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ANIMAL STUDY



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Manuscrii

Diagnostic Value of SjR2 Gene in Colonic Tissue from Schistosoma Japonicum Infected Hosts

Autho Datais Data I nuscrip Lite Fur	rs' Contribution: Study Design A ata Collection B stical Analysis C nterpretation D ot Preparation E rature Search F Idds Collection G	ABEFG 1 ABCDEF 2 AG 1 CDE 1 CE 1	Xu Zhao Kongzhen Gu Qingren Zeng Lu Gao Da Cheng	 Department of Parasitology, Xiangya School of Medicine, Central South University (CSU), Changsha, Hunan, P.R. China Faculty of Laboratory Medicine, Xiangya School of Medicine, Central South University (CSU), Changsha, Hunan, P.R. China
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	Back	ground:	The prevalence and intensity of schistosomiasis infe Therefore, more accurate methods are critically nee- miasis infection. For chronic schistosomiasis patients tissues is commonly used. This work aimed to explor (<i>S. japonicum</i>) retrotransposon (<i>SjR2</i>) gene in colon to ideal method for genetic diagnosis of low-intensity s	ection in China has decreased markedly in recent years. ded to ensure further control of low-intensity schistoso- , the detection of schistosome eggs in colorectal mucosa e differences in sensitivity of the <i>Schistosoma japonicum</i> issue from <i>S. japonicum</i> infected hosts and to develop an chistosomiasis.
	Material/M	lethods:	Serum and colon samples were collected from mice a treatment (PT). Colorectal biopsy specimens from out from mice and patients, including serum as well as co were examined using the polymerase chain reaction	at different time points, either post-infection (PI) or post- patients with schistosomiasis were collected. All samples Ion tissue containing eggs and tissue containing no eggs, technique.
	Conc	Results: :lusions:	The results showed that the <i>SjR2</i> gene could be detect for when the egg was completely degraded. The posi from egg-free colon tissue from around the eggs wer The results indicate that detection of the gene in col- would be a practical and ideal method for genetic of this method can be a sensitive assisted examination infection.	ted in all colon tissue containing at least one egg, except tive rate of gene detection in serum was low. The results we more consistent with the actual parasitism <i>in vivo</i> . on tissue located within a 0.5 cm distance from the eggs diagnosis of schistosomiasis. After the colorectal biopsy, to the clinical diagnosis of low-intensity schistosomiasis
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Background

Schistosomiasis japonica is a tropical parasitic disease that generally receives little attention; this disease seriously endangers human health, causing tissue and organ damage induced by S. japonicum eggs. Schistosomiasis is found in 74 countries in the tropical and subtropical band, particularly in developing countries [1,2]. Currently, 779 million people are under threat of infection and nearly 230 million people are infected worldwide [3,4]. And the number of patients infected with schistosomiasis in China was about 77 000 at the end of 2015 [5]. Due to decades of positive prevention action, the prevalence of human schistosomiasis and the number of fatalities has dropped significantly [6]. However, with a reduced prevalence of schistosomiasis, the people infected with S. japonicum in endemic areas have become low-worm-load chronic patients and worm carriers. This increases the difficulty in diagnosing and discovering sources of infection in endemic areas.

Finding eggs or hatched miracidia from an infected patient is the golden standard for schistosomiasis diagnosis. The etiological methods of diagnosing hepatic, intestinal schistosomiasis is the Kato-Katz method, the nylon silk screening egg method, the hatching miracidia method, and the colorectal mucosal biopsy [7,8]. Since there is fibrosis of the intestinal wall in chronic patients with a low degree of infection, there is a reduction in the number of excreted eggs, and thus, the chances of detecting eggs are also significantly reduced. Therefore, the stool method is primarily applied to the examination of moderatesevere hepatic intestinal schistosomiasis and is not suitable for screening patients with low infection levels. Furthermore, the fecal test requires high levels of time and effort and has a high rate of missed detection [9,10].

In order to improve the diagnostic efficiency, a number of immune techniques for schistosomiasis diagnosis have been developed. However, antibody detection cannot judge whether or not there is a living worm in an infected person, because antigen detection can be interfered by idiotype antibody. On the other hand, many scholars use molecular biology techniques to detect schistosome cell-free DNA from the serum or urine of schistosomiasis patients [11–13]. The sensitivity and specificity of these method are higher than the immunological methods, because these techniques are not affected by changes in the host immune state or antigens and antibodies [14–16]. However, the overall detection rate with the use of serum samples is low. This is probably related to the low level of schistosome-free DNA in body fluid, especially in chronic and low-grade infections.

For chronic schistosomiasis patients, the detection of schistosome eggs in colorectal mucosa tissues is commonly used [17,18]; it is based on the discovery of active *S. japonicum* eggs in biopsy tissue. When there are few eggs in the tissue or mistakes are made in obtaining the materials, missed diagnosis may occur. Further, a fault in observer judgment with regard to the activity of eggs can also lead to misdiagnosis and missed diagnoses.

What is the best way to make use of the biopsy samples to improve the sensitivity of *S. japonicum* detection? After eggs die, there is some time before DNA degeneration. Antigen proteins and DNA can be released from eggs and penetrate into tissue around the eggs, though the content in this surrounding tissue is lower than the content in the eggs. Under the effects of host organization enzymes, the DNA and protein antigens degenerate faster than in the eggs. Therefore, we believe that detecting DNA in the tissue adjacent to *S. japonicum* eggs might reflect the state of active infection in a patient's body.

SjR2 is an RTE-like, non-long terminal repeat retrotransposon from S. japonicum, and it was first described by Laha et al. [19]. Xia et al. detected its 230-bp sequence in host serum by normal polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), and his results showed that both of these methods could detect the specific S. japonicum gene in rabbit serum after a week of infection [20]. In followup studies, he found that the sensitivity of DNA detection was positively related to the complete and partial copy number of target retrotransposons sequences, activeness of ESTs (expressed sequence tags), and their proportion in the whole genome. Only SjR2 and SjCHGCS19 show high specificity and could simultaneously meet all the important requirements aforementioned [21]. This indicates that SjR2 could act as a symbolic diagnostic target and could be used for early schistosomiasis diagnosis and evaluation of treatment.

In our study, we chose the *SjR2* gene as our target. Then we used serum and colon tissue samples (including tissue with eggs deposited in it and tissues containing no eggs) from schistosomiasis animal models and from clinical patients. Satisfactory results were obtained and are detailed the results section.

Material and Methods

Establishment of S. japonicum infected mouse models

Seventy 7–8 weeks old SPF Kunming mice (half male and half female) were purchased from the Experimental Animal Department of Central South University, and infected snails were gathered from Dongting Lake by professionals. *S. japonicum* cercariae were released using the conventional method [22]. Then 60 Kunming mice were infected transdermally (at 24°C), using 14 ± 2 cercariae for each mouse. Then, after 7 to 8 weeks, 30 mice were randomly selected for

praziquantel (PQZ; Nanjing pharmaceutical factory of China, 20110307) treatment for 2 weeks (once a week, 300 mg/kg each time). The mice were divided into 7 groups: group 1 was 45 days after infection (45d Pl); group 2 was 90 days after infection (90d Pl); group 3 was 180 days after infection (180d Pl); group 4 was 45 days after treatment (45d PT); group 5 was 90 days after treatment (90d PT); group 6 was 180 days after treatment (180d PT); and group 7 was the control group with no infection and treatment.

Collection of colon tissue and serum from model mice

Blood was drawn from the eyeball of each mouse after ether anesthesia and worms were washed using the conventional method [23]. The number of residual worms and male-female worm pairs in the hepatic portal vein and mesenteric vein of each mouse were counted. Serum was separated and conserved at -20°C in preparation for DNA extraction. The colon mucosa tissues were collected and cut into pieces. The number and types of eggs in each piece were recorded. Six pieces of colon tissue deposited with eggs and 6 pieces containing no eggs were obtained from every mouse. Then, the tissues were flattened on glass slides and observed under microscope. The mucosa tissue around the eggs at different distances were measured by a microscopic scale and cut using eye scissors. Three tissues (containing no eggs) within and beyond 5 cm, respectively, from the eggs, were collected. After weighing, the tissues were conserved at -20°C for DNA extraction.

Collection of colorectal colonoscopy biopsy specimens from outpatients with schistosomiasis

Colorectal biopsy specimens from outpatients with schistosomiasis were acquired at Xiangyue Hospital, Schistosomiasis Control and Prevention Institution of Hunan Province. An Image Acquisition System was used for the observation of eggs. Then, the samples were conserved at -20°C for DNA extraction. In total, 52 samples were found to contain eggs, and all clinical reports suggested that these eggs were partially and completely degraded; 30 samples were found to contain no eggs. The images of the eggs were re-observed and classified according to the monograph criterion [24] to allow more in depth analysis: faint yellow mature eggs which contained clear miracidia and immature eggs containing clear blastocytes were classified as living eggs; eggs containing unclear miracidia or having an unclear structure and eggs containing black spots or reticulate structures were classified as partially degraded eggs; deep black or disrupted eggs were classified as completely degraded. Thus, the biopsy samples were divided into 4 groups: 1) no eggs; 2) mature eggs; 3) mainly partially degraded eggs; 4) mainly completely degraded eggs.

DNA extraction from serum and colon tissues

Cell-free DNA from serum was extracted using a Circulating DNA Kit (OMEGA Bio-Tek, Norcross GA, number: D3091-02). DNA from colon tissues was extracted using a Tissue DNA Kit (OMEGA Bio- Tek, Norcross, Georgia, USA, number: D3396-02). These 2 procedures were almost the same and were carried out as follows. Tissue or serum was mixed with 200 mL digestive buffer and 25 uL OB protease was added. Then, the mixtures were heated for 2 hours at 55°C until completely digested. Anhydrous alcohol was used for DNA precipitation in supernatants. The liquid was transferred to a HiBind silicagel column and centrifuged at 10 000 g for 2 minutes at room temperature to allow the DNA to adhere to the silicagel column. After the impurities on the silica membranes were washed out, solution buffer was used for DNA elution. Finally, total DNA concentrations were determined using a Nanodrop 2000 (Thermo, Beijing). DNA was conserved at -20°C for PCR.

PCR amplification and agar gel electrophoresis

Primers targeting 3184-3705 bp (an ORF) of the SjR2 gene were designed by the software Primer Premier 5.0 and were synthesized by the Nanjing Jinpurui Company. The forward primer was 5'-ACGCGTTTATTGTGCAGCAGTC-3' and the reverse primer was 5'-GGGCCCCAACCAGGTAGTCTA-3'. The amplified segment length was 408 bp. Routinely, PCR reactions were performed in a final volume of 25 µL containing 2.5 µL of 10× reaction buffer, 0.2 µL of 5 U/L rTaq polymerase, 0.5 µL of each 5 Mm primer, 2 µL dNTPs, and 2 µL of template DNA. PCR cycling parameters [21] consisted of 3 minutes at 94°C followed by 30 cycles of 60 seconds at 94°C, 60 seconds at 58°C, and 30 seconds at 72°C with a final extension at 72°C for 7 minutes. The amplified products were visualized by electrophoresis on ethidium bromide-staining 1.5% agarose gels (90 V, 30 minutes) and then observed using a molecular gel imaging system (BIO-RAD).

Statistical analysis

SPSS version 19 was used to analyze the data. The χ^2 test was used to determine differences between the various positive rates of detection. A *P* value less than 0.05 was considered statistically significant.

Ethics statement

Prior to the commencement of this study, the study protocol was reviewed and approved by the Ethics Committee of the Hunan Institute of Parasitic Disease (reference number: 2014-S003). Before participating in the study, all subjects were given detailed explanations about the objectives and methodologies of this research. Written informed consent to participate and

		45d Pl	90d Pl	180d PI	30d PT	90d PT	180d PT
Number of mice in each group		10	10	10	10	10	10
Adult worms	Mean ± SD	13.2±3.3	7.6±1.7	4.5±0.7	0	0	0
	Male-Female worm pairs	5.4	3.8	1.6	0	0	0

Table 1. The number of adult worms in different groups of model mice before and after treatment.



Figure 1. Schistosoma japonicum eggs in colon tissue from mice. A–F show S. japonicum eggs in colon tissues from mice of 45d PI, 90d PI, 180d PI, 30d PT, 90d PT and 180d PT respectively. Immature eggs, mature eggs, partially degraded eggs, completely degraded eggs and egg-shell were pointed out by blue, green, red, yellow and black arrows respectively.

publish was obtained from all participants who voluntarily provided colorectal mucosa biopsy samples. All participants were adults. The study was performed in accordance with the recommendations of the Chinese Code of Practice for the Care and Use of Animals for Scientific Purposes. The Ethical Committee of the Center for Parasitology Research (ECCPR) approved all experimental procedures, including animal handling, under animal license number: syxk (Xiang) 2011-0001 and in accordance with strict ethical standards. Laboratory animal quality conformance license number: 43006700002928.

Results

Distribution of *S. japonicum* adult worms and eggs in model mice at different points of time

The number of adult worms found in different groups of model mice is shown in Table 1. As can be seen, the number of adult worms decreased with increases in the number of infected

days; there were no adult worms found in model mice after PZQ treatment. The distribution of different eggs in the colon tissues was as follows (Figure 1). In the 45d PI group, there were a great number of mature and immature eggs found in the colon tissues; in the 90d PI group, there were partially degraded eggs as well as mature and immature eggs; in the 180d PI group, there were light black partially degraded eggs and deep black completely degraded eggs as well as mature and immature eggs; in the 30d PT group, there were many empty egg shells, partially degraded eggs and a few completely degraded eggs; in the 90d PT group, there were primarily partially degraded eggs; in the 180d PT group, there were primarily completely degraded eggs and a few empty egg shells.

PCR results in serum and colon tissue from model mice at different points in time

Before DNA extraction, all samples to be detected by PCR were observed under the microscope to record the number and types

Morphology results	lmma eg	ature gs	Mat eg	ture gs	Partially eg	degraded gs	Completely eg	y degraded gs
	м	S	м	S	м	S	м	S
Number of samples	10	10	10	10	10	10	10	10
Number of positive	10	10	10	10	10	7	10	0

Table 2. The results of SjR2 gene detection in different types of Schistosoma japonicum eggs.

M - multiple eggs; S - single egg.

Table 3. The results of different distances from colon samples to egg.

	45d Pl	90d Pl	180d PI	30d PT	90d PT	180d PT
Distance from eggs						
<0.5 cm	+	+	+	+	-	-
>0.5 cm	+	+	-	-	-	-

Table 4. The detected rates of different colon samples from model mice before and after treatment.

Groups	Control	45d PI	90d Pl	180d PI	30d PT	90d PT	180d PT
Serum:							
Number of samples	10	10	10	10	10	10	10
Number of positive	0	7	5	3	1	0	0
Positive rate of SjR2 (%)	0.0	70.0	50.0	30.0	10.0	0.0	0.0
Tissues contained eggs:							
Number of samples		30	30	30	30	30	30
Number of positive	-	30	30	30	30	28	26
Positive rate of SjR2 (%)		100	100	100	100	90.33	86.67
*Р	-	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tissues contained no egg:							
Number of samples	30	30	30	30	30	22	22
Number of positive	0	27	22	16	11	0	0
Positive rate of eggs (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Positive rate of <i>SjR2</i> (%)	0.0	90.0#	73.3#	53.3#	36.7#	0.0	0.0
*Р	-	<0.01	<0.01	<0.01	<0.01	-	-

* *P* compared with serum groups at the same time points; # *P*<0.01, compared with results of pathogenic detection (positive rate of eggs) in the same groups.

of eggs in the tissues. The samples were detected in batches according to their corresponding purpose. The detection results of the four different types of egg samples are shown in Table 2. The *SjR2* gene could be detected in all types of colon samples, except for samples containing only 1 completely degraded egg. The detection results for the different distances between the colon samples and the eggs are shown in Table 3. In the 45d PI, 90d PI, 180d PI, and 30d PT groups, detection of the *SjR2* gene was positive if the egg-free tissue was less than 0.5 cm away from the eggs. However, if the distance was more

than 0.5 cm, the result was positive only in the 45d PI and 90d PI group. There were 30 colon samples deposited with eggs, 30 colon samples containing no eggs, and 10 serum samples detected in parallel. The detection results for different colon samples from model mice are shown in Table 4. The positive rate of detection of the *SjR2* gene was the highest in the specimens containing eggs, followed by the egg-free tissues; the rate was lowest in the serum specimens. There are significant differences between pathogenic detection and *SjR2* detection in 45PI, 90d PI, 180d PI and 30d PT groups (P<0.01). Further,



Figure 2. DNA detecting results of colon tissues and serum samples from *Schistosoma japonicum* infected model mice. The amplified target segment length was 408 bp. (a) 1~7: tissue samples deposited with eggs from 45d PI, 90d PI, 180d PI, 30d PT, 90d PT, 180d PT and Control groups respectively. Target segments was detected in 1~6. (b) 8~14: tissue samples contained no eggs from these 7 groups. (c) 15~21: serum from these 7 groups. Target segments were only detected in 45d PI, 90d PI, 180d PI and 30d PT groups. And amplicons of colon tissue samples were much brighter than those of serum samples.



Figure 3. Schistosoma japonicum eggs in colon mucosa from schistosomiasis japonica patients. Some S. japonicum eggs in colon mucosa from parts of patients with schistosomiasis japonica. Immature eggs, mature eggs, partially degraded eggs and completely degraded eggs were pointed out by blue, green, red and yellow arrows respectively.

the SjR2 gene segment in colon tissues deposited with eggs was much brighter than those containing no eggs. The amplicons of serum were the darkest (Figure 2).

PCR results for biopsy samples from suspected schistosomiasis patients

The distribution of different eggs in biopsy samples from suspected schistosomiasis patients is shown in Figure 3. The rate of

Biopsy tissue samples groups	No egg	Mature eggs	Mainly partially degraded eggs	Mainly completely degraded eggs
Number of patients (n)	30	28	22	2
Number of positive in tissues deposited with eggs (n)	0	28	22	2
Positive Rate (%)	0	100	100	100
Number of positive in tissues contained no egg (n)	0	25	9	0
Positive Rate of Eggs (%)	0.0	0.0	0.0	0.0
Positive Rate (%)	0	89.28*#	40.91#	0.00
Р		0.118	<0.01	<0.01

Table 5. SjR2 detecting results in biopsy tissue samples from suspected schistosomiasis patients.

* P<0.01, compared with "mainly partially degraded eggs" group and "mainly completely degraded eggs" group; # P<0.01, compared with positive rate of pathogenic detection (positive rate of eggs) in the same groups.

SjR2 gene detection in colon tissue samples deposited with eggs was significantly higher than for tissue containing no eggs. In the egg-free colon tissue from around the eggs, the positive rate of detection in the mature eggs group was significantly higher than that in the mostly partially degraded eggs group and the mostly completely degraded eggs group (P<0.01). The *SjR2* gene was not detected in the negative control group (Table 5). In the egg-free colon tissue from around the eggs, the positive rate of *SjR2* was significantly higher than that of pathogenic detection (P<0.001) (Table 5).

Discussion

Etiological and immunological methods have been used to diagnose schistosomiasis for many years. The gold standard of schistosomiasis diagnosis is to find S. japonicum eggs in patients' bodies. However, the detection rate of traditional etiological methods is low, and both missed diagnosis and misdiagnosis occurs. Meanwhile, the sensitivity and specificity of immunological methods does not meet clinical standards. With the rapid development of molecular biology in recent years, researchers have increasingly applied specific gene segment detection for the diagnosis of schistosomiasis [25]. Fernandez-Soto and colleagues used different methods to extract DNA for PCR from long-term frozen urine samples from sub-Saharan African patients who were immigrants to Spain. The highest positive rate of the specific S. japonicum gene, CF2-CR2, was only 13.7% (10 out of 73 patients), while the positive rate of the SmF-SmR gene was lower, at only 4.1% (3 out of 73 patients) [26]. Sady et al. used real-time PCR to detect the COX1 gene in stool and urine samples from patients suffering from schistosomiasis to evaluate the prevalence rate and infection levels of Schistosoma mansoni and Schistosoma haematobium in Yemen rural areas [27]. Randrianasolo et al. extracted DNA from vaginal secretion and urine samples from 118 women suffering from reproductive tract schistosomiasis in Madagascar. Using real-time PCR, they found that 42% of women with schistosoma-negative urine specimens had at least 1 genital specimen test positive for schistosoma by PCR, while 13% of urine samples were positive by PCR but negative by microscope. All of the urine samples of severe infection were positive by PCR while only 62% of low-grade infections were positive [28]. Meurs et al. used real-time PCR to detect the S. mansoni ITS2 gene in stool samples from patients. At the same time, they investigated the presence of eggs in stools under microscope. The infective rate of S. mansoni was 72% (PCR) and 57.4% (fecal examination) in Senegal, while it was 32.4% (PCR) and 19.2% (fecal examination) in Kenya. The sensitivity of detecting the ITS2 gene in stools of patients infected with S. mansoni was 13% to 15% higher than for fecal examination. Thus, gene detection may be suitable for the diagnosis of S. mansoni in low-grade infection areas [29]. Vinkeles et al. used fluorogenic quantitative PCR and microscopy positive as a combined "gold standard" for the diagnosis of S. haematobium in order to enhance the diagnosis accuracy [30].

Our study explored a new method to improve the sensitivity of gene detection, and 3 types of samples (colon tissue deposited with eggs, tissue containing no eggs, and serum) were chosen for the research. First, we compared different quantities and morphotypes of eggs in colon tissues from established model mice before and after treatment. The result (Table 2) illustrates that the target gene (*S. japonicum* DNA) could be detected in tissues deposited with more than one *S. japonicum* egg, regardless of whether these eggs were living eggs or degenerated eggs. Although this method did not have diagnosis value for active infection, it did show sensitivity. Further, the target gene could be detected in tissue samples deposited with only 1 egg, except if the egg was a completely degraded egg. This result suggests that the target gene in the completely degraded eggs was degraded. Then, we detected colon tissue and serum samples from model mice before and after PQZ treatment. As shown in Table 4, in colon tissue deposited with eggs, the positive rate was 100% in all groups, except for the 90d PT group (90.33%) and the 180d PT group (86.76%). In colon tissue containing no eggs, the positive rate decreased as the time of infection increased, and it turned negative 3 months after treatment. This could be explained by the diminution of worm burden (Table 1) and the differences among samples obtained from different positions. The negative results of the inactive infection period after treatment in model mice shows the diagnostic significance of efficacy. The positive rate of the serum samples was lower than that of the tissue containing no eggs, among all groups. This illustrates that the sensitivity of colon tissue was higher than serum. The colon samples containing no eggs were the closest to the state carrying live worms in the human body. Table 4 shows that the positive rate of SjR2 detection is higher than the pathogenic detection, and it can change according to the actual state of infection.

Finally, in order to verify the clinical practicality of detecting colon tissue samples containing no eggs, we examined 52 colon mucosa biopsy samples from chronic schistosomiasis patients who tested positive (having eggs) under rectoscope. The results (Table 5) indicated that the detection of colon tissue deposited with eggs did not reflect the actual egg states and the infection states while the detection of colon tissue containing no eggs was closer to the morphological observations. As can be seen in Table 5, positive rate of *SjR2* detection

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is higher than the pathogenic detection if the tissue is around the mature egg or mainly partially degraded egg. This could be explained by specific gene degradation. Sometime after the eggs die, the specific gene from the worm body surface debris, metabolites, and eggs that have infiltrated into the tissue, degrades. Therefore, the positive rate of colon tissue around active eggs is higher than that around partially degraded eggs, and the gene cannot be detected in tissue around completely degraded eggs. Thus, it would be an ideal method to detect the schistosomiasis gene in colon tissues within a 0.5 cm distance from the eggs. Combined with rectoscope, detection of the *SjR2* gene will be valuable to the diagnosis of active schistosomiasis infection.

Conclusions

Our study is the first genetic diagnosis research using colon mucosa biopsy samples from *S. japonicum* infected patients. Our results demonstrate that the positive rate of DNA detection of colon tissue was much higher than DNA detection of serum. The results for colon tissue containing no eggs can reflect the actual state of infection. After the colorectal biopsy, this method can be a sensitive assisted examination to the clinical diagnosis of low-intensity schistosomiasis infection.

Conflict of Interest

None.

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