the maximum detection rate of 100% of IgG

antibodies was not reached until at least 1–3 months after human infection with the parasite (Bruschi *et al.*, 1990; Wang *et al.*, 1998). There is an obvious time lag (window period) between clinical symptoms and positive serology during the acute stage of trichinellosis.

The majority of *T. spiralis* ML ES antigens come from the cuticles and secretory granules of stichocyte and are ML stage-specific (Bioreau *et al.*, 1997; Ortega-Pierres *et al.*, 1996), and ML ES antigens are not recognized by specific antibodies induced by the parasites during the intestinal stage (Tang *et al.*, 2015). So, the false-negative results may occur during the early stage of *Trichinella* infection when the ML ES antigens were used for serodiagnosis of trichinellosis (Wang *et al.*, 2012). Hence, it is

necessary to exploit the new sources of early diagnostic antigens from intestinal stage worms during *Trichinella* infection.

Once ingested, ML develop to adult worms (AW) in the small intestine 31 h after infection, live in the intestinal mucosa and persist for 10–20 days in mice and rats or 4–6 weeks in humans (Campbell 1983). During enteral stage of *Trichinella* infection, the AW antigens were early exposed to the immune system and elicit the production of specific anti-*Trichinella* antibodies by the host (Sun *et al.*, 2015; Yang *et al.*, 2015). Theoretically, the AW might contain the early expressed antigens and the window period of trichinellosis could be shortened by using *T. spiralis* AW diagnostic antigens. The aim of this study was to assess the early diagnostic antigenic bands in soluble proteins from *T. spiralis* AW by Western blot with early infection sera.

## MATERIALS AND METHODS

### Parasite

*Trichinella spiralis* isolate (ISS534) used in this study was obtained from a domestic pig in Nanyang city of Henan province, China. The isolate was maintained by serial passages in Kunming mice in our laboratory.

### Experimental Animals

Specific pathogen-free (SPF) BALB/c mice aged 6 weeks were purchased from the Experimental Animal Centre of Henan Province, China. Experimental procedures for *T. spiralis* infection in this study were approved by the Life Science Ethics Committee of Zhengzhou University (no. 2011–016). Ten female BALB/c mice were orally infected with 300 ML. About 100 µl of tail blood was collected from infected mice during 6–15, 26 and 42 days post infection (dpi) and serum samples were isolated (Wang *et al.*, 2012).

### Collection of *T. spiralis* AW

The mice were infected per os with 1000–5000 ML of *T. spiralis* and euthanized 2–4 dpi. The small intestine from experimentally infected mice was cut along its entire length,

and washed in pre-warmed PBS. Then, the small intestine was cut into pieces and incubated in PBS at 37°C for 1.5 h on a 300 µm sieve. The released AW was separated from the intestinal debris by filtration through a 200 µm sieve and differential sedimentation for 30 min. After several washes in PBS supplemented with 100 U penicillin/ml and 100 µg streptomycin/ml, the AW were centrifuged at 600 × g for 10 min and collected (Long *et al.*, 2014; Sun *et al.*, 2015). The average number of AW recovered was calculated and compared among different groups of infected mice.

### Preparation of AW soluble proteins

The soluble proteins (crude antigens) of *T. spiralis* AW were prepared as previously described (Cui *et al.*, 2013). Briefly, adults were resuspended in deionized water. The suspension was subjected to 5 freeze-thaw cycles. The worms were homogenized on ice in a glass tissue grinder. Then, the worm fragments were further homogenized with ultrasonication (99 times 3-s cycles, 100 W, 0°C). The supernatant was collected after centrifugation at 15,000 × g for 1 h at 4°C. The protein concentration of the AW soluble proteins was determined by the Bradford assay (Bradford, 1976).

### ELISA

The optional dilutions of various reagents were determined using checkerboard titration. ELISA was performed as previously described (Long *et al.*, 2015). Briefly, 96-well ELISA plates (Corning, USA) were coated with AW soluble proteins (1.5 µg/ml) in 100 µl of bicarbonate buffer (pH 9.6) overnight at 4°C. After blocking with PBS-0.1% Tween 20 (PBST) containing 5% skimmed milk at 37°C for 2 h, the following reagents were sequentially added and incubated at 37°C for 1 h: (1) mouse sera diluted 1:100 in PBST, and (2) HRP-conjugated goat anti-mouse IgG (Sigma, USA) diluted 1:5000. The reactions were detected by the addition of the substrate o-phenylenediamine dihydrochloride (OPD; Sigma, USA) plus H<sub>2</sub>O<sub>2</sub> and stopped with 50 µl/well of 2 M H<sub>2</sub>SO<sub>4</sub>. Optical density (OD) values at 490 nm were measured with a microplate reader (TECAN, Austria). All

samples were run in duplicate. The ratios < 2.1 of the samples to be tested/negative sample (OD values of the samples to be tested divided by OD of the negative, S/N < 2.1) were regarded as negative, whereas S/N  $\geq$  2.1 was regarded as positive (Cui *et al.*, 2011).

### SDS-PAGE

AW soluble protein samples (8  $\mu$ g) were diluted with loading buffer (250 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 5% 2-mercaptoethanol, 0.5% bromophenol blue) and boiled for 5 min. After cooling, the proteins were separated by SDS-PAGE on 5% stacking gels and 10% resolving gels (83 $\times$ 73 $\times$ 1.0 mm) in a Mini-PROTEAN 3 Cell electrophoresis unit (Bio-Rad, USA) at 120 V for 2.5 h (Wang *et al.*, 2011). After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue (CBB) R-250 (Sigma, USA) for 4 h, and then bleached with the eluate (100 ml acetic acid, 50 ml ethanol, 850 ml dH<sub>2</sub>O). A second gel was prepared with the above-mentioned proteins for the following Western blot.

### Western blot

After electrophoresis, proteins were transferred to PVDF membrane (Millipore, USA) at 17 V for 35 min (Liu *et al.*, 2014). After blotting, the membranes were stained with Ponceau S to verify transfer and to locate the protein marker and cut into strips. Each strip was blocked with 5% skimmed milk in Tris-Buffered Saline with Tween-20 (TBST) at 37°C for 1 h, and incubated with 1:100 dilutions of the mouse infection sera or normal mouse sera at 4°C overnight. After washing, the strips were incubated at 37°C for 1 h with HRP-conjugated goat anti-mouse IgG (1:5000 dilution), and finally with 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma). The reaction was finally stopped by washing the strips with distilled water.

### Statistical analysis

Data are expressed as the mean  $\pm$  standard deviation. All statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). Differences of recovered adult worms between male and female mice were

compared by using Student's t-test. The differences among the groups of different infecting dose and different time of adult worm recovery post infection were analyzed using the one-way ANOVA method. For post hoc testing, when variances were unequal, the Dunnett T3 test was used.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of mouse sex on recovery of AW

Ten female and ten male BALB/c mice were orally infected with 3000 ML, respectively. The infected mice were euthanized 3 dpi, the AW were collected from intestine and numbered. The results showed that although the difference has no statistical significance ( $t=1.18, P > 0.05$ ), the number of AW recovered from female mice (1345 $\pm$ 289) was higher than those from male mice (1172 $\pm$ 361). So, the female mice were used to collect the AW in the following experiment.

### Optimal infecting dose to recover AW

Fifty female BALB/c mice were randomly divided into five groups of 10 animals each. Each group was orally infected with 1000, 2000, 3000, 4000 or 5000 ML. The AW was isolated from the small intestines of infected mice at 3 dpi. As shown in Figure 1, the recovery AW from the mice infected with 1000 and 2000 ML was statistically lower than that from mice infected with 3000, 4000 or 5000 ML ( $P < 0.05$ ), but the values was not statistical different among the three groups of mice infected with 3000, 4000 or 5000 ML ( $P < 0.05$ ). Therefore, 3000 ML was selected to inoculate mice for collecting more AW in the following experiment.

### Optimal time to recover AW

Thirty female BALB/c mice were randomly divided into three groups of 10 animals each and each mouse was infected with 3000 ML. The AW was isolated from the small intestines of infected mice at 2, 3 or 4 dpi, respectively. As shown in Figure 2, the recovered AW at 3 dpi was significantly high than that at 2 and 4 dpi ( $P < 0.05$ ). Hence, we selected the 3 dpi as the optimal time for recovering AW.

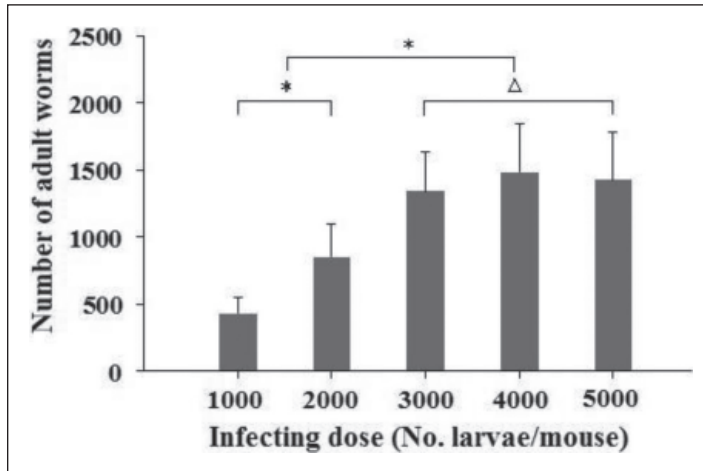


Figure 1. Determination of the optimal infecting dose to recover adult worms. Fifty female mice were divided into five groups of 10 animals each. Each group was orally infected with different dose of *T. spiralis* muscle larvae. The adult worms were collected from the small intestines of infected mice at 3 dpi.

\* Data are significantly different from the other groups ( $P < 0.05$ ).

Δ Data are not significantly different among three groups ( $P > 0.05$ ).

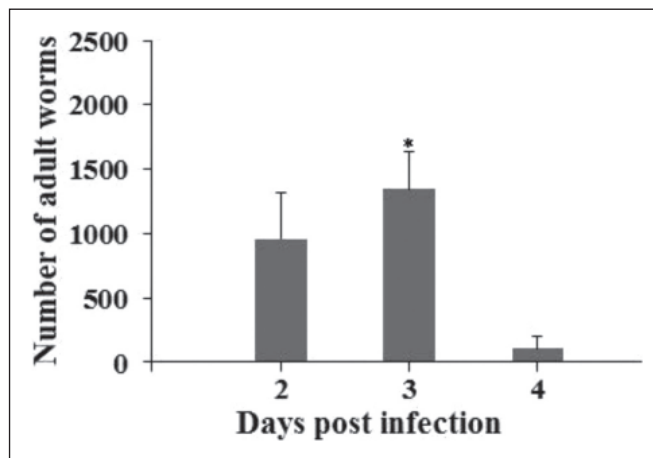


Figure 2. Determination of the optimal time to recover AW. Thirty female mice were divided into three groups of 10 animals each, and each mouse was infected with 3000 *T. spiralis* muscle larvae. The adult worms were isolated from the small intestines of infected mice at different time after infection.

\* Data are significantly different from the other groups ( $P < 0.05$ ).

### Detection of anti-*Trichinella* antibodies in infected mice by ELISA

The levels of anti-*Trichinella* IgG in the sera of infected mice at different time points after infection were determined by ELISA with AW soluble antigens (Figure 3).

The results showed that anti-*Trichinella* antibodies were firstly detected at 6 dpi, and then serum antibody levels increased gradually. The antibody positive rate reached 100% at 14 dpi. The positive sera at 6-12dpi were used for the Western blot analysis.

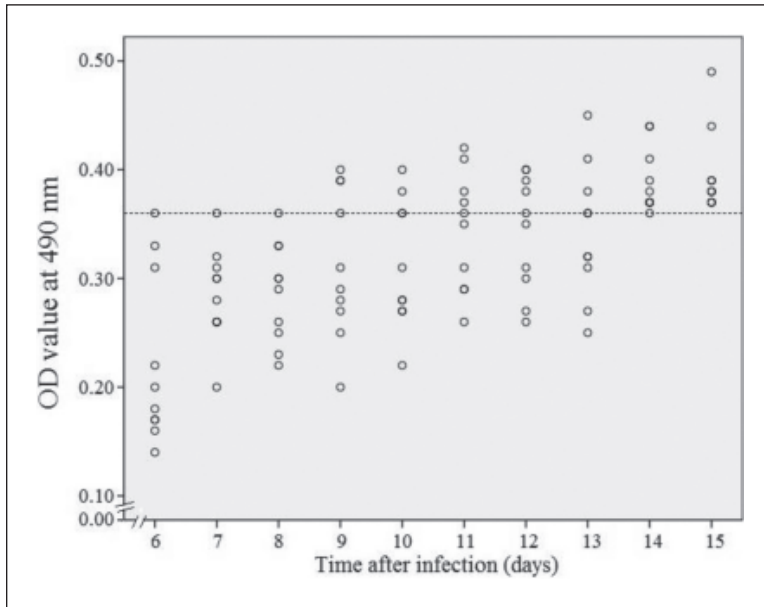


Figure 3. Optical density values of mice infected with 300 *T. spiralis* muscle larvae during 6-15 dpi by ELISA with adult worm soluble antigens. The cut-off value is represented by the dotted line.

### SDS-PAGE and Western blot analysis

The soluble proteins of *T. spiralis* AW were separated by SDS-PAGE and the results were shown in Figure 4A. Image analysis revealed that the AW soluble proteins had 27 bands with a molecular weight of 95, 81.3, 73.4, 63.6, 61.4, 55, 50.6, 47, 45.1, 43.7, 41.9, 40.6, 39, 37.4, 35.1, 33.3, 31, 30.4, 27.8, 26.6, 25.4, 24, 21, 19.2, 17.9, 15.4, and 14.4 kDa. The results of Western blotting analysis showed that seven protein bands (31, 35.1, 39, 40.6, 41.9, 47, 50.6 kDa) could be weakly recognized by mouse infection sera at 8–10 dpi (Figure 4B). The seven protein bands were strongly reacted with infection sera at 11-12 and 26 dpi. Furthermore, 19 protein bands (17.9, 19.2, 24, 21, 27.8, 31, 33.3, 35.1, 39, 40.6, 41.9, 47, 50.6, 55, 61.4, 63.6, 73.4, 81.3 and 95kDa) in soluble proteins of *T. spiralis* AW were recognized by infection sera at 42 dpi. But, all protein bands in soluble proteins of *T. spiralis* AW can not be recognized by normal mouse sera.

### DISCUSSION

Following ingestion of infested meat, *T. spiralis* ML are released from their capsules in the stomach, migrate to the small intestine and enter intestinal columnar epithelial cells where they molt and develop to adults (Campbell, 1983). This represents the earliest interaction between the parasite and host, and therefore, it is important to identify the antigens expressed by adults during this stage (Tang *et al.*, 2015). In order to collect more adults for preparing adult antigens, the affecting factors of adult recovery were observed in this study. The results showed that the maximum number of adults was collected from small intestine when the female BALB/c mice were orally infected with 4000 ML and sacrificed at 3 dpi.

The results of ELISA indicated that some antigenic epitopes of AW soluble proteins were recognized by infection sera at 8–10 dpi, suggesting that the excretory-

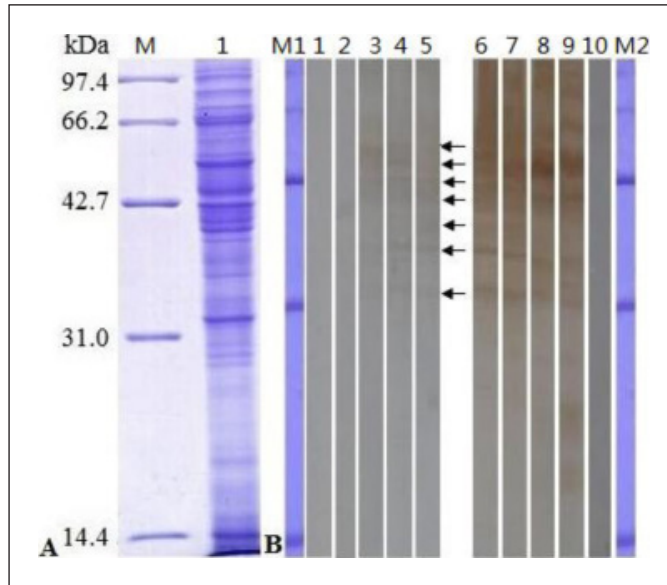


Figure 4. SDS-PAGE and Western blot analysis of soluble proteins of *T. spiralis* AW. (A) An amount of 8  $\mu$ g of AW soluble proteins was separated on a 10% polyacrylamide separating gels. M, Protein marker with low molecular weights; lane 1, soluble proteins of *T. spiralis* AW. (B) Western blot analysis of AW soluble proteins probed by mouse infection sera. M1 and M2, protein marker with low molecular weights; lane 1-7, mouse infection sera at 6-12 days post infection; lane 8, mouse infection sera at 26 days post infection; lane 9, mouse infection sera at 42 days post infection; lane 10, normal mouse sera.

secretory (ES) or surface antigens might be secreted by the parasite into the peripheral blood circulation of host at early infection stage, induced an early antibody response (Cui *et al.*, 2015; Sun *et al.*, 2015). As shown in Figure 3, anti-*Trichinella* antibodies in 50% of infected mice were detected by ELISA with AW soluble proteins as soon as 11 dpi. In order to identify the early diagnostic antigenic bands in soluble proteins from *T. spiralis* AW, the AW soluble proteins were analyzed by SDS-PAGE and Western blot with early infection sera. The results of SDS-PAGE showed the AW soluble proteins had 27 protein bands with a molecular weight of 14.4-95kDa. But on Western blotting analysis, only seven protein bands (31, 35.1, 39, 40.6, 41.9, 47 and 50.6 kDa) could be weakly recognized by early infection sera at 8–10 dpi. The seven protein bands were strongly reacted with mouse infection sera at 11–12, 26, and 42 dpi. All protein bands

in soluble proteins of *T. spiralis* AW had no cross reaction with normal mouse sera. Recently, other studies showed that the recombinant protein from *T. spiralis* pre-adult stage at 20 hours after infection was recognized by pig antiserum in Western blot as early as 15–20 dpi (Zocevic *et al.*, 2014), and the AW crude antigens can be recognized in ELISA or Western blot with mouse infection sera at 8–12 days post infection (dpi) (Sun *et al.*, 2015; Yang *et al.*, 2015). Our results suggested that the seven protein bands might be the early expressed antigens during intestinal stage of *Trichinella* infection and had potential for the early diagnosis of trichinellosis.

However, the seven protein bands should be identified and characterized by shotgun LC-MS/MS (Liu *et al.*, 2015), and their sensitivity and specificity needs to be further validated with the sera of the patients with trichinellosis and other helminthiasis.



*Acknowledgments.* This work was supported by the National Natural Science Foundation of China (no. 81672043 and 81572024).

## REFERENCES

- Bioreau, P., Vayssier, M., Fabien, J.F., Perret, C., Calamel, M. & Soule, C. (1997). Characterization of eleven antigenic groups in *Trichinella* genus and identification of stage and species markers. *Parasitology* **115**: 641-651.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.
- Bruschi, F., Moretti, A., Wassom, D. & Fioretti, D.P. (2001). The use of a synthetic antigen for the serological diagnosis of human trichinellosis. *Parasite* **8**: S141-S143.
- Campbell, W. (1983). *Trichinella* and trichinosis. Plenum Press, New York, p75.
- Cui, J., Li, N., Wang, Z.Q., Jiang, P. & Lin, X.M. (2011). Serodiagnosis of experimental sparganum infections of mice and human sparganosis by ELISA using ES antigens of *Spirometra mansoni* spargana. *Parasitology Research* **108**: 1551-1556.
- Cui, J., Liu, R.D., Wang, L., Zhang, X., Jiang, P., Liu, M.Y., *et al.* (2013). Proteomic analysis of surface proteins of *Trichinella spiralis* muscle larvae by two-dimensional gel electrophoresis and mass spectrometry. *Parasite & Vectors* **6**: 355.
- Cui, J., Wang, L., Sun, G.G., Liu, L.N., Zhang, S.B., Liu, R.D., Zhang, X., Jiang, P. & Wang, Z.Q. (2015). Characterization of a *Trichinella spiralis* 31kDa protein and its potential application for the serodiagnosis of trichinellosis. *Acta Tropica* **142**: 57-63.
- Dupouy-Camet, J., Kociecka, W., Bruschi, F., Bolas-Fernandez, F. & Pozio, E. (2002). Opinion on the diagnosis and treatment of human trichinellosis. *Expert opinion on pharmacotherapy* **3**: 1117-1130.
- Gamble, H.R., Pozio, E., Bruschi, F., Nockler, K., Kapel, C.M.O. & Gajadhar, A.A. (2004). International commission on trichinellosis: Recommendations on the use of serological tests for the detection of *Trichinella* infection in animals and man. *Parasite* **11**: 3-13.
- Gottstein, B., Pozio, E. & Nockler, K. (2009). Epidemiology, diagnosis, treatment, and control of trichinellosis. *Clinical Microbiology Reviews* **22**: 127-145.
- Liu, L.N., Jing, F.J., Cui, J., Fu, G.Y. & Wang, Z.Q. (2013). Detection of circulating antigen in serum of mice infected with *Trichinella spiralis* by an IgY-IgM mAb sandwich ELISA. *Experimental Parasitology* **133**: 150-155.
- Liu, R.D., Cui, J., Liu, X.L., Jiang, P., Sun, G.G., Zhang, X., Long, S.R., Wang, L. & Wang, Z.Q. (2015). Comparative proteomic analysis of surface proteins of *Trichinella spiralis* muscle larvae and intestinal infective larvae. *Acta Tropica* **150**: 79-86.
- Liu, R.D., Cui, J., Wang, L., Long, S.R., Zhang, X., Liu, M.Y. & Wang, Z.Q. (2014). Identification of surface proteins of *Trichinella spiralis* muscle larvae using immunoproteomics. *Tropical Biomedicine* **31**: 579-591.
- Long, S., Wang, Z., Liu, R., Liu, L., Li, L., Jiang, P., Zhang, X., Zhang, Z., Shi, H. & Cui, J. (2014). Molecular identification of *Trichinella spiralis* nudix hydrolase and its induced protective immunity against trichinellosis in BALB/c mice. *Parasites & Vectors* **7**: 600.
- Ortega-Pierres, M.G., Yopez-Mulia, L., Homan, W., Gamble, H.R., Lim, P.L. & Takahashi, Y., *et al.* (1996). Workshop on a detailed characterization of *Trichinella spiralis* antigens: a platform for future studies on antigens and antibodies to this parasite. *Parasite Immunology* **18**: 273-284.
- Sun, G.G., Wang, Z.Q., Liu, C.Y., Jiang, P., Liu, R.D., Wen, H., Qi, X., Wang, L. & Cui, J. (2015). Early serodiagnosis of trichinellosis by ELISA using excretory-secretory antigens of *Trichinella spiralis* adult worms. *Parasites & Vectors* **8**: 484.

- Tang, B., Liu, M., Wang, L., Yu, S., Shi, H. & Boireau, P., *et al.* (2015). Characterisation of a high-frequency gene encoding a strongly antigenic cystatin-like protein from *Trichinella spiralis* at its early invasion stage. *Parasites & Vectors* **8**: 78.
- Wang, S.W., Wang, Z.Q. & Cui, J. (2011). Protein change of intestinal epithelial cells induced *in vitro* by *Trichinella spiralis* infective larvae. *Parasitology Research* **108**: 593-599.
- Wang, Z.Q., Cui, J., Wu, F., Mao, F.R. & Jin, X.X. (1998). Epidemiological, clinical and serological studies on trichinellosis in Henan Province, China. *Acta Tropical* **71**: 255-268.
- Wang, Z.Q., Fu, G.Y., Jing, F.J., Jin, J., Ren, H.J., Jiang, P. & Cui, J. (2012). Detection of *Trichinella spiralis* circulating antigens in serum of experimentally infected mice by an IgY-mAb sandwich ELISA. *Foodborne Pathogens and Disease* **9**: 727-733.
- Yang, J., Pan, W., Sun, X., Zhao, X., Yuan, G., Sun, Q., Huang, J. & Zhu, X. (2015). Immunoproteomic profile of *Trichinella spiralis* adult worm proteins recognized by early infection sera. *Parasites & Vectors* **8**: 20.
- Zocevic, A., Lacour, S.A., Mace, P., Giovani, B., Grasset-Chevillot, A., Vallee, I. & Boireau, P. (2014). Primary characterization and assessment of a *T. spiralis* antigen for the detection of *Trichinella* infection in pigs. *Veterinary Parasitology* **205**: 558-567.