

# Serodiagnosis of experimental sparganum infections of mice and human sparganosis by ELISA using ES antigens of *Spirometra mansoni* spargana

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**Abstract** We conducted a study of serodiagnosis of experimental sparganum infections of mice and human sparganosis by enzyme-linked immunosorbent assay (ELISA) using excretory–secretory (ES) antigens of *Spirometra mansoni* spargana and compared the sensitivity and specificity of crude and ES antigens for detecting the specific anti-sparganum IgG antibodies. By crude antigen ELISA and ES antigen ELISA, anti-sparganum IgG was detected in all of 30 serum samples of the infected mice; no cross-reactions were observed in serum samples of the mice infected with *Trichinella spiralis*, *Schistosoma japonicum*, *Toxoplasma gondii*, and normal mice. Anti-sparganum IgG was detected by ES antigen ELISA in sera of mice infected with one, two, four, six, and eight spargana at 3 weeks post-infection (wpi), with a detection rate of 100%, and lasted to 18 wpi when the experiment was ended. The difference in anti-sparganum antibody levels among five groups of the infected mice was statistically significant ( $F=245.296$ ,  $p<0.05$ ); the antibody levels were correlated with infecting doses of spargana ( $r=0.323$ ,  $p<0.05$ ). The sensitivity of both ELISA in detecting the serum samples of patients with sparganosis was 100% (20/20), but 96.72% (59/61) of specificity of ES antigen ELISA in detecting serum samples of patients with cysticercosis, echinococcosis,

paragonimiosis, clonorchiosis, and schistosomiasis, and healthy persons was significantly greater than 72.13% (44/61) of crude antigen ELISA ( $\chi^2=14.027$ ,  $p<0.05$ ). Our finding indicates that ELISA using ES antigens of *S. mansoni* spargana may be applied to the specific early serodiagnosis of sparganosis.

## Introduction

Sparganosis is a serious parasitic zoonosis caused by infection with spargana, the plerocercoid larvae of some diphyllbothroid tapeworms belonging to the genus *Spirometra* (Nithiuthai et al. 2004). The most important species of the genus *Spirometra* tapeworms with plerocercoids that can produce sparganosis in human include *Spirometra mansoni* (syn. *Spirometra erinacei*) which is the most common in Asia, and *Spirometra mansonioides* which is mainly distributed in North America (Bogitsch et al. 2005; Roberts et al. 2009). The adults are intestinal parasites of some species of Canidae and Felidae; the first intermediate hosts are freshwater copepods (cyclops), whereas the second intermediate or paratenic hosts belong to different species of vertebrates (snakes, frogs, pigs, and so on). Human is an accidental host. Human infection results mainly from drinking raw water contaminated with cyclops harboring proceroids, ingesting raw flesh of frogs and snakes infected with plerocercoids, or placing frog or snake flesh on open wound for treatment of skin ulcers or eye inflammations (Fukushima and Yamane 1999; Magnino et al. 2009). Sparganosis poses a serious threat to human health; the larvae usually lodge in the subcutaneous tissues and sometimes invades the abdominal cavity, eye, and central nervous system causing blindness, epilepsy, paralysis, and even death (Yang et al. 2007; Shirakawa et

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al. 2010). Human sparganosis is reported in many countries but is most common in eastern Asia and the Far East (Wiwantikit 2005). In the People's Republic of China, sparganosis is an important food-borne parasitic zoonosis, with more than 1,000 human cases reported in 27 out of 34 provinces, autonomous regions, or municipal districts during 1927–2009 (Qiu and Qiu 2009).

Generally, the clinical diagnosis of sparganosis is rather difficult and often misdiagnosed because the specific signs or symptoms are lacking. A definite diagnosis of human sparganosis is established by detection of the larvae in a biopsy specimen obtained from the subcutaneous nodule, but the confirmative diagnosis is very difficult for visceral and cerebral sparganosis since the larva is found only by surgical removal (Murata et al. 2007). Because of most of cases with sparganosis are sporadic, there are a few of reports on serodiagnosis of human sparganosis (Kim et al. 1984). Hence, serological tests and diagnostic antigens for detection of specific anti-sparganum antibodies are needed to be studied.

The enzyme-linked immunosorbent assay (ELISA) is rapid and is the most commonly used serological method for the detection of sparganum infection in humans; the sensitivity and specificity of ELISA are largely dependent on the quality of the antigens used in the test (Yeo et al. 1994). The excretory–secretory (ES) antigens of the tissue-dwelling parasites have been used in ELISA for serodiagnosis of the parasitosis (trichinellosis, cysticercosis, etc.) with high sensitivity and specificity (Gamble et al. 2004; Nkouawa et al. 2010). However, the application of sparganum ES antigens to the diagnosis of sparganosis has been not reported. In addition, the dynamics of serum anti-sparganum IgG at different time intervals after infections are not clear.

The aim of this study was to compare the sensitivity and specificity of crude antigen ELISA and ES antigen ELISA for detecting the specific serum anti-sparganum IgG in experimentally infected mice with different-level infections and patients with sparganosis.

## Materials and methods

### Parasites

The spargana of *S. mansoni* was used in this study because most of cases with sparganosis in China are caused by the plerocercoid larvae of this tapeworm. Wild frogs were captured from Zhoukou city of Henan province, China. By necropsy, the spargana were collected from the muscles of the naturally infected wild frogs (*Rana limnocharis* and *Rana nigromaculata*). Most of spargana were located in the muscles of hind legs and backside, some parasitized in the

muscles of abdominal wall and forelegs of frogs. Spargana dissected from frog muscles were wrinkled, whitish, and ribbon-shaped worms, which continuously crept in normal saline. These spargana were 1–13 cm long and 1–2.5 mm wide.

### Experimental infection of mice

Forty-day-old specific pathogen-free (SPF) Kunming mice weighing 20 to 25 g were purchased from the Experimental Animal Center of Henan province and bred in plastic micro-isolator cages were used for the study. Thirty male SPF Kunming mice were inoculated each with five spargana by gastric intubation with physiological saline with the animals under ether anesthesia. Blood samples were taken from the infected mice at 5 weeks post-infection (wpi) when these animals were scarified by deep ether anesthesia, the spargana were recovered for preparation of antigens.

Additional 40 male SPF Kunming mice were randomly divided into five groups of eight mice each. After their blood being collected, the mice in each group were inoculated by gastric intubation with one, two, four, six, and eight spargana of *S. mansoni*, respectively. From 1 to 18 wpi, 100  $\mu$ l of tail blood was collected weekly from each animal. All of the infected mice were sacrificed at 18 wpi by deep ether anesthesia, and their carcasses were then skinned. The spargana were recovered and enumerated.

### Serum samples

Twenty-five serum samples of mice infected with 300 muscle larvae of *Trichinella spiralis* were collected 42 days post-infection. Fifteen serum samples of mice infected with *Schistosoma japonicum* and 14 serum samples of mice infected with *Toxoplasma gondii* were gifted by Prof. JL Shen (Anhui Medical University) and Prof. GR Yin (Shanxi Medical University), respectively. Thirty serum samples from normal mice were used as negative control.

Twenty serum samples were obtained from the sparganosis patients who attended our department during August 2006–August 2008. All the 20 patients had the history of ingesting living tadpoles 1–3 weeks before the onset of the disease. They had the different clinical manifestations of sparganosis, such as fever, migratory subcutaneous nodules of the abdominal wall, eruption, and eosinophilia (10–49% of leukocytes). Serum anti-sparganum IgG antibodies were detected in all the 20 patients by dot immunogold-filtration assay using crude antigens of plerocercoids of *S. mansoni*, but the specific antibodies for cysticercosis, echinococcosis, paragonimiasis, clonorchiosis, and schistosomiasis were negative by ELISA. Seven of 20 patients were confirmed

by finding the plerocercoids in biopsy of the subcutaneous nodule with the permission of the patients.

The serum samples of patients with cysticercosis (11 cases), echinococcosis (ten cases), paragonimiasis (20 cases), clonorchiosis (ten cases), and schistosomiasis (11 cases) were collected from our department with parasitological or serological diagnosis. Normal control sera were obtained from 22 healthy persons who had no parasite eggs in their stools and no serum antibodies for the parasitic diseases above-mentioned. All of the serum samples had been stored at  $-80^{\circ}\text{C}$  until used.

#### Preparation of crude and ES antigens of spargana

The crude antigens of spargana collected from experimentally infected mice were prepared as described previously (Kong et al. 1994), with some modification. Briefly, spargana were homogenized in sterile normal saline solution with a glass tissue grinder with for 1 h and then, sonicated with an ultrasonic disintegrator at  $4^{\circ}\text{C}$  for 5 min. The supernatant was obtained by centrifugation at  $15,000\times g$  for 1 h; the dialysis against deionized water was conducted at  $4^{\circ}\text{C}$  for 2 days. The protein concentration (1.425 mg/ml) of the supernatant was assayed by the method of determined by Bradford (1976).

The ES antigens of spargana were prepared referring to the preparation of ES antigens of *T. spiralis* muscle larvae (Mahannop et al. 1992; Kapel and Gamble 2000). After washing thoroughly in sterile normal saline solution and serum-free RPMI-1640 medium supplemented with 100 U penicillin per milliliter and 100 U streptomycin per milliliter, the spargana were incubated in a  $75\text{ cm}^2$  culture dish with the same medium at concentration of two worms per milliliter for 18 h at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . After incubation, the media that contained the ES products were filtered through a  $0.2\text{-}\mu\text{m}$  membrane into a 50-ml conical tube and then centrifuged at  $4^{\circ}\text{C}$ ,  $15,000\times g$  for 30 min. The supernatant was dialyzed and then lyophilized by a vacuum concentration and freeze-drying (Heto Mxi-Dry-Lyo, Denmark). The ES antigens were diluted to a concentration of 2.755 mg/ml and stored at  $-20^{\circ}\text{C}$  before use.

#### Enzyme-linked immunosorbant assay

ELISA with crude and ES antigens was performed as previously described (Wang et al. 2006). In brief, 96-well ELISA plates (Corning, USA) were coated with  $2.5\text{ }\mu\text{g}$  of crude or ES antigens in  $100\text{ }\mu\text{l}$  of bicarbonate buffer (pH 9.6) overnight at  $4^{\circ}\text{C}$ , were washed three times with 0.1% Tween-20 in PBS (PBS-T), and blocked in 3% skimmed milk in PBS-T for 2 h at  $37^{\circ}\text{C}$ . After washing three times, the following reagents were sequentially added and incubated for 1 h at  $37^{\circ}\text{C}$ : (1) human or mouse sera

diluted at 1:100 in PBS-T, and (2) HRP-conjugated anti-human or anti-mouse IgG (Sigma, USA) diluted at 1:5,000. After the final wash, color was developed by incubating with a  $50\text{-}\mu\text{l}$  aliquot of *ortho*-phenylene diamine (5 mg/10 ml of citrate–phosphate buffer) and  $5\text{ }\mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$  for 30 min. The reaction was stopped by adding  $50\text{ }\mu\text{l}$  of 2M  $\text{H}_2\text{SO}_4$ . Optical density at 492 nm was measured with a microplate reader (TECAN, Austria). All samples were run in duplicate. Test sera/negative sera OD values  $<2.1$  were regarded as negative and those  $\geq 2.1$  as positive. The cut-off values of crude antigen ELISA and ES antigen ELISA for detection of experimentally infected mice were 0.44 and 0.34, respectively. The cut-off values of both ELISA for detection of patients with sparganosis were 0.61 and 0.48, respectively.

#### Statistical analysis

All statistical analyses of data were done with SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL). The sensitive and specific of crude and ES antigens were compared with Student's *t* test. The groups were compared among each other by the analysis of variance, later the Student Newman–Keuls test (*q* test) was made for multiple comparisons. The comparison of coefficients of correlation was used in this study. All statistical tests were considered significant at *P* value of 0.05.

## Results

#### Comparison of crude antigen ELISA and ES antigen ELISA for detection of anti-sparganum IgG in experimentally infected mice

Serum anti-sparganum IgG antibodies in experimentally infected mice were assayed by crude antigen ELISA and ES antigen ELISA; the results are shown in Table 1. The sensitivity and specificity of both ELISA for detecting anti-sparganum IgG were 100% (30/30) and 100% (84/84), respectively. The crude and ES antigens of *S. mansoni* did not have cross-reaction with sera from mice infected with *T. spiralis*, *S. japonicum*, and *T. gondii*.

#### Serum anti-sparganum antibody dynamics in mice experimentally infected with different-level infections

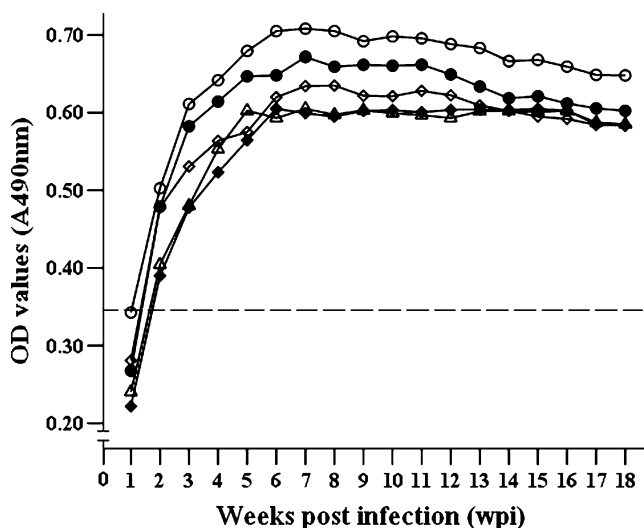
The results of ES antigen ELISA for detecting anti-sparganum IgG in sera of mice infected with one, two, four, six, and eight spargana were shown in Fig. 1. No positive reactions were seen in serum samples taken before inoculation. The antibody positive rate at 2 wpi was 87.5% (7/8) in group of mice infected with two spargana and 100% (8/8) in groups of mice infected with one, four, six,

**Table 1** Detection of specific anti-sparganum IgG antibodies in serum samples of experimentally infected mice by crude antigen ELISA and ES antigen ELISA

Sera of mice infected with	No. of serum samples	Crude antigen ELISA		ES antigen ELISA	
		OD value	No. of positive serum samples (%)	OD value	No. of positive serum samples (%)
<i>S. mansoni</i>	30	0.67±0.03	30 (100.00)	0.61±0.04	30 (100.00)
<i>T. spiralis</i>	25	0.15±0.07	0 (0)	0.17±0.03	0 (0)
<i>S. japonicum</i>	15	0.25±0.06	0 (0)	0.22±0.05	0 (0)
<i>T. gondii</i>	14	0.26±0.06	0 (0)	0.18±0.05	0 (0)
Normal mice	30	0.21±0.05	0 (0)	0.16±0.05	0 (0)

and eight spargana. The serum anti-sparganum antibody levels in five groups of infected mice increased rapidly at 2–4 wpi and reached their peak during 5–7 wpi. The seroconversion was observed in all groups of mice at 3 wpi and lasted to 18 wpi when the experiment was ended. The difference in serum anti-sparganum antibody levels among five groups of the infected mice at 1–18 wpi was statistically significant ( $F=245.296$ ,  $p<0.05$ ); the antibody levels were significantly correlated with infecting doses of spargana ( $r=0.323$ ,  $p<0.05$ ) and showed an increasing trend with an increasing infecting dose.

After the five groups of the infected mice were killed at 18 wpi, the number of spargana recovered was equal to that inoculated. All the spargana recovered were located in subcutaneous or muscle tissues.



**Fig. 1** Serum anti-sparganum IgG antibody dynamics in mice experimentally infected with different doses: mice infected with one (open triangle), two (closed diamond), four (open diamond), six (closed circle), and eight (open circle) spargana of *S. mansoni*; serum cut-off value is represented by the dotted line

Comparison of crude antigen ELISA and ES antigen ELISA for detection of anti-sparganum IgG in patients with sparganosis and other parasitosis

The sensitivity of both crude antigen ELISA and ES antigen ELISA in detecting the serum samples of patients with sparganosis was 100% (20/20) (Table 2). However, the specificity of ELISA in detecting serum samples of patients with other parasitosis and healthy persons was 72.13% (44/61) by crude antigen ELISA and 96.72% (59/61) by ES antigen ELISA. The differences of the specificity of both ELISA was statistically significant ( $\chi^2=14.027$ ,  $p<0.05$ ).

## Discussion

It is difficult to make a confirmed diagnosis of sparganosis because the larvae have no predilection site in humans. Therefore, the serodiagnosis is very useful and important for sparganosis. In order to compare the sensitivity and specificity of crude and ES antigens of *S. mansoni* spargana for detecting the specific serum anti-sparganum IgG, crude, and ES antigen ELISA were used for detection of experimentally infected mice and patients with sparganosis. Our results showed that the sensitivity and specificity of both crude antigen ELISA and ES antigen ELISA for detecting anti-sparganum IgG were 100% in all of experimentally infected mice. The sensitivity of both ELISA in detecting serum samples of patients with sparganosis was 100%, but the specificity (96.72%) of ES antigen ELISA was significantly greater than that (72.13%) of crude antigen ELISA. Although the crude antigen ELISA has high sensitivity, the main disadvantage is that it usually has the cross-reactions with serum samples from patients with other parasitic diseases (cysticercosis, paragonimiasis, clonorchiosis, ect.; Nishiyama et al. 1994). The specificity of the ELISA was partially improved when the antigens purified from the crude extract by affinity chromatography was used; but the purifying procedure is

**Table 2** Detection of specific anti-sparganum IgG antibodies in serum samples of patients with sparganosis and other parasitosis by crude antigen ELISA and ES antigen ELISA

Sera of patients with	No. of serum samples	Crude antigen ELISA		ES antigen ELISA	
		OD value	No. of positive serum samples (%)	OD value	No. of positive serum samples (%)
Sparganosis	20	0.68±0.03	20 (100.00)	0.60±0.03	20 (100)
Cysticercosis	11	0.55±0.12	3 (27.27)	0.25±0.11	1 (9.09)
Echinococcosis	10	0.59±0.09	5 (50.00)	0.18±0.09	0 (0)
Paragonimiasis	20	0.51±0.23	9 (45.00)	0.21±0.11	1 (5.00)
Clonorchiosis	10	0.39±0.10	0 (0)	0.33±0.05	0 (0)
Schistosomiasis	11	0.30±0.10	0 (0)	0.28±0.07	0 (0)
Healthy persons	22	0.29±0.06	0 (0)	0.23±0.07	0 (0)

complicated and inefficient, and the cross-reactions still occur (Cho et al. 1990; Kong et al. 1991). Our results indicated that ES antigen ELISA is superior to crude antigen ELISA for serodiagnosis of sparganosis.

An earlier study had found that the serum anti-sparganum IgG antibody levels at 4 wpi increased in mice infected with five spargana by ELISA using crude antigens (Hong et al. 1989). However, no detailed data have been reported about the results of ELISA in detecting anti-sparganum IgG levels at different infective doses in mice or about the sensitivity of ELISA for detecting the anti-sparganum IgG in the period after infection, particularly in the case of mild infection. Hence, we infected mice with various doses of spargana, of which one sparganum is the smallest infective dose, and collected blood weekly from 1 to 18 wpi to better assess the efficacy of ES antigen ELISA for the early diagnosis of sparganosis. The results showed the anti-sparganum antibodies at 2 wpi was detected in 100% of mice infected with one sparganum and in 87.5% of mice infected with two spargana. The sero-conversion was observed in all groups of mice at 3 wpi and lasted to 18 wpi, suggesting that ES antigen ELISA can also be used to the early serodiagnosis of sparganosis with low-level infections.

Parasites are designed by evolution to invade and survive in hosts; they release a variety of molecules that help them to penetrate the defensive barriers and avoid the immune attack of the host (Dzik 2006). The ES antigens of living plerocercoids can induce a strong humoral response involving the generation of specific antibodies and may be very important for serodiagnosis as they are easily targeted by the immune system. In addition, the antigenic composition of sparganum from different hosts (snakes or mice) were different (Yang 2004), and the differential proteins were expressed at different development stages in its host (Kim et al. 2009). Hence, we firstly inoculated into mice with spargana collected from wild frogs, then prepared the ES antigens with the spargana recovered from the infected mice. The results showed that the ES antigens prepared

with the spargana recovered from the infected mice are ideal diagnostic antigens for sparganosis.

In conclusion, our finding indicates that ELISA using ES antigens of *S. mansoni* sparganum may be used to the specific early serodiagnosis of sparganosis with low-level infections.

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**Conflict of interest** There are no conflicts of interest.

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