Expression of the Antigenic Gene TspE1 of *Trichinella spiralis* in Skin and Muscle of BALB/c Mice

Jing Cui[1], Zhongquan Wang[1], Hongwei Zhang[1], Guoqiang Zhao[2], Haiyan Wei[1], Huamin Han[1]

1. Department of Parasitology, Zhengzhou University, Zhengzhou, Henan 450052, China
2. Department of Microbiology and Immunology, Basic Medical College, Zhengzhou University, Zhengzhou, Henan 450052, China

Abstract: Trichinellosis is a serious zoonosis with a worldwide distribution and pork is still the most common source of human trichinellosis. Vaccines are needed to control this disease in swine. The recombinant eukaryotic expression plasmid, pcDNA3-TspE1, containing a gene encoding a 31 kDa antigen from *T. spiralis* was constructed. BALB/c mice were immunized with plasmid DNA vaccine by intramuscular injection and via gene-gun delivery. The transcriptional activity of the pcDNA3-TspE1 in skin and muscle at the site of inoculation was detected by RT-PCR using gene specific primers. Protein expression from the TspE1 gene in skin and muscles at the site of inoculation was detected by immunohistochemistry and indirect fluorescencent antibody test (IFAT), respectively. The results indicated that the recombinant plasmid pcDNA3-TspE1 was successfully transcribed and expressed in skin and muscle at the site of inoculation of mice. Thus, the plasmid encoding 31 kDa antigen may be of value for further development of a DNA vaccine against swine trichinellosis. [Life Science Journal. 2005;2(1):30-36] (ISSN: 1097-8135).

Keywords: *Trichinella spiralis*; DNA vaccine; gene-gun delivery; expression; mice

1 Introduction

Trichinellosis, one of the most serious helminthic zoonosis, is still considered to be endemic in many parts of the world. Human acquire the disease by ingesting raw or insufficiently cooked meat containing *Trichinella* larvae. Outbreaks of trichinellosis have occurred in many areas around the world over the past 20 years, even though the veterinary public health efforts have focused on the control and eradication of the disease for more than a century. The global prevalence of trichinellosis is difficult to evaluate, but it is estimated that as many as 11 million people may be infected (Dupouy-Camet, 2000). More than 10000 cases of human trichinellosis were reported by the International Commission on Trichinellosis from 1995 to June 1997 and about 10000 porcine infections were reported by the Office International des Epizooties in 1998 (Dupouy-Camet, 2000). Thus, trichinellosis has been regarded as emerging or re-emerging disease in both developed and developing regions (Murrell and Pozio, 2000). The disease is particularly problematic in some parts of China. Since the first patient with trichinellosis was recorded in Tibet in 1965 (Huang, 1965), there have been 548 outbreaks reported from 12 provinces, autonomous regions or municipalities (P/A/M) of China during 1966-1999 (Wang and Cui, 2001a). Pork is the main source of *Trichinella* infection for humans in China (Wang et al., 1998). The transmission of *T. spiralis* via household garbage is a main feature of the epidemiology of swine trichinellosis in China (Wang and Cui, 2001b). Vaccination of swine against *T. spiralis* could provide an alternative to prevent the risk of human infection. The development of vaccines capable of preventing swine from becoming infected would thus make a substantial contribution towards the ultimate goal of disease elimination.

In order to control trichinellosis, vaccines have been developed based upon irradiated- or ultraviolet-attenuated infective first stage larvae (Agyei-Frempong & Catty, 1983; Nakayama et al., 1998), autoclaved larvae (Eissa et al., 2003), antigens from different life-cycle stages (Darwish et al., 1996; Aucouturier et al., 2001), and a synthetic peptide (Robinson et al., 1995; McGuire et al., 2002). In each case, high levels of specific antibodies against *Trichinella* were induced in experimental animals, but failed to protect these animals fully against infection. In addition, the attenuated vaccine is impractical for field use.

DNA vaccination has been used to deliver a variety of parasite antigens to both small and large animal species, and the protective efficacy of antibodies generated in response to DNA vaccines has been shown in several challenge models (Donnelly et al., 1997; Rothel et al., 1997; Zhang et al., 2001;

Dumonteil et al., 2003). DNA vaccination has the attraction of enabling antigen expression to remain in a eukaryotic cell, increasing the probability that expressed antigen will be correctly glycosylated and generate conformation-specific antibodies. *Trichinella* antigens used as recombinant-protein vaccines have been identified, and demonstrated capable of eliciting host protective humoral immune responses (Arasu, et al., 1994; Sun, et al., 1994). In our previous study, the structural gene (TspE1) encoding 31 kDa antigen of *T. spiralis* was cloned and expressed in the prokaryotic expressing vector, pGEMEX-1. High specific antibody against the recombinant 31 kDa protein was identified by Western blot, and *Trichinella*-specific antibody was induced in mice immunized with the fusion protein (Cui et al., 2002). Production and analysis of a DNA vaccine encoding the 31 kDa antigen of *Trichinella spiralis* in skin and muscle of BALB/c mice is described in this paper.

2 Materials and Methods

2.1 Mice

Male BALB/c mice aged 4 – 6 weeks were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) and were raised in plastic micro-isolator cages before and after immunization. Anti-*Trichinella* antibodies were not detected by ELISA using secretory-excretory (ES) antigens of *T. spiralis* muscle larvae in all mice before immunization.

2.2 Parasite

T. spiralis used in this study was obtained from a swine source in the Henan Province of China and was maintained by serial passage in Sprague-Dawley (SD) rats every 6 – 8 months. Each rat was orally infected with 500 *T. spiralis* larvae.

2.3 Generation of anti-31 kDa fusion protein polyclonal sera

Anti-31 kDa fusion protein polyclonal mouse sera, used as positive control sera in IFAT, were obtained from BALB/c female mice injected intraperitoneally on two occasions 2 weeks apart with 60 μg (20 μg each injection) of 31 kDa fusion protein in complete Freund's adjuvant. Sera were collected 28 days after the second immunization and stored at −80℃ until use.

2.4 Plasmid construction

The recombinant eukaryotic expression plasmid pcDNA3-TspE1 was constructed as previously described (Cui et al., 2004). In brief, the larvae of *T. spiralis* in skeletal muscles were collected by acid-pepsin digestion. The target gene encoding the 31 kDa protein was prepared by RT-PCR from *T. spiralis* muscle larval RNA and cloned into the pUC18 vector. The positive clones were examined for the presence of a correct size insert by double digestion with *BamH* I and *Hind* III and single digestion with *Eco* RI, and also by PCR using gene specific primers. The target gene was sub-cloned into the eukaryotic expression vector pcDNA3 (Invitrogen, CA, USA). The pcDNA3 vector, which contains the human cytomegalovirus (CMV) promoter and an ampicillin-resistance gene, was used for the DNA vaccination studies. After transforming *Escherichia coli* strain JM109, the recombinant clones were selected on LB plates containing 100 μg/ml ampicillin overnight at 37℃ and plasmid DNA was extracted by the alkaline lysis method (Maniatis et al., 1982). The colonies containing inserts of the appropriate size in the right orientation were identified by electrophoresis of PCR products using gene specific primers. An 876 bp fragment was obtained after the recombinant plasmid pcDNA3-TspE1 was digested by *BamH* I and *Hind* III and as expected. Nucleotide sequencing was carried out to confirm the authenticity of the insert.

The recombinant plasmids were maintained and propagated in *E. coli* JM109 cells. The endotoxin-free plasmid DNA was purified from bacterial cells using a plasmid purification kit (Qiagen China Representative Office, Shanghai, China). The pcDNA3 plasmid without insert was also purified for use as a control. The purified plasmids were stored at −20℃ until use.

2.5 Production of DNA coated gold beads for immunization

Using protocols developed for the Hilos Gene Gun System (Bio-Rad, CA, USA), pcDNA3-TspE1 DNA and the control, pcDNA3 DNA, were precipitated onto gold beads (1.6 μm average diameter) and used to coat the inner surface of plastic tubing. The tubing was cut into a half inch length and stored dry at 48℃ until required. The quantity of gold and DNA comprising each immunizing "shot" was adjusted to produce the 1 mg DNA/0.5 mg gold "shots" for use in the immunization.

2.6 Immunization of mice

Male BALB/c mice, aged 4 – 6 weeks (10 per experimental group), were anesthetized and immunized. For intramuscular vaccination, 50 μg of pure plasmid DNA (in 50 μl of PBS) were injected into the left and right quadriceps of each leg, using a 1 ml tuberculin syringe fitted with a 28 G needle; The control animals received only PBS or pcDNA3. The mice were sacrificed 2 weeks after the primary intramuscular inoculation of DNA vaccine or pcD-

NA3 alone. For vaccination by gene-gun delivery, mice were anesthetized, their abdomens shaved, wiped with damp gauze and "shot" with the Gene Gun using 300 psi of helium gas. DNA vaccine doses of 3 μg (three shots) per mouse were used for epidermal inoculation. Controls mice were immunized similarly with pcDNA3 alone. The mice were sacrificed 4 h, 8 h, 24 h, 3 d, 5 d and 7 d after the initial inoculation of DNA vaccine or pcDNA3 alone by gene-gun delivery.

2.7 Detection of transcriptional activity of the pcDNA3-TspE1 in skin and muscles

To determine the duration and site of antigen presentation to the host, RNA was extracted from the skin and muscles of immunized mice and subjected to RT-PCR to detect evidence of transcription of the parasite DNA in transfected skin and muscle cells. Skin and muscles at the site of inoculation were excised at various intervals after DNA immunization, frozen on dry ice and stored at −80℃. The tissue was pulverized with mortar and pestle into powder and total messenger RNA was extracted using a commercial kit (Invitrogen, CA, USA). The extracted mRNA was treated with DNase I (RNase-free, Promega) to prevent PCR priming from plasmid a genomic DNA for 30 min at 37℃ and subjected to AMV reverse transcription reaction (Promega, WA, USA) for 60 min at 42℃. The reverse transcribed material was PCR amplified with primers specific to the encoding region of 31 kDa protein (using the thermal cycle protocol: 2 min 94℃; 35 cycles of 94℃ for 30 sec, 55℃ for 30 sec and 72℃ for 40 sec, and terminating with a 5 min extension step of 72℃). The resultant RT-PCR products were examined in 1.5% agarose electrophoresis gels.

2.8 Immunohistochemical staining

The expression of TspE1 gene in skin at the site of inoculation was assayed by immunohistochemical staining. The abdominal skin of vaccinated mice was excised and embedded in paraffin and 5 μm thickness of sections were prepared. After deparaffinization, the sections were processed to unmask the antigens by conventional microwave oven heating in 10 mg/ml citric acid buffer (pH 6.0) and subsequent detergent treatment using polyoxyethylene sorbitan monolaurate in PBS for 30 min. In order to observe the expression of TspE1 antigen in epidermis, immunohistochemical staining of 5 μm paraffin sections was performed with the Strept-Avidin- Biotin-Peroxidase complex (SABC) method (DGBio Co, Bejing, China). Briefly, 5 μm paraffin sections were treated with 3% hydrogen peroxide at room temperature for 10 min to remove endogenous peroxidase and incubated with 10% normal goat serum at room temperature for 5 min to block non-specific binding. The sections were then incubated with sera from mice infected with *T. spiralis* (diluted 1:50 in PBS) or normal mouse serum at 4℃ overnight. Section were washed in 0.01 M PBS three times and exposed to biotinylated goat anti-mouse IgG, followed by treatment with the SABC and stained with diaminobenzidine (DAB) with 0.15% hydrogen peroxide. Counterstaining was performed with haematoxylin.

2.9 Indirect fluorescencent antibody test (IFAT)

The expression of the TspE1 antigens in muscles at the site of inoculation in mice was detected by IFAT. The mice were sacrificed 2 weeks after the initial intramuscular inoculation of DNA vaccine and the muscles at the site of inoculation were separated and stored at −80℃. Then, 4 μm thick of frozen sections were cut, collected on gelatinized slides, and fixed with cold acetone for 10 min to prevent detachment of the sections during the following procedures. IFAT with frozen sections of muscles was carried out as previously described (Cui et al., 1999). Briefly, the frozen sections were incubated with 1:10 dilution of sera from mice immunized with recombinant fusion protein or infected with *T. spiralis* at 37℃ for 30 min. After washing with PBS, the sections were incubated with 1:16 dilution of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) at 37℃ for 30 min. Sera from normal mice were used as negative control. Slides were examined under fluorescent microscope (Olympus, Tokyo, Japan). When a yellow green fluorescence staining appeared on the section, the reaction was defined as IFAT positive.

3 Results

3.1 Detection of mRNA for TspE1 in skin and muscle of DNA immunized mice

RT-PCR products were obtained only from the skin samples of the pcDNA3-TspE1 immunized mice at 8 h post-immunization (Figure 1), but not obtained at 4 h, 24 h and 72 h post-immunization. As expected, there was no TspE1 transcriptional activity in the skin samples from the control mice immunized with pcDNA3. These results demonstrate that detectable transcriptional activity of DNA transfected skin cells is very transient and confined to the site of immunization. mRNA was extracted from muscle of pcDNA3-TspE1 immunized mice 2 weeks following injection and was analyzed by RT-PCR using 31 kDa antigen-specific primers. The transcriptional activity of TspE1 gene

was clearly visible in muscle tissue from mice injected with pcDNA3-TspE1 but not in those mice injected with control plasmid (Figure 2).

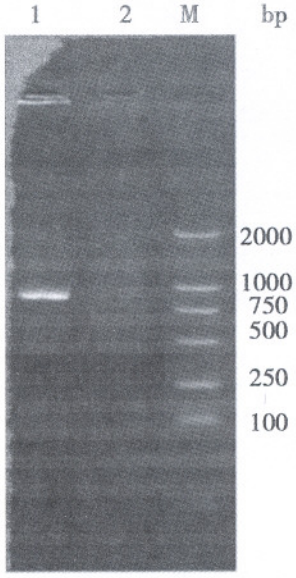

Figure 1. RT-PCR analysis of mRNA in skin of BALB/c mice immunized with pcDNA3-TspE1 and pcDNA3 plasmid. Mice were immunized with 3 μg of indicated plasmid DNA by gene-gun delivery. Skin at the site of inoculation was excised 8 h after the initial inoculation of DNA vaccine or pcDNA3 alone. The mRNA was isolated from the muscles and used for RT-PCR.
Lane 1: pcDNA3-TspE1; Lane 2: pcDNA3; Lane M: high molecular weight biomarker.

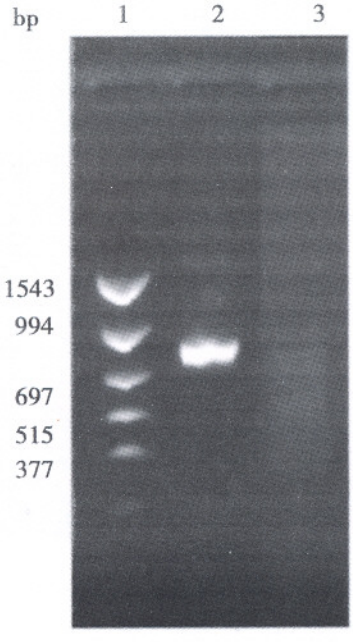

Figure 2. RT-PCR assay on RNA extracted from muscle two weeks after immunization with pcDNA3-TspE1 and pcDNA3 plasmid (1.5% agarose gel electrophoresis). Lane 1: DNA marker; Lane 2: RNA extracted from quadriceps muscle after immunization with pcDNA3-TspE1; Lane 3: RNA extracted from quadriceps muscle after immunization with pcDNA3.

3.2 Detection of TspE1 protein in skin at the site of inoculation

The results of immunohistochemical staining showed that specific brown round particles were seen among cells and in the cytoplasm of epidermial cells in all of the mice immunized with pcDNA3-TspE1 at 8 h post-immunization, but not in the mice immunized with pcDNA3 (Figure 3). Sections of *T. spiralis* muscle larvae as positive control were reacted with sera from the infected mice. No deep brown particles were observed when sections from the immunized and the infected mice were reacted with PBS or normal mouse sera (not showed in the figure).

3.3 Expression protein in muscle at inoculation sites of mice

The results of IFAT showed that the frozen section of inoculation site of mouse muscle 2 weeks after the initial intramuscular inoculation of pcDNA3-TspE1 reacted with sera from mice immunized with recombinant fusion protein or infected with *T. spiralis*. The fluorescence did not appear on the frozen section of inoculation site of mouse skin with only empty plasmid pcDNA3 (Figure 4).

4 Discussion

Both conventional protein vaccines and DNA vaccines (normally constituted of a naked DNA plasmid) elicit antibody responses, however, DNA vaccines have the additional advantage of stimulating cytotoxic T cells because the host is producing the antigenic protein intracellularly, thereby facilitating presentation of the antigen in the context of MHC class I molecules. Development of cellular immunity is important in fighting intracellular pathogens, giving DNA vaccines a clear advantage over protein vaccines. Another advantage of a DNA vaccine is its ease of administration and production. DNA is easily produced in large quantities with great purity, minimizing the risk of vaccine contamination with potential pathogens (McDonnell and Askari, 1996). Moreover, DNA can be readily introduced into tissues by DNA-coated microprojectiles through particle-mediated epidermal delivery (PMED), which facilitates easy epidermal administration and avoids the use of needles (Williams et al, 1991). Additionally, DNA vaccines may result in expressed antigens that resemble native antigens more closely than do antigens in conventional vaccines because manufacturing techniques can alter epitopes and reduce antigenicity. DNA vaccines may also be safer than some live attenuated vaccines, particularly in immunocompromised hosts.

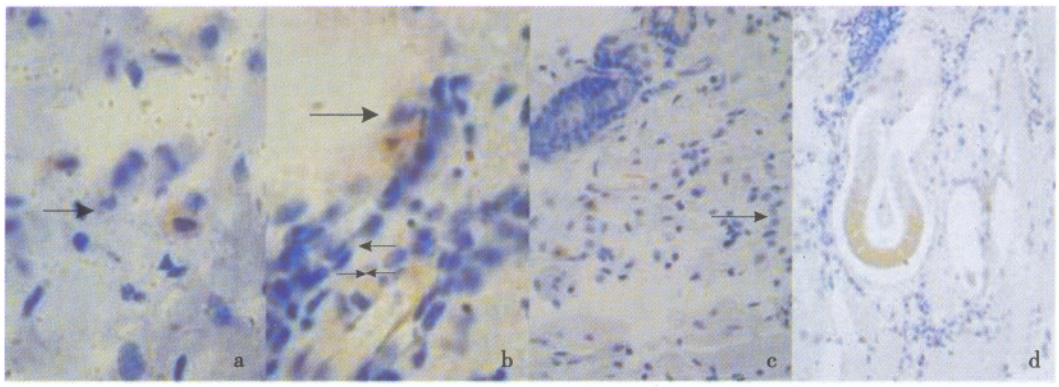

Figure 3. Immunohistochemical staining in skin of BALB/c mice inoculated with pcDNA3-TspE1 by gene-gun delivery.
a－b. Immunohistochemical staining of skin tissue of mouse at 8 h after gene-gun injection (1000×), arrows showing brown particles in cytoplasm (a) and extracells (b).
c. Negative control of pcDNA3 expressed in skin tissue of mouse at 8 h after gene-gun injection (400×).
d. Positive control of immunohistochemical staining of skeletal muscle of mouse infected with *T. spiralis* (400×), arrow showing the larva stained as brown.

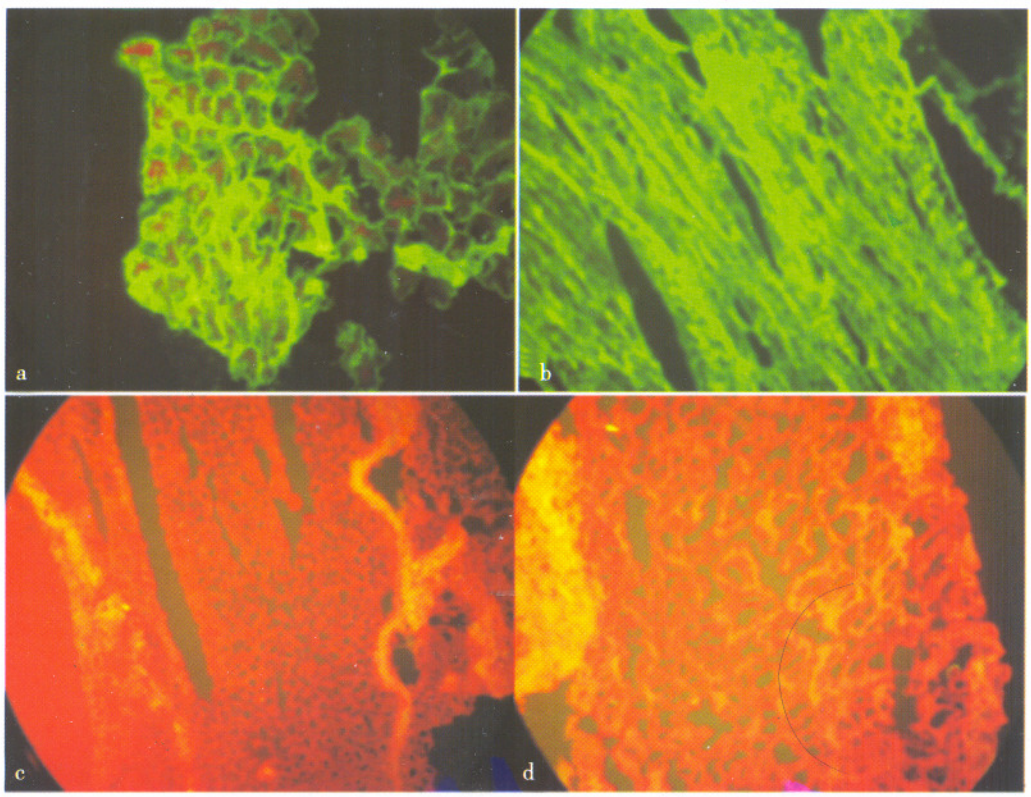

Figure 4. IFAT showing the antigenic protein expression of TspE1 gene in muscles 2 weeks after the initial intramuscular inoculation of recombinant plasmid pcDNA3-TspE1.
a. Section of muscle carrying pcDNA3-TspE1 reacted with sera from mice immunized with the recombinant fusion protein (200×).
b. Section of muscle carrying pcDNA3-TspE1 reacted with sera from mice infected with *T. spiralis* (200×).
c. Section of muscle carrying empty plasmid pcDNA3 reacted with sera from mice immunized with the recombinant fusion protein (100×)
d. Section of muscle carrying empty plasmid pcDNA3 reacted with sera from mice infected *T. spiralis* (200×).

Furthermore, several antigen genes could be included on one plasmid, reducing the total number of vaccinations that must be administered. Initial studies suggest that DNA vaccines can be administered safely and without overt toxicity (Tacket et al., 1999; Roy et al., 2001).

DNA immunization represents a novel method for generating the in situ expression of vaccine antigens and is now a widely reported means of generating immune responses. The administration of plasmid DNA via intramuscular, intradermal or gene gun delivery routes has been shown to stimulate T helper cells, cytotoxic T cells (CTL) and antibodies specific to the plasmid encoded antigen (Donnelly et al., 1997). The protective role for antigen-specific antibodies induced by genetic immunization has been manifestly demonstrated in a variety of challenge models against parasitic infection (Zhang et al, 2001; Dumonteil et al., 2003). However, up to date, DNA vaccines against Trichinella infection have not been reported in the literature.

We selected DNA vaccine as a strategy to prevent swine trichinellosis. The recombinant eukaryotic expression plasmid pcDNA3-TspE1 contained the gene encoding a 31 kDa antigen of *T. spiralis* was constructed. BALB/c mice were immunized with plasmid DNA vaccine by intramuscular injection and via gene-gun delivery. The transcriptional activity of the pcDNA3-TspE1 in skin and muscles at the site of inoculation was detected by RT-PCR using gene specific primers. The expression of TspE1 gene in skin and muscles at the site of inoculation was confirmed by immunohistochemistry and iFAT, respectively. These results showed that the recombinant plasmid pcDNA3-TspE1 was successfully transcribed and expressed in skin and muscles at the site of inoculation of mice. Thus, the plasmid encoding 31 kDa antigen may be of value for further development of DNA vaccine against challenge infection with *T. spiralis*.

Acknowledgments

This study was supported by a grant (20023100014) from Department of Education, and grant (0524410037) from Department of Science and Technology, Henan Province, China.

Correspondence to:
Jing Cui
Department of Parasitology
Medical College, Zhengzhou University
Zhengzhou, Henan 450052, China
Fax: 01186-371-6699-7182
Email: cuij@zzu.edu.cn

References

1. Agyei-Frempong M, Catty D. The measurement of antigens released by radiation-attenuated *Trichinella spiralis* larvae. Parasite Immunol 1983;5(3):289-303.
2. Arasu P, Ellis LA, Iglesias R, Ubeira FM, Appleton JA. Molecular analysis of antigens targeted by protective antibodies in rapid expulsion of *Trichinella spiralis*. Mol Biochem Parasitol 1994;65(2):201-11.
3. Aucouturier J, Deville S, Perret C, Vallee I, Boireau P. Assessment of efficacy and safety of various adjuvant formulations with a total soluble extract of *Trichinella spiralis*. Parasite 2001; 8 (2 Suppl):S126-S132.
4. Cui J, Wang ZQ, Han HM, Wei HY, Zhang HW, Li YL. Expression of DNA vaccine against *Trichinella spiralis* in mammalian cells. Chin J Parasitol Parasit Dis 2004; 22(5): 266-70.
5. Cui J, Wang ZQ, Wang HZ, Zhao GQ, Zhang HW. Cloning and expression of the antigen structural gene TspE1 of *Trichinella spiralis* pre-encysted larvae. Chin J Parasitol Parasit Dis 2002;20(5):278-80 (in Chinese).
6. Cui J, Wang ZQ, Zhu W, Zhang RG. Seroepidemiologicl study of *Trichinella spiralis* infection in central China. Helminthologia 1999;36(4):235-9.
7. Darwish RA, Sanad MM, Youssef SM. Immunization against *Trichinella spiralis* using antigens from different life-cycle stages experimental study in mice. J Egypt Soc Parasitol 1996;26(1):19-26.
8. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu Rev Immunol 1997;15:617-48.
9. Dumonteil E, Maria Jesus RS, Javier EO, Maria del Rosario G. M. DNA vaccines induce partial protection against *Leishmania mexicana*. Vaccine 2003; 21 (17-18):2161-8.
10. Dupouy-Camet J. Trichinellosis: a worldwide zoonosis. Vet Parasitol 2000;93(3-4):191-200.
11. Eissa MM, el-Azzouni MZ, Baddour NM, Boulos LM. Vaccination trial against experimental trichrinellosis using autoclaved *Trichinella spiralis* larvae vaccine (ATSLV). J Egypt Soc Parasitol 2003;33(1):219-28.
12. Huang FC. Report of one case of human trichinellosis. Chin J Intern Med 1965;13(4):392.
13. McDonnell WM, Askari FK. DNA vaccines. N Engl J Med 1996;334(5):42-5.
14. McGuire C, Chan WC, Wakelin D. Nasal immunization with homogenate and peptide antigens induces protective immunity against *Trichinella spiralis*. Infect Immun 2002;70(12):7149-52.
15. Murrell KD, Pozio E. Trichinellosis: the zoonosis that won't go quietly. Int J Parasitol 2000; 30(12-13): 1339-49.
16. Nakayama H, Inaba T, Nargis M, Chisty M, Ito M, Kamiya H. Immunization of laboratory animals with ultraviolet-attenuated larvae against homologous challenge infection with *Trichinella britovi*. Southeast Asian J. Trop Med Public Health 1998; 29(3): 563-6.
17. Robinson K, Bellaby T, Chan WC, Wakelin D. High levels of protection induced by a 40-mer synthetic peptide vaccine against the intestinal nematode parasite *Trichinella spiralis*. Immunology 1995;86(4):495-8.
18. Rothel JS, Boyle DB, Both GW, Pye AD, Waterkeyn

JG, Wood PR, Lightowlers MW. Sequential nucleic acid and recombinant adenovirus vaccination induces host-protective immune responses against Taenia ovis infection in sheep. Parasite Immunol 1997;19(5):221－7.

19. Roy MJ, Wu MS, Barr LJ. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 2000;19(7－8):764－78.
20. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual, 3rd ed. NY: Cold Spring Harbour Laboratory. 2001:1.31－1.42.
21. Sun S, Xu W, He N, Sugane K. An antigenic recombinant fusion protein from *Trichinella spiralis* induces a protective response in BALB/c mice. J Helminthol 1994;68(1):89－91.
22. Tacket CO, Roy MJ, Widera G, Swain WF, Broome S, Edelman R. Phase I safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. Vaccine 1999;17(22):2826－9.
23. Wang ZQ, Cui J. Epidemiology of human trichinellosis in China during 1964－1999. Parasite 2001a;8 (2 Suppl):S63－S66.
24. Wang ZQ, Cui J. Epidemiology of swine trichinellosis in China. Parasite 2001b;8 (2 Suppl):S67－S70.
25. Wang ZQ, Cui J, Li HS, Jin XX, Wu F, Xue CG, Mao FR. Some observations on trichinosis in China. Helminthologia 1998;35(1):27－9.
26. Williams RS, Johnston SA, Riedy M, DeVit MJ, McElligott SG, Sanford JC. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc Natl Acad Sci USA 1991;88(7):2726－30.
27. Zhang Y, Taylor MG, Johansen MV, Bickle QD. Vaccination of mice with a cocktail DNA vaccine induces a Th1-type immune response and partial protection against Schistosoma japonicum *infection*. *Vaccine* 2001;20(5－6):724－30.