JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Occurrence and multilocus genotyping of *Giardia duodenalis* in pets and zoo animals in Shanghai, China

Hua Liu[1,2,3,4], Yujuan Shen[1,2,3,4], Aiqin Liu[5], Jianhai Yin[1,2,3,4], Zhongying Yuan[1,2,3,4], Yanyan Jiang[1,2,3,4], Wei Pan[1,2,3,4], Yumei Zhang[1,2,3,4], Wei Zhao[5], Jianping Cao[1,2,3,4]

[1] *National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai, China*
[2] *Key Laboratory of Parasite and Vector Biology, MOH, Shanghai, China*
[3] *National Center for International Research on Tropical Diseases, Shanghai, China*
[4] *WHO Collaborating Center for Tropical Diseases, Shanghai, China*
[5] *Department of Parasitology, Harbin Medical University, Harbin, Heilongjiang, China*

Abstract

Introduction: High prevalence of *Giardia* infections occurs in humans and animals, partly because of the increasing numbers of pets. We determined the presence and genotypes of *G. duodenalis* in pets and zoo animals.

Methodology: A total of 84 specimens were collected from dogs and cats from a pet hospital, and 54 specimens from a zoo, which included deer, tigers, yaks, and others. All the specimens were examined by microscopy and by polymerase chain reaction (PCR) amplification and subsequent sequencing of glutamate dehydrogenase (*gdh*), beta-giardin (*bg*), and triose phosphate isomerase (*tpi*) genes.

Results: *Giardia* infection was confirmed in 5.95% and 15.48% of animals by microscopy and by PCR, respectively; the detection levels were 13.33% and 26.67% for pets, and 1.85% and 9.26% for zoo animals. Four assemblages were identified: assemblage C in dogs, cats, and a sheep; D in dogs, a wolf, a yak, and a leopard; E in a sheep; and F in a cat and a leopard. PCR gave the highest amplification rate at the *gdh* locus. Eight, five, and four sequences were novel at the *gdh*, *bg*, and *tpi* loci, respectively. Two *tpi* sequences of dog-derived assemblage C had 100% homology with amino acid sequences from human-derived isolates.

Conclusions: The molecular characterization of *G. duodenalis* in pets and zoo animals in China is described. Assemblage D was identified in a yak and a leopard for the first time. Multilocus genotyping analysis identified the same *tpi* gene sequences of assemblage C in dogs and humans, indicating potential zoonotic transmission.

Key words: *Giardia duodenalis*; multilocus genotyping; *gdh*; *tpi*

J Infect Dev Ctries 2017; 11(6):479-486. doi:10.3855/jidc.8421

(Received 19 March 2016 – Accepted 22 October 2016)

Introduction

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is one of the most common flagellated protozoan parasites; it colonizes and reproduces in the small intestine of hosts, causing giardiasis. Humans and many other mammals, including livestock, pets, wildlife, and even a few species of birds can become infected [1,2]. Giardiasis is accompanied by a wide spectrum of clinical signs, including diarrhea, abdominal pain, bloating, and can retard the growth and development of children, even in asymptomatic cases [2]. Because of the great number of asymptomatic giardiasis cases, human giardiasis is often underestimated [3]. Meanwhile, asymptomatic sufferers are usually ignored and become sources of infection [4,5]. Chief pathways of human infection include ingestion of untreated sewage, which is particularly common in many developing countries; contamination of natural waters has been reported in watersheds where intensive grazing occurs [6,7]. Case-control studies of human giardiasis have confirmed that contact with farm animals is associated with an increased risk of infection for adults [8].

Based on the differences in genetic characterization and host ranges or host specificity, *G. duodenalis* is a complex species, and at least eight distinct assemblages (A to H) have been defined [9]. Among them, assemblages A and B have the widest host ranges, and are mainly found in humans and a variety of other mammals [9-11]. Assemblages C, D, E, and F seem to be host-specific for non-human species, with assemblages C and D in dogs, assemblage E in hoofed livestock, assemblage F in cats, assemblage G in rodents, and assemblage H in marine mammals [12,13].

The presence of the same *G. duodenalis* gene sequences in humans and animals, mainly from assemblages A and B, indicates the possibility of zoonotic transmission, and the role of host animals in the transmission of human giardiasis is therefore a potential public human health concern. Additionally, molecular epidemiological data on giardiasis revealed that assemblages C, D, E, and F have also occasionally been found in humans [14].

Polymerase chain reaction (PCR)-based molecular analysis techniques have been widely used to genotype and subtype *Giardia* isolates to trace sources of infection or contamination and to assess the human giardiasis burden attributable to animal origin. Although a single-locus PCR has been adopted in most epidemiological studies of giardiasis [15], multilocus genotyping (MLG) is being used increasingly to characterize *G. duodenalis* isolates from humans and animals. MLG data can contribute greatly to understanding the transmission routes and dynamics of human giardiasis by providing a more detailed genetic description [16,17]. To date, among the many genes of *G. duodenalis* reported, *gdh*, *bg*, and *tpi* are the most common genotyping and subtyping markers [18,19].

In China, although there have been some epidemiological studies of human and livestock giardiasis [20,21], few reports assessed pets or zoo animals, and little is known about their potential for zoonotic transmission [22]. In urban areas of Shanghai, a recent study conducted on dogs and cats showed the presence of zoonotic giardiasis [23]. In recent years, there has been an increase in the number of pet animals, especially dogs and cats. In fact, Shanghai, Beijing, Chongqing, Guangzhou, and Wuhan have been recognized as "pet cities" of China, and currently, there are an estimated 150 million pet dogs in China. This has increased the incidence of human-animal contact. Meanwhile, more attention should be paid to the health of protected animal species and endangered/threatened animal species in China. We have previously molecularly identified *Cryptosporidium* in pets and zoo animals [24]; it is also necessary to detect other intestinal pathogens in these animals. The present study mainly focused on the molecular epidemiological investigation of *G. duodenalis* in pet and zoo animals from Shanghai, China. The aims were to understand the occurrence of *G. duodenalis* in these animals by PCR amplification of genes *gdh*, *tpi*, and *bg*, and to assess the potential for zoonotic transmission by aligning sequences obtained in the present study with those derived from humans available from GenBank.

Methodology

Specimen collection and processing

The species and identification of each animal were recorded at the time of sampling. All the specimens were transported to the laboratory in a cooler with ice packs within 24 hours of collection and processed as previously described [25]. Briefly, 20 g of feces were transferred to a 50 mL centrifuge tube and washed three times with distilled water. After centrifugation, the supernatant was discarded and the deposit was resuspended in distilled water and used directly to make three smears for iodine wet mount staining. *Giardia* cysts were examined using a bright-field microscope at 100 × and 400 × magnification after staining. Remaining fecal material was stored in 2.5% potassium dichromate solution prior to use in molecular biological characterizations.

DNA extraction

The potassium dichromate was washed off the 84 fecal specimens with distilled water by centrifugation at room temperature. Genomic DNA was extracted from 200 mg of fecal specimens using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, USA) following the manufacturer-recommended procedures. DNA was eluted in 200 μL of AE elution buffer and stored at -20°C until needed for PCR.

PCR amplification of gdh, bg, and tpi genes

Each *G. duodenalis* DNA preparation was characterized using three distinct protocols for nested PCR depending on the target to be amplified. Portions of the *gdh* (530 bp), *tpi* (530 bp), and *bg* (380 bp) genes were individually amplified using primers as previously reported [26-28]. For all three genes, primary PCR was performed with 12.5 μL of 2 × PCR master mix (Promega, Madison, USA), 1 μL of each primer (10 μM), and 1 μL of DNA in a total reaction volume of 25 μL. For secondary PCR, 1 μL of product from the primary PCR was used as template and amplified using the same reaction volumes and reagents. The cycle parameters for PCR amplifications of *gdh* and *tpi* genes were as follows: 35 cycles at 94°C for 50 seconds, 57°C for 45 seconds, and 72°C for 1 minute, with an initial hot start at 95°C for 5 minutes and a final extension step at 72°C for 10 minutes. Nested PCR cycling conditions were identical to the primary PCR conditions. The cycle parameters for PCR amplification of the *bg* gene were the same as those of the *gdh* and *tpi* genes, except the annealing temperature was 60°C. A negative control with no DNA was included in all the PCR tests. PCR

products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide.

DNA sequencing and analysis

For accurate analysis, all the genes were amplified at least three times, and all PCR-positive products were sequenced in both directions using the secondary primers on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) using a Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems Foster, USA). ContigExpress was used to evaluate the wave peak and assemble the sequences. All the nucleotide sequences obtained in the present study were subjected to the basic local alignment search tool (BLAST), and then were aligned with *G. duodenalis* reference sequences downloaded from GenBank and analyzed using Clustal X version 1.83.

Nucleotide sequence accession numbers

The new nucleotide sequences described here have been deposited in GenBank under accession numbers KF993728 to KF993739 (*gdh* gene), KF993721 to KF993727 (*tpi* gene), and KF993712 to KF993720 (*bg* gene).

Ethics statement

Prior to collection of fecal specimens, permission was obtained from zoo managers and pet owners in Shanghai, China. No specific permits were required for the described field studies. All animals were initially kept isolated and fed alone for one day, and fecal specimens were collected from the ground using plastic bags the following day. Animals were not harmed in any way during the procedure. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. The protocol was approved by the Laboratory Animal Welfare & Ethics Committee (LAWEC), National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (permit number: IPD 2012-6).

Results

Prevalence of G. duodenalis in pets and zoo animals

A total of 84 fecal specimens were collected between August 2012 and December 2012, including 30 (19 from dogs and 11 from cats) from a pet hospital in Shanghai, and 54 from animals in a Shanghai zoo (11 from sheep, 9 from deer, 9 from tigers, 5 from yaks, 5 from leopards, 2 from elephants, and 13 from wolves, a panda, and various other species) (Table 1). Fecal specimens from each of the 84 animals representing 15 pet and zoo species were examined for the presence of *G. duodenalis* cysts by microscopy and were also investigated by PCR. In pet animals, 15.79% (3/19) and 31.58% (6/19) of dog fecal specimens were positive for *G. duodenalis* by microscopy and by PCR, respectively, while 9.09% (1/11) were positive by microscopy, versus 18.18% (2/11) by PCR in cats. In zoo animals, *G. duodenalis* cysts were only detected in one fecal specimen (a sheep) by microscopy, whereas five fecal specimens were positive for *G. duodenalis* by PCR, from two sheep, one leopard, a yak, and a wolf. In general, infection rates of *G. duodenalis* in zoo animals were 1.85% (1/54) by microscopy versus 9.26% (5/54) by PCR (Table 1).

Table 1. Prevalence of *G. duodenalis* by microscopy and PCR.

Animal source (n)	host	No. examined	No. of positive	
			By microscopy	By PCR
Pet hospital (30)	Dog	19	3	6
	Cat	11	1	2
Zoo (54)	Sheep	11	1	2
	Deer	9	0	0
	Tiger	9	0	0
	Leopard	5	0	1
	Yak	5	0	1
	Zebra	4	0	0
	Elephant	2	0	1
	Wolf	2	0	0
	Camel	2	0	0
	Others[1]	5	0	0

[1]Others included a rhinoceros, a takin, a lion, a panda, and a fox (one each).

PCR amplification rates of G. duodenalis at the gdh, bg, and tpi loci

All fecal specimens were subjected to nested PCR amplifications of the *gdh*, *bg*, and *tpi* genes. Thirteen fecal specimens were identified as *G. duodenalis* positive by PCR and sequencing of one or more of the three genes. Twelve, nine, and seven fecal specimens were successfully amplified and subtyped at the *gdh* (92.3%), *bg* (69.2%), and *tpi* (53.8%) loci, respectively. It was observed that six, three, and four *G. duodenalis* isolates were successfully amplified at three, two, and one loci, respectively (Table 2).

Molecular identification of G. duodenalis at the gdh, bg, and tpi loci

Among the twelve *gdh* gene sequences obtained, four assemblages were identified: C (n = 3), D (n = 6), E (n = 1), and F (n = 2). Three assemblages were detected based on nine *bg* sequence analyses: C (n = 2), D (n = 6), and F (n = 1). Assemblages C (n = 6) and F (n = 1) were identified from the *tpi* locus of seven *G. duodenalis* isolates. Assemblages C and D were detected in dogs, while assemblages C and F were present in cats. In zoo animals, assemblage D was identified in one wolf and one yak, while assemblages C and E were identified in one sheep, and assemblages D and F were present in one leopard (Table 2). Meanwhile, the phenomenon named "assemblage-swapping" (different assemblages at different loci in the same isolate) [29] was observed in five fecal specimens: the four from dogs and one from a leopard (Table 2).

Genetic characterization of assemblages C, D, E, and F

Among the eleven gene sequences of assemblage C at the *gdh* locus, the three sequences (one from cat, two from dogs) were identical to each other and had 100% homology with those from dogs in Australia, Brazil, Croatia, and Japan [30-32]. At the *bg* locus, two sequences of assemblage C from dogs (KF993712 and KF993714) were different from each other and have not been described previously. At the *tpi* locus, two sequences (KF993723 and KF993724) of assemblage C from dogs differed at two base positions and have been described previously in dogs. The remaining four nucleotide sequences (KF993721, KF993722, KF993725, and KF993727) were novel. Although two (KF993721 and KF993722) differed at two base positions, both were identical (at the amino acid level) with previous human isolates in China [24].

Six gene sequences of assemblage D were successfully amplified at both the *gdh* and *bg* loci. At the *gdh* locus, three sequences (KF993728, KF993734, and KF993736) of assemblage D were identified in one yak, one wolf, and a dog, and all were identical to each other and to those previously characterized from dogs in Brazil and Croatia [31,32]. The other three sequences (KF993729, KF993731, and KF993738) from dogs have not been reported previously. At the *bg* locus, three sequences of assemblage D (KF993713, KF993718 and KF993720) were identical to each other and have been described in dogs and *Nyctereutes procyonoides*, while the other three (KF993715, KF993716, and KF993717), from a dog, wolf, and leopard are novel.

Assemblage E was only identified in one sheep based on sequence analysis of the *gdh* gene, with the sequence being novel. Two *G. duodenalis* isolates were identified as assemblage F based on sequence analysis of two, one, and one *gdh*, *bg*, and *tpi* loci, respectively. Of these, both *gdh* sequences (KF993735 and KF993737) amplified from a leopard and a domestic cat

Table 2. *G. duodenalis* assemblages based on *gdh*, *bg* and *tpi* genes.

Host (n)	Isolate ID	*gdh* gene		*bg* gene		*tpi* gene	
		Assemblages	Accession no.	Assemblages	Accession no.	Assemblages	Accession no.
Dog (6)	DWY7	D	KF993729*	C	KF993712*	C	KF993721*
	DWY9	D	KF993731*	D	KF993713	C	KF993722*
	DWY12	C	KF993732	C	KF993714*	C	KF993723
	DWY22	C	KF993733	D	KF993715*	C	KF993724
	DWY43	D	KF993736	D	KF993718	C	KF993727*
	DWY52	D	KF993738*	D	KF993720		
Cat (2)	DWY8	C	KF993730	—	—	—	—
	DWY48	F	KF993737*	F	KF993719	—	—
Wolf (1)	DWY34	D	KF993734	D	KF993716*	—	—
Leopard (1)	DWY40	F	KF993735*	D	KF993717*	F	KF993726*
Sheep (2)	DWY67	E	KF993739*	—	—	—	
	DWY26	—	—	—	—	C	KF993725*
Yak (1)	DWY2	D	KF993728	—	—	—	—

* These sequences were novel.

were novel, but the *bg* sequence (KF993719) was identical to a previous cat-derived sequence from Italy [33]. The *tpi* sequence (KF993726) from a leopard was also novel.

Discussion

Previous reports on the prevalence and molecular characterization of *G. duodenalis* revealed the occurrence of this parasite in humans, domestic animals, and wildlife, showing the widespread distribution of hosts and the zoonotic potential of *G. duodenalis* [19,20,30,33,34]. However, few studies have been conducted on *G. duodenalis* in pets and zoo animals in China [22]. To investigate the occurrence and genotypic characterization of *G. duodenalis*, samples from animals from a pet hospital and zoo were analyzed by MLG analysis in this study. By microscopy and PCR, respectively, 5.95% (5/84) and 15.48% (13/84) of fecal specimens were found to be positive for *Giardia* infection (Table 1), with 13.33% (4/30) and 26.67% (8/30) for pets, and 1.85% (1/54) and 9.26% (5/54) for zoo animals. The ratios of PCR detection of *Giardia* to microscopic detection were different in pets (2.0) and in zoo animals (5.0). The higher ratio in zoo animals might be attributable to the fact that all the zoo animals were healthy at the time of sampling; in contrast, the animals from the pet hospital were suffering from diseases. *Giardia* cysts in feces in healthy hosts are generally low in number, and infection may be missed using relatively insensitive conventional morphological methods. In contrast, PCR has a much higher sensitivity than microscopy in the detection of *Giardia* cysts in fecal specimens. For example, 8.7% (144/1647) and 44% (220/500) of sheep specimens were identified as containing *G. duodenalis* cysts using morphological analysis and PCR, respectively [35]. Similarly, in a study of prevalence and molecular characterization of *Giardia* species and genotypes in sheep, *Giardia* was detected in 12.7% (8/63) of sheep samples by microscopy, versus 25.4% (16/63) by PCR [36]. However, in a study conducted on the prevalence and molecular typing of *Giardia* spp. in captive mammals at the zoo of Zagreb, Croatia, the parasite was detected in 38 of the 131 animals (29%) tested using epifluorescence microscopy, and positive PCR results were obtained for 23 of 27 (85%) samples [37]. Results concerning the presence of *Giardia* in nonhuman primates housed in two Spanish zoological gardens showed an infection rate of 60% (12/20) by microscopy, versus 70% (14/20) by PCR [38]. Mukarati *et al*. also found that the prevalence of *Giardia* spp. was higher by microscopy than by molecular assay in their study [39]. These differences could be attributable to the high host density present in captive conditions and different sample sources.

The present study revealed that the infection rates of *Giardia* was higher in pets than in zoo animals, regardless of whether PCR or microscopy was used. This may be due to the relatively strict feeding management of zoo animals and decreased accessibility to pathogens compared with pets that are not as geographically limited. In the present study, fecal specimens were tested for the presence of *G. duodenalis* by PCR amplification of *gdh*, *bg*, and *tpi* genes. The *gdh* locus gave the highest amplification rate (92.3%, 12/13), followed by the *bg* locus (69.2%, 9/13) and the *tpi* locus (53.8%, 7/13). These results were similar to those of a study on mammalian *G. duodenalis* in the United States, in which 91.0% (172/189), 84.8% (145/171), and 19.8% (34/172) of specimens were confirmed to be *Giardia*-positive at the *gdh*, *bg*, and *tpi* loci, respectively [16]. In another comparative study of the *tpi* and *gdh* genes for detection and genotyping of human-derived *G. duodenalis* isolates, the *tpi* gene was amplified from 96.2% (25/26) of samples, whereas only 81% (21/26) were positive when the *gdh* gene was targeted [40]. The difference in PCR amplification rates is probably associated with different assemblages, since assemblage A has been reported to give a higher amplification rate than assemblage B with the *gdh* locus (100% versus 71.4%) or the *bg* locus (100% versus 85.7%) [29].

G. duodenalis isolates from different animal origins were characterized by DNA sequencing of the *gdh*, *bg*, and *tpi* genes, and four assemblages (C, D, E, and F) were identified in the present study. In pets, assemblages C and D were detected in dogs, whereas assemblages C and F were present in cats. In zoo animals, assemblage D was identified in one wolf and a yak, while assemblages C and E were detected in a sheep, and assemblages D and F were present in one leopard (Table 2). Assemblages C, D, E, and F are all known to exhibit strong host specificities and narrow host ranges. Assemblages C and D are mainly found in dogs and other canines (foxes and coyotes) and canine-related animals (seals) [1,2]. In addition, both assemblages were occasionally reported in cats [41] and humans [42], and assemblage D was also reported in pigs [14]. In this study, assemblage D was identified in a yak and a leopard for the first time. This finding indicated that assemblage D might have a broader host range than previously believed. It can be speculated that assemblage D might be transmitted between these animals though water or food sources, and further study

should be adopted to confirm it. Assemblage E is mainly found in cloven-hoofed domestic mammals (cattle, water buffalo, sheep, goats, and pigs) [1], and it has been occasionally reported in cats [43]. Assemblage F is mainly found in cats and other carnivores (leopards), and a few cases have been reported in pigs [44] and humans [45].

"Assemblage-swapping" [33] was observed in five *G. duodenalis* isolates at all three loci: between assemblages C and D identified in four dog-derived *G. duodenalis* isolates, and between assemblages D and F identified in one leopard-derived *G. duodenalis* isolate. This could be due to genetic recombination, mutation of inter- and intra-assemblages, or the occurrence of mixed infections in fecal specimens [1,26]. In the present study, six isolates of assemblage C were identified out of seven successfully amplified *tpi* gene sequences, including one being negative at both the *gdh* and *bg* loci, one identified as assemblage C at all three loci, two identified as assemblage D at both the *gdh* and *bg* loci, and two identified as assemblage D or C at either the *gdh* or *bg* loci. This finding may also reflect primer specificity. In future studies, assemblage-specific PCR assays should be performed to more accurately assess the occurrence of mixed infections. In a recent study of prevalence and genotyping of *G. duodenalis* isolated from sheep in Henan Province, central China, some fecal samples contained *G. duodenalis* isolates that were also ascribed to different assemblages at different loci. This might be the result of mixed infections in the animals or genetic exchange between assemblages [46].

Meanwhile, the inconsistency in genotyping *G. duodenalis* at different loci suggests that a single gene is insufficient to confirm the true infection status or for accurate genetic characterization. Recently, MLG has been proved to be a powerful tool for detecting *G. duodenalis* and assessing the zoonotic potential of giardiasis by improving the resolution of the current genotyping tools [1,17,18,26]. A high degree of genetic variation was observed at the three loci studied following alignment with published sequences from GenBank. In total, 16 novel gene sequences were identified: six from assemblage C, six from assemblage D, one from assemblage E, and three from assemblage F. This result may reflect the genetic characterization of *G. duodenalis* isolates in the investigated areas. Indeed, epidemiological studies from India and Thailand suggested the possibility of zoonotic transmission of assemblages A and B between dogs and humans living in the same locality [47,48]. The possibility of zoonotic transmission needs to be investigated further by molecular analysis of a large number of giardiasis cases from humans and animals. In addition, the zookeepers and the water sources around the zoo animals, in addition to the other animals in contact with them should be investigated.

Conclusions

The present study described the occurrence and molecular characterization of *G. duodenalis* in pets and zoo animals in Shanghai, China. Assemblage D was identified in a yak and a leopard for the first time. Meanwhile, the epidemiology of *G. duodenalis* was described for pets in Shanghai, and MLG analysis identified the same *tpi* gene sequences of assemblage C in dogs and humans, indicating potential zoonotic transmission. The burden of human giardiasis attributable to animals needs to be assessed by systematic molecular epidemiological investigations of *G. duodenalis* in a large number of samples from humans and animals living in close contact.

Acknowledgements

We would like to thank technician Yuxin Xu (National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention) for his involvement in this work. This work was supported by grants from the Chinese Special Program for Scientific Research of Public Health (No. 201502021 to JC, No. 201302004 to YS), the National Key Research and Development of China (No.2016YFC1201900 to JC), the Project Sponsored by Shanghai Municipal Commission of Health and Family Planning (No. 20164Y0225 to HL) and the Fourth Round of Three-Year Public Health Action Plan of Shanghai, China (No. 15GWZK0101 to JC), and the Project Sponsored by the Young Scholar Scientific Research Foundation of China CDC (No. 2016A102 to HL). The funders had no role in study design, data collection and analysis, decision to publish, or preparation.

Authors' contributions

YS, JC, and HL conceived and designed the experiments. HL, YS, JY, ZY, YJ, WP, YZ, and WZ performed the experiments. YS, HL, JY, JC, and AL analyzed the data. JC and YS contributed reagents/materials/analysis tools. HL, YS, and JC wrote the paper. All authors read and approved the final version of the manuscript.

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Corresponding author

Professor Jianping Cao
Deputy Director of National Institute of Parasitic Diseases,
Chinese Center for Disease Control and Prevention
207 Rui Jin 2nd Road, Shanghai 200025, China
Tel: +8621 64735258
Fax: +8621 64376308
Email: caojp@yahoo.com

Conflict of interests: No conflict of interests is declared.