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Detection of circulating antigens in serum samples of mice experimentally infected with *Trichinella spiralis* by a sandwich ELISA based on IgY

F. J. JING[1,2], J. CUI[1*], R.D. LIU[1], L. N. LIU[1], P. JIANG[1], Z. Q. WANG[1*]

[1]Department of Parasitology, Medical College, Zhengzhou University, China,
*E-mail: *cuij@zzu.edu.cn, wangzq@zzu.edu.cn*; [2]Department of Laboratorial Medicine, Wuhu Hospital of Traditional Chinese Medicine, Anhui Province, China

Summary

In the present study, a sandwich ELISA based on IgY (egg yolk immunoglobulin) was developed for detection of circulating antigens (CAg) in sere of mice experimentally infected with *Trichinella spiralis*. The IgY-sandwich ELISA assay involved the use of chicken antibody IgY against excretory-secretory (ES) antigens of *Trichinella spiralis* muscle larvae as a capture antibody and mouse polyclonal antibody IgG to ES antigens as a detecting antibody. This method was able to detect as little as 3 ng/ml of ES antigens added to normal mouse serum. A group of sixteen mice was orally inoculated with 500 *T. spiralis* muscle larvae per animal. The serum samples from the infected mice were taken during 1 – 35 days post-infection (dpi). The CAg was detectable as early as 8 dpi in the sera of infected mice. The level of CAg increased dramatically during 13 – 15 dpi and reached a peak at 22 dpi and remained a plateau for 3 days, then declined gradually. Another peak of CAg occurred at 31 dpi. The anti-*Trichinella* antibodies was first detected in 14.3 % of the infected mice at 2 weeks post-infection (wpi), and reached a peak positive rate of 100 % at 5 wpi. Moreover, the infected mice were treated with abendazole at 5 wpi and the serum CAg levels increased significantly during 2 – 6 days post-treatment (dpt) and then declined rapidly during 8 – 14 dpt. By 42 dpt, the CAg levels decreased to the undetected level, but the detection rate of antibodies was still 100 %. The IgY- sandwich ELISA appears to be a sensitive for detection of antigenemia of *T. spiralis* and valuable to judge the efficacy of chemotherapy in trichinellosis.

Keywords: *Trichinella spiralis*; circulating antigens; sandwich ELISA; IgY; abendazole

Introduction

Trichinellosis is a parasitic zoonosis caused by the tissue-dwelling nematode *Trichinella*. Humans acquire the disease by ingesting raw or insufficiently cooked meat of pigs or other animals containing the *Trichinella* larvae (Dvorožňáková *et al*., 2010, 2013). The adult worms live in the intestinal mucosa of flesh-eating animals and humans, while the larvae live in skeletal muscles of the same hosts (Kołodziej-Sobocińska *et al*., 2012). The clinical manifesttations of trichinellosis can be divided into two phases: an enteral phase and a muscular (parenteral or acute) phase, which can coexist for a period lasting from a few days to weeks. The hallmark of the intestinal phase is a non-specific gastroenteritis. Diarrhea, abdominal pain, nausea, and vomiting can occur. The muscular phase is associated with an inflammatory and allergic response to muscle invasion by the migrating larvae. Fever, eyelid or facial edema, myalgia, and eosinophilia are the main clinical manifestations (Pozio *et al*., 2003; Dvorožňáková *et al*., 2012).

Trichinella infection in animals and humans is widely distributed in the world. Of all of the countries of the world, *Trichinella* spp. infection has been documented in domestic animals (mainly pigs) and wildlife in 43 (21.9 %) and 66 (33.3 %) countries, respectively. Human trichinellosis has been documented in 55 (27.8 %) countries around the world (Pozio, 2007). In China, the overall seroprevalence of *Trichinella* infection in humans was 3.19 %. From 2004 to 2009, 15 outbreaks of human trichinellosis, consisting of 1,387 cases and four deaths, were reported in southwestern China (Cui *et al*., 2011). So, trichinellosis is a major food-borne zoonosis with health, social, and economic impacts in China (Cui *et al*., 2013).

The diagnosis of trichinellosis is rather difficult because fever, myalgia and eosinophilia are nonspecific, and this disease may be misdiagnosed. At present, muscle biopsy and serologic testing are used for diagnosing human trichinellosis (Yera *et al*., 2003; Gómez-Morales *et al*., 2008), but the biopsy technique is not sensitive to infections with small numbers of *T. spiralis*, and serologic tests (e.g., enzyme-linked immunosorbent assay (ELISA) using muscle

larval excretory-secretory (ES) antigen or the synthetic antigen 3,6-dideoxy-D-arabinohexose [tyvelose]) for detecting IgG specific for *Trichinella* are not positive in pig and mice infected experimentally until 3 to 4 weeks after infection (Kapel & Gamble, 2000; Gamble *et al*., 2004; Oltean *et al*., 2012; Wang *et al*., 2012;). Several studies have shown that the maximum positivity of 100 % of ELISA in detecting anti-*Trichinella* antibodies was not reached until at least 1 to 3 months after human infection with the parasite (Bruschi *et al*., 1990; Morakote *et al*., 1991; Wang *et al*., 1998). In addition, the diagnostic methods based on detecting antibodies cannot provide a definite diagnosis of acute trichinellosis because the antibody detection is impossible to distinguish acute or past infection (Wang *et al*., 1998).

The detection of *Trichinella* circulating antigen (CAg) seems an effective way to discriminate between previous exposure and current infection. In this respect, the detection of *Trichinella* CAg takes a prominent place. Various methods have been developed for detecting *Trichinella* CAg, including counterimmunoelectrophoresis (Smith & Kennedy, 1984), latex agglutination test (Choy et al., 1988), immunoradiometric assay (Ivanoska *et al*., 1989), Direct enzyme-linked immunosorbent assay (ELISA) (De-La-Rosa *et al*., 2001) and sandwich ELISA (Arriaga *et al*., 1995; Hegazy *et al*., 1996; Rodriguez-Osorio *et al*., 1999; Li and Ko, 2001). However, because the serum levels of circulating antigens of *T. spiralis* are quite low, the detection rate of CAg in serum samples was usually only 30 % – 50 % in the patients with clinical trichinellosis (Nishiyama *et al*., 1992). Hence, the serological methods available for detecting CAg cannot be used to serodiagnosis of trichinellosis (Dupouy-Camet *et al*., 2002; Gamble *et al*., 2004).

Chicken IgY has been recognized as an excellent source of polyclonal antibodies for over decades (Tini *et al*., 2002). Specific antibodies produced in chickens offer several important advantages over producing antibodies in mammals, for example, avoiding the interference in immunological assays caused by the rheumatoid factors (Larsson & Sjouist, 1988), complement system (Larsson *et al*., 1992), or anti-mammalian antibodies (Juliarena *et al*., 2007). Furthermore, chicken IgY recognize more epitopes than the corresponding mammalian antibodies (Svendsen & Hau, 2006). Because of the above advantages, IgY has been widely used to diagnosis of different diseases (Sunwoo *et al*., 2006; Dias da Silva & Tambourgi, 2010).

In the present study, we developed a sandwich ELISA based on IgY (IgY- sandwich ELISA) for detection of CAg in serum samples of mice experimentally infected with *Trichinella spiralis*. The assay consisted of chicken IgY against the excretory-secretory (ES) antigens of *T. spiralis* muscle larvae as a capture antibody and mouse anti-ES antigen polyclonal antibodies as a detecting antibody. Moreover, the serum levels of CAg of the infected mice after treatment with albendazole was also investigated. In comparison, antibodies were assayed by indirect ELISA using ES antigens of *T. spiralis* muscle larvae.

Materials and methods

Parasite

The isolate (ISS534) of *T. spiralis* used in the study were obtained from domestic pigs in Nanyang city of Henan Province, China. This isolate was maintained by serial passage in Kunming mice at 6–8-month intervals. Muscle larvae were recovered by acid pepsin digestion as described previously (Li *et al*., 2010).

Mice and serum samples

BALB/c mice aged 6 weeks were provided by the Experimental Animal Center of Henan province. Sixteen mice weighing 20 to 25 g were orally infected with 500 larvae per animal of *T. spiralis* larvae. Blood was collected daily until 35 days post infection (dpi). Moreover, to evaluate the effect of chemotherapy on antigen levels, the infected mice were divided into two subgroups: albendazole treated group (11 mice) and its corresponding untreated group (5 mice). Albendazole (Hanjiang Pharmaceutical Factory of Shanxi, China) suspended in distilled water was administered intragastrically at 35 dpi in a dose of 370 mg/kg body weight, twice daily, for 7 consecutive days (Dupouy-Camet *et al*., 2002). The untreated group was given only distilled water. Blood was collected at 2 days-interval after treatment. The sera of mice before infection were also collected. All sera were stored at −20 °C until use. At the end of the study, all the infected mice were euthanized, skinned, and eviscerated. The carcasses of each mouse were individually digested by artificial digestion method as described previously (Li *et al*., 2010). The larvae were collected and counted from each mouse. Results were expressed as reduction rates, calculated as the percent of recovered larvae versus those recovered from control mice.

Preparation and characterization of chicken anti-ES IgY

The ES antigens of *T. spiralis* muscle larvae were prepared as described previously (Wang *et al*., 2011). The preparation and characterization of chicken IgY against ES antigens were developed according to the method established in our laboratory (Li *et al*., 2010). In brief, 24-week-old Roman hens that were maintained in a standard SPF condition were subcutaneously immunized with ES antigens for four times. Each hen was immunized with 500 μg of ES antigens firstly and then boosted with 250 μg of ES antigens for three times at 10-day intervals. Eggs were collected daily before immunization and after the last immunization. IgY was extracted from yolk according to the water dilution method (Akita & Nakai, 1993). The protein content of IgY was determined by the method described by Bradford (1976). The purified IgY was analyzed by SDS-PAGE.

Preparation of poloclonal IgG antibodies

Poloclonal IgG antibodies against ES antigens of *T. spiralis* muscle larvae were produced in female BALB/c mice (6 weeks old), according to the methods as described previously (Liu *et al*., 2013). Normal mouse serum served as the negative control.

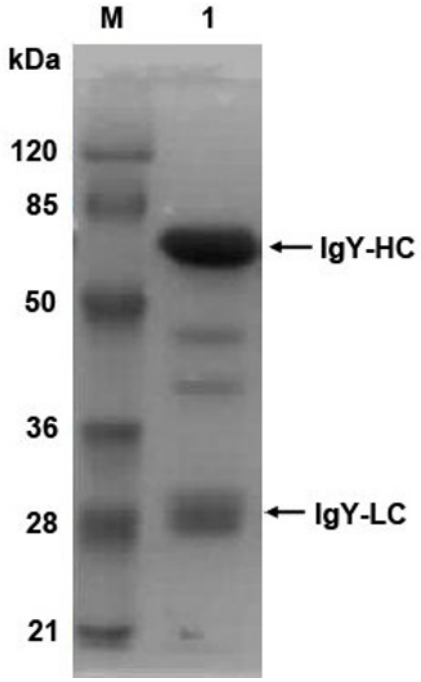

Fig. 1. Analysis of IgY by 12% SDS-PAGE in reducing condition. 15 µg of protein was loaded for each lane. *M*: Protein marker with low molecular weights; *1* Purified IgY from immunized egg yolk. IgY-HC = IgY heavy chain; IgY-LC = IgY light chain

IgY-sandwich ELISA for detection of circulating antigen

An IgY-sandwich ELISA was developed using IgY to capture free antigens present in serum samples, and polyclonal IgG to selectively react with ES components of muscle larvae. In brief, 96-wells ELISA plates (Corning, USA) were coated with 7.5 µg IgY proteins/well in 100 µl of bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed three times with 0.05 % Tween-20 in PBS (PBST). After blocking with 5 % skimmed milk in PBST, serum samples were diluted at 1:100 and IgG was used at 1:200 dilutions. For the detection of bound IgG, HRP-conjugated sheep anti-mouse IgG (Sigma, USA) was used at 1:5,000 dilutions. The reactions were detected by addition of the substrate O-phenylendiamine dihydrochloride (Sigma, USA) plus H_2O_2 and stopped with 50 µl/well of 2M H_2SO_4. Optical density (OD) values at 490 nm were measured with a microplate reader (TECAN, Austria). All samples were run in duplicate. Murine sera before infection and PBS were used as negative control and blank control, respectively. All the values were recorded after appropriate blank correction. The reactivity of CAg was expressed as the lower detection limit (LDL) in nanograms of ES antigens/ml. The ES antigen standard was serially diluted in normal serum, ranging from 1 ng to 5 mg/ml and BSA was used as negative control.

Indirect ELISA for detection of antibodies

Anti-*Trichinella* antibodies in the infected mice before infection, 1 – 5 weeks post-infection (wpi) and 1 – 6 weeks post-treatment (wpt) were weekly assayed by indirect ELISA with ES antigens. It was performed as previously described (Wang *et al*. 2006).

In both ELISAs, Test sera/negative sera OD values <2.1 were regarded as negative and those ≥2.1 as positive. The cut-off values of IgY-sandwich ELISA for detecting CAg and indirect ELISA for detecting antibodies were 0.21 and 0.20, respectively.

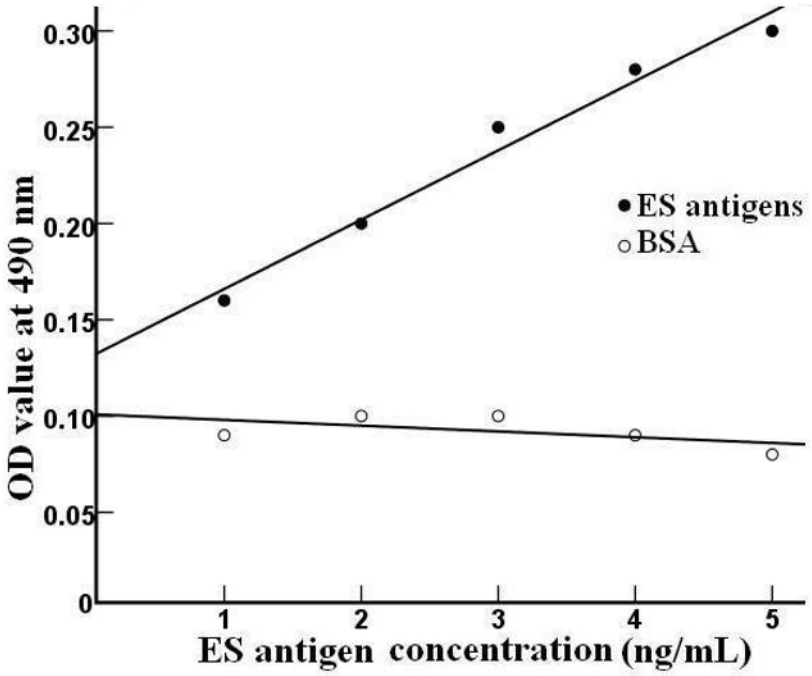

Fig. 2. Relationship between ES antigen concentration and OD value. BSA was used as negative control. Antigen levels are expressed as mean OD values measured with IgY-sandwich ELISA

Statistical analysis

All statistical analyses of data were done with SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL). Chi Square Test and Student's *t*-test was used to determine the differences between CAg and antibody at various periods post-infection, difference between the treated group and untreated group, and differences between different time points in the same group with respect to the detection rate and serum level of CAg and antibody. A *P* Value of < 0.05 was considered as significant. Wilcoxon rank sum test was used to compare the differences of the number of recovered larvae between treated group and untreated group.

Results

Analysis of IgY

On average, 70 mg of IgY was extracted from each immunized egg yolk. SDS-PAGE analysis showed two major protein bands with molecular weight of 67 kDa and 29 kDa characteristic of the IgY (Fig. 1).

IgY-sandwich ELISA

To establish the sensitivity and linearity of IgY-sandwich ELISA, a standard dilution curve of ES antigen concentrations was set up. As clearly illustrated in Figure 2, there was a significant correlation between the OD value and ES antigen concentration, with correlation coefficients being 0.988. The LDL of ES antigens detected by the assay was 3 ng/ml with a linear increase in OD from 1 ng to 5mg/ml. Furthermore, the results indicated that IgY was specifically bound to ES antigens.

Kinetics of circulating antigens and antibodies in sera of infected mice

The levels of CAg were determined with all serially collected sera by IgY-sandwich ELISA. Figure 3 shows the CAg levels of different time point. CAg could be detected at 8 dpi with a detection rate of 21.4 %. The level of CAg increased dramatically during 13 – 15 dpi and reached a peak at 22 dpi with 92.8 % of CAg detection rate and remained a plateau for 3 days, then declined gradually. Another peak of CAg occurred at 31 dpi with 58 % of CAg detection rate.

The antibodies against ES antigens was first detected in 14.3 % of the infected mice at 2 wpi, and reached a peak at 5 wpi with 100 % of antibody positive rate (Fig. 4). The difference in the detection rate between CAg and antibodies was statistically significant at 2 – 5 wpi ($\chi^2 = 9.956$, $P < 0.05$)

Effect of chemotherapy on the levels of circulating antigens and antibodies

The CAg levels in the untreated and treated groups showed no difference before treatment ($t = 0.093$, $P > 0.05$), but they had a statistical difference during 2 – 6 days after treatment ($t = 4.243$, $P < 0.05$) (Fig. 5). Compared with

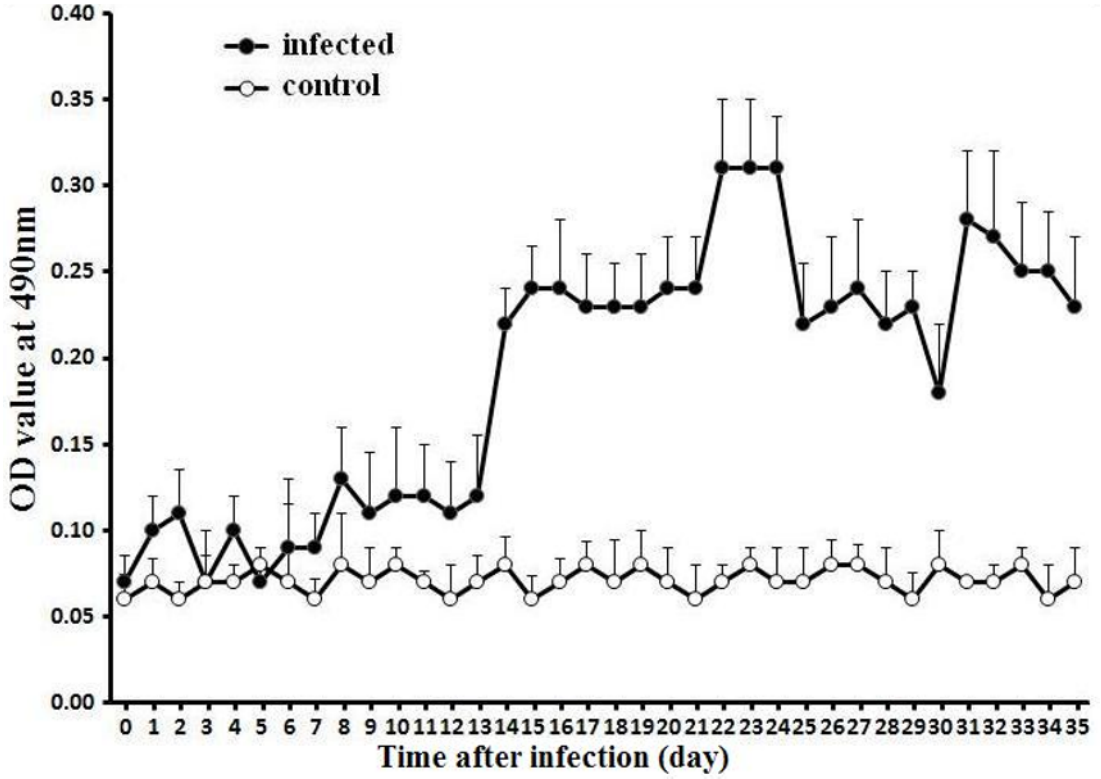

Fig. 3. Kinetics of circulating antigens in sera of mice experimentally infected with *T. spiralis* at different time intervals after infection. Circulating antigens were detected by a sandwich ELISA with IgY as the capturing antibodies and mouse polyclonal antibody IgG to ES antigens as a detecting antibody. Each mouse was given an oral dose of 500 infective larvae. The sera of no-infected mice were used as negative control. Antigen levels are expressed as the mean OD value±SD of the individual serum sample

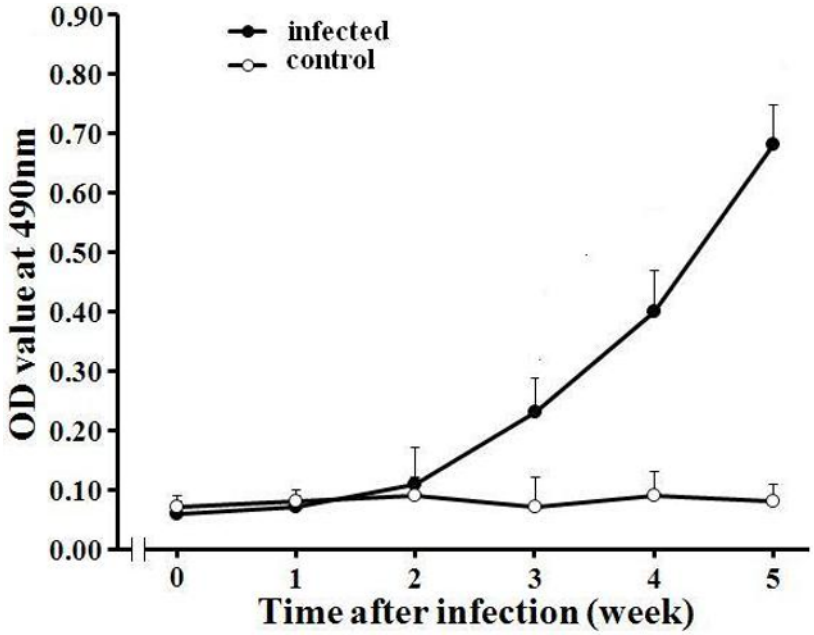

Fig. 4. Kinetics of circulating antibodies in sera of mice experimentally infected with *T. spiralis* at different time intervals after infection. Circulating antibodies were detected by an indirect ELISA with ES antigens of *T. spiralis* muscle larvae. Each mouse was given an oral dose of 500 infective larvae. The sera of no-infected mice were used as negative control. Antibody levels are expressed as the mean OD value ± SD of the individual serum sample.

that of untreated group, the serum levels of CAg in treated group increased significantly during 2 – 6 days post-treatment and then showed rapid decline during 8 – 14 days post-treatment. By 42 days after treatment, the CAg levels decreased to the undetected level.

However, as clearly illustrated in Figure 6, the serum levels of antibodies in the treated groups decreased slowly at 1 – 6 wpt, but the antibody levels in the treated and untreated groups showed no difference ($t = -2.219$, $P > 0.05$). By 6 wpt, the detection rate of antibodies was still 100 % in both groups.

At the end of the study (6 wpt), all the infected mice were slaughtered. The number of larvae recovered from the treated group was 382 ± 96 larvae, which was significantly

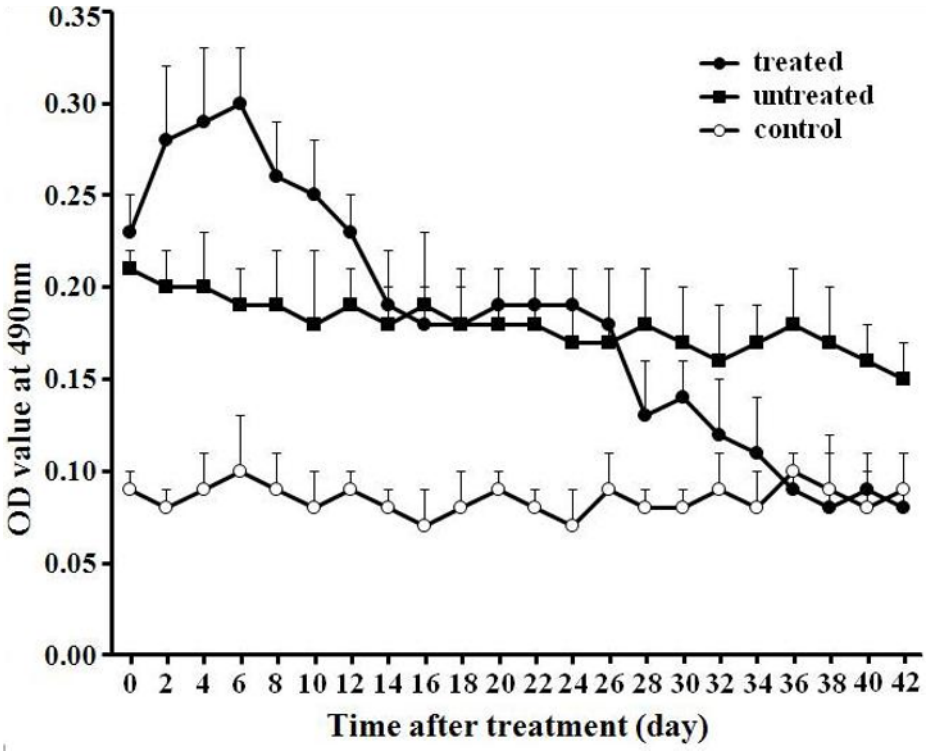

Fig. 5. Comparison of circulating antigen levels in sera of infected mice treated and untreated with albendazole. Albendazole was intragastrically administered at 5 weeks post-infection (0 week after treatment). The sera of no-infected mice were used as negative control. Antigen levels are expressed as the mean OD values ± SD of the individual serum sample.

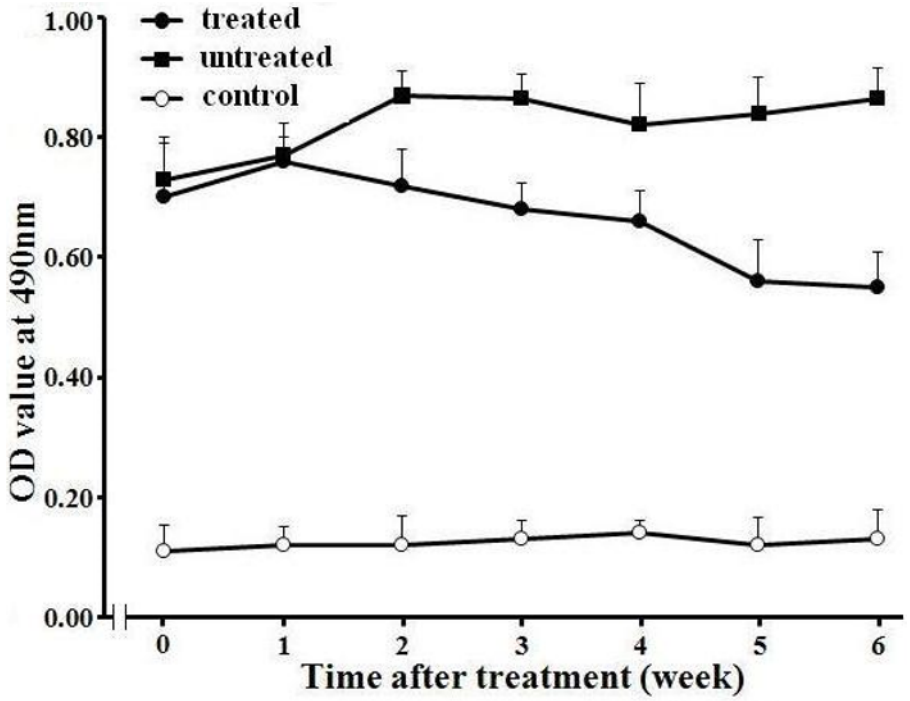

Fig. 6. Comparison of circulating antibody levels in serum samples of infected mice treated and untreated with albendazole. Albendazole was intragastrically administered at 5 weeks post-infection (0 week after treatment). The sera of no-infected mice were used as negative control. Antibody levels are expressed as the mean OD value ± SD of the individual serum.

lower than 103720 ± 8699 larvae recovered from untreated group (Z = -3.139, P < 0.01), and the larval reduction rate in the treated group was 99.6 %.

Discussion

The CAg that is excreted into the host body of macromolecular particles by live worm, mainly worms shedding secretion and excretion, can distribute throughout in various tissues. Hence, the positive CAg implies persistence of living parasites, the amount of CAg was closely parallel to the worm burden in the host. Compared with detection of circulating antibodies, antigen detection might be a useful confirmatory test of parasite infection because it is direct demonstration of parasite products in the circulation (Smith *et al.*, 1984; Ivanoska *et al*., 1989). The level of CAg in serum fluctuates widely at various periods post-infection. Therefore, for serodiagnosis, it is necessary to use an extremely sensitive and stable method which can detect slight quantities of CAg. Otherwise, the false-negative results may easily be obtained. This may account for detection CAg in only 13 %, 29.9 % and >30 % of the clinical cases with trichinellosis by immunoradiometric assay, sandwich ELISA and dot-blot (Ivanoska *et al*., 1989; Nishiyama *et al*., 1992; Dzbenski *et al*., 1994).

In this work, immunization of Roman hens with ES antigens of *T. spiralis* muscle larvae presented specific IgY in the egg yolks. The average amount of 70 mg of IgY against ES antigens was harvested for each egg, which indicated that IgY was transited to egg yolk at high levels. The analysis of the recovered IgY by SDS-PAGE showed a good purity. It indicates that Roman hens, as the host for the production of IgY to ES antigens, show the remarkable ability to rapidly and efficiently generate an abundant IgY and provide specific IgY in a noninvasive way.

Monoclonal antibodies (mAbs) have been used in sandwich ELISA, since the reactivity pattern of each mAb is addressed to a specific molecule epitope (Youssef *et al*., 1989). The LDL of CAg was 35 ng antigen/ml (Arriaga *et al*., 1995). Although we used polyclonal IgY and IgG instead of a mAb, the sensitivity of IgY-sandwich ELISA could reach 3 ng antigens/ml. The assay can be used to evaluate the existence and level of ES antigens in the serum of infected mice because the OD values increase linearly with ES antigen concentrations. Apparently, the application of IgY to ES antigens is advantageous for the detection of circulating antigens in experimentally infected mice. The factors contributing to the advantage include that chicken IgY reacts with more epitopes on mammalian antigens and then produce an amplification of the signal due to evolutionary difference (Carlander *et al*., 1999; Larsson & Sjouist, 1990).

T. spiralis CAg of the experimentally infected mice was continuously detected by IgY-sandwich ELISA with an early and a late peak of CAg during 8 – 35 dpi. The serum CAg of the infected mice could be detected by a mAb-sandwich at 11 dpi (Youssef *et al*., 1989). Similar observations regarding the two peaks of CAg have also been found in experimental infected mice and pigs (Arriaga *et al*., 1995; Li & Ko, 2001). In this study, the level of CAg increased dramatically during 13 – 15 dpi,

which was probably related to the massive migration of the newborn larvae released by gravid females to the skeletal muscles. The two peaks of CAg occurred at 22 dpi and 31 dpi when the larvae have developed to the infective stage and the encapsulated of the parasite had been completed. The increase of CAg levels, as detected in this study, seems to correlate with the establishment of muscle larvae in infected animals (Murrell, 1985). The subsequent decline in CAg level may be due to the formation of immune complexes which in turn are eliminated by the host (Arriaga *et al*., 1995).

Compared with detection of the circulating antigen, circulating antibodies were detected in 12.3 % of the infected mice at 2 wpi, the antibody detection rate was only 35.7 % at 3 wpi, and it reaches 100 % at 5 wpi. Although the sensitivity of the antibody detection was higher than antigen detection at 5 wpi, it appears relatively late and cannot discriminate between the past and present infection. CAg is present in the serum only during active infection, and the levels of CAg continue to decrease after successful chemotherapy. Thus, detection of CAg can also be used as an effective way to evaluate the efficacy of chemotherapy. Our result shows that the serum levels of CAg increased significantly during 2 – 6 days post-treatment. The sharp increasing of the CAg might be induced by the larvae destroyed by albendazole. At the end of the experiments, the larval reduction rate in the treated group was 99.6 %, which indicated that the chemotherapy was efficient. Our results show that the CAg drops down to the undetected level by 6 weeks after treatment with albendazole, which indicates that the novel assay can be used to evaluate the efficacy of chemotherapy. Similar observations regarding the killing effects of albendazole on the encapsulated *T. spiralis* larvae in muscles have been reported. A reduction of 75.5 % in the parasitic load was observed, when the mice infected with 10 *T. spiralis* muscle larvae were treated by using under-dosed with albendazole (20 mg/kg for 7 days) at 40 dpi (de-la-Rosa *et al*., 2007). While albendazole (200 mg/kg for 7 days) was given to the mice infected with 100 larvae at 35 dpi, the larval reduction rate was 71.8 % at 15 days after treatment (Xue *et al*., 2010). The efficacy of albendazole against the larvae in muscle tissues depend on the time between infection and treatment and could be dose-dependent. The later the treatment is prescribed, the higher the probability that the infected will harbor viable larvae in their muscles (Dupouy-Camet *et al*., 2002).

In conclusion, the IgY- sandwich ELISA appears to be a sensitive and feasible assay for detection of antigenemia of *T. spiralis* and valuable to assess the efficacy of chemotherapy in murine trichinellosis. However, the method for detection of circulating *T. spiralis* antigens did not allow the identification of the infected mice during the first week after infection, although it was able to detect as little as 3 ng antigens/ml. The IgY and monoclonal antibodies against the early antigens of *T. spiralis* larvae needs to be studied and applied to the antigen detection.

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