

Parasitological and molecular detection of human fascioliasis in a young man from Guizhou, China

Chen, M.X.^{1,2†}, Zhang, R.L.^{2#}, Xu, X.N.¹, Yu, Q.¹, Huang, D.N.², Liu, W.¹, Chen, S.H.¹, Song, P.¹, Lu, Y.¹, Cai, Y.C.¹, Ai, L.^{1*} and Chen, J.X.^{1*}

¹National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, WHO Collaborating Center for Tropical Diseases, Key Laboratory of Parasite and Vector Biology, National Health and Family Planning Commission, Shanghai 200025, PR China

²Shenzhen Center for Disease Control and Prevention, Shenzhen 518055, PR China

[†]Chen, M.X. and Zhang, R.L. contributed equally to this work.

*Corresponding author e-mails: ailin08306@163.com (L. Ai); chenjiayu1962@163.com (J.X. Chen)

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Abstract. A 24-year-old man born in Guizhou province was diagnosed with obstructive jaundice and bile duct stones in 2013. Four living trematodes were found during laparotomy and cholecystectomy. Based on the morphology and molecular genetics analysis of internal transcribed spacer and *cox1* genes of the flatworm specimens, the trematodes from the patient were confirmed to be *Fasciola hepatica*. This report provided the clinical and molecular diagnosis information on human fascioliasis, which is an emerging sanitary problem still ignored in China. Human fascioliasis constantly occurs due to climatic changes and frequency of human travel. Therefore, it deserves more attention from physicians working in both developing and developed countries.

BACKGROUND

Fascioliasis is a food-borne parasitic disease caused by liver fluke species of the genus *Fasciola* in many countries (Mas-Coma *et al.*, 2014a, b). *F. hepatica* is the main causative agent widely distributed in Europe, Africa, Asia, Oceania, and the Americas (Mas-Coma, 2004; 2005). In the last decades, zoonotic fascioliasis has emerged or reemerged in more than 60 countries (Kim *et al.*, 2015). Human infection occurs mainly via consuming raw fresh water plants with metacercaria of *Fasciola* spp. The total number of *Fasciola* infections in humans was estimated to be less than 3000. However, conservative estimates on its burden indicate that the number of individuals infected worldwide is at least 2.65 million recently (Fürst *et al.*, 2012). Based on the emergence, pathogenicity, and immunological interactions (Haçarız *et al.*, 2015; Figueroa-Santiago & Espino, 2014), the World

Health Organization has listed human fascioliasis among so-called neglected tropical diseases (World Health Organization, 2008), which are chronic, debilitating, and poverty-promoting, and also among the most common causes of illness in developing countries (Gil *et al.*, 2014; Pavlovic *et al.*, 2014; Losada *et al.*, 2015).

In terms of infection and transmission, the emergence of fascioliasis seems to be related to climatic changes and travels. This involves mainly anthropogenic modifications of the environment, increasing short- and long-distance travels, and import/export facilities available nowadays (Ashrafi *et al.*, 2014), which has increased the number of human fascioliasis cases. Therefore, the disease deserves more attention from physicians working in both developing and developed countries.

Since January 2012, 29 human fascioliasis cases with symptoms of intermittent fever, hepatalgia, and eosinophilia were

reported from local hospitals in Binchuan, southwest China. This was the first fascioliasis outbreak in China that gained the attention of the Chinese government toward the prevention and control of this disease.

The aim of this study was to report parasitological and molecular detection of human fascioliasis in a young man in Guizhou, China.

Subjects and methods

Medical history

Mr. He, aged 24 years, was born in Guizhou province in southwest China. From 2008 to 2013, he worked in Taizhou city, Zhejiang Province. He preferred consuming raw aquatic plants, especially water bamboo (one to two times per week on average). Occasionally he ate fried snails in restaurants and swam in a lake that was near his workplace. In June 2013, Mr. He showed symptoms of abdominal pain, diarrhea, fatigue, discomfort, skin and scleral jaundice, and tea-like urine. Especially, the patient's face turned "green" in color. The laboratory tests revealed mild normocytic normochromic anemia, high eosinophilia, and high serum activity of alkaline phosphatase, which were maintained during all hospitalizations, with normal alanine aminotransferase and aspartate aminotransferase levels. The ultrasonic examination revealed multiple stones in the bile duct, secondary extrahepatic bile duct dilatation, chronic cholangitis, chronic cholecystitis, incarcerated calculus in the gallbladder neck, and enlarged spleen. Due to obstructive jaundice and bile duct stones, laparotomy and cholecystectomy were performed, and four living trematodes were found.

The parasitic material from the patient was submitted for macro- and microscopic examinations, DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing analysis in the Department of Parasitology, Zunyi Medical College, and National Institute of Parasitic Diseases (NIPD), Chinese Center for Disease Control and Prevention.

After the surgery, triclabendazole (Egaten) was administered orally for 2 days consecutively (500 mg/day). In the following stool detection, no eggs were found, and the patient's positive signs disappeared.

MATERIAL AND METHODS

Ethics statement

This research study was approved by the Medical Ethics Review Committee of NIPD, Chinese Center for Disease Control and Prevention. The Medical Ethics Review Committee of NIPD, Chinese Center for Disease Control and Prevention exempted the individual informed consent targeting the molecular identification of adult flukes because the specimens were obtained after the surgery.

Parasitological analyses

The trematodes were repeatedly washed in physiological saline solution, macroscopically examined and measured, explored by microscopy examination, and morphologically identified according to the morphological keys (Periago *et al.*, 2006). Then, one part was fixed in 75% ethanol and sent to NIPD for species classification and genotyping by molecular methods.

DNA extraction and PCR amplification

Genomic DNA was extracted from a portion of the specimen by treating with sodium dodecyl sulfate/proteinase K (Ai *et al.*, 2010a, b), column-purified using Wizard DNA Clean-Up System (TAKARA BIO INC., Japan), and then eluted into 65 μ L of H₂O according to the manufacturer's recommendations. The DNA samples were stored at -20°C until further use.

The DNA region comprising internal transcribed spacer (ITS)-1, 5.8S rDNA, and ITS-2 plus primer flanking sequences (ITS+) was amplified by PCR from trematode DNA using primers BD1 (forward: 5'-GTCGTAACAAGGTTTCCGTA-3') and BD2 (reverse: 5'-TATGCTTAAATTCAGCGGGT-3') (Luton *et al.*, 1992). A portion of the *cox1* gene (*pcox1*) was amplified with primers JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3')

and JB4.5 (5'-TAAAGAAAGAACATAATG AAAATG-3') (Bowles *et al.*, 1992). PCR reaction system and conditions were as described in previous studies (Luton *et al.*, 1992; Bowles *et al.*, 1992).

Based on the comparison of the known ITS-2 sequences for the parasites (Huang *et al.*, 2004), a species-specific reverse primer known as DSJ3 (5'-CCA ATG ACA AAGTGA CAG CG-3') and another known as DSJ4 (5'-CCA ATG ACA AAG TAA CAGCA-3') were designed to amplify part of the ITS-2 sequences of *F. hepatica* and *F. gigantica*, respectively. A single forward primer, known as DSJf (5'-ATA TTGCGG CCA TGG GTT AG-3'), based on a genus-specific sequence of the 5.8S rDNA region of *Fasciola*, was used for both species. The PCR protocol was as described in a previous study (Huang *et al.*, 2004).

Two *Fasciola* DNA samples (one was *F. hepatica* and the other was *F. gigantica*), as well as a sample without genomic DNA, were included in each amplification run as positive and negative controls, respectively. An aliquot (5 µL) of each amplicon was examined on 1% agarose-TBE (65mM Tris-HCl, 22.5mM boric acid, and 1.25mM EDTA, pH 9.0) gels, stained with ethidium bromide, and photographed using a gel documentation system (Bio- Red, USA). The DNA size marker DL2000 was used to estimate the length. Positive amplicons were selected, purified, and sequenced using an ABI 377 automated DNA sequencer (using BigDye Terminator chemistry) employing the same

primers (individually) as used in the PCR. The ITS and *pcox1* sequences were obtained from GenBank under the accession numbers shown in Table 1.

Sequence analysis and reconstruction of phylogenetic relationships

The ITS and *pcox1* sequences were separately aligned using the Clustal X 1.83 software (Thompson *et al.*, 1997). Sequence differences (*D*) were calculated by pair-wise comparison using the formula $D = 1 - (M/L)$, in which *M* is the number of alignment positions at which the two sequences have a base in common, and *L* is the total number of alignment positions over which the two sequences were compared (Chilton *et al.*, 1995).

To study the phylogenetic relationships between this sample and other seven trematode species belonging to six genera in four families, the *pcox1* sequence of this sample, as well as that of *F. hepatica*, *F. gigantica*, *Clonorchis sinensis*, *Opisthorchis felineus*, *Schistosoma japonicum*, *S. mekongi*, and *Paragonimus westermani* obtained from GenBank (Table 1), was used for phylogenetic analyses, with *P. westermani* (AF540958) as the outgroup (GenBank accession number given in Table 1). Three methods, namely neighbor-joining (NJ), maximum likelihood (ML), and maximum parsimony (MP), were used for phylogenetic re-constructions. NJ and MP analyses were carried out using the PAUP 4.0 Beta 10 program (Swofford, 2002), and

Table 1. *Fasciola* sample of the patient from Guizhou, China, used in the present study, and the GenBank accession numbers for sequences of the ITS of nuclear rDNA, a portion of mtDNA cytochrome *c* oxidase subunit 1 (*pcox1*), and other reference *pcox1* sequences

Sample code	Location	Stage	Identification by morphology	GenBank accession number	
				ITS	<i>pcox1</i>
Fhs	China	Adult	<i>Fasciola hepatica</i>	KU555843	KU555842
Fh	Australia	Adult	<i>F. hepatica</i>	/	NC_002546
Fg	Zambia	Unknown	<i>F. gigantica</i>	/	AB983839
Cs	Unknown	Adult	<i>Clonorchis sinensis</i>	/	NC_012147
Of	Unknown	Unknown	<i>Opisthorchis felineus</i>	/	NC_011127
Sj	Unknown	Unknown	<i>Schistosoma japonicum</i>	/	NC_002544
Sm	Puerto Rico	Unknown	<i>Schistosoma mekongi</i>	/	AF216698
Pw	South Korea	Unknown	<i>Paragonimus westermani</i>	/	AF540958

ITS, Internal transcribed spacer; mtDNA, mitochondrial DNA; rDNA, ribosomal DNA.

ML analyses were performed using PUZZLE 4.1 (Strimmer and von Haeseler, 1996) under the default settings. The consensus tree was obtained after bootstrap analysis of 1000 replications, and values more than 50% were reported. Phylograms were drawn using the TreeView program version 1.65 (Page, 1996).

RESULTS

Macroscopic and microscopic analyses of the surgically removed material evidenced, besides fragments of other damaged specimens, a flat, brownish leaf-shaped organism measuring 1.0 cm × 0.8 cm, with a cone-shaped projection followed by a body enlargement, a spiny tegument, and two suckers (Fig. 1). It was identified as *Fasciola* spp.

The three ITS + PCR products were subjected to direct sequencing, and two sequences of 945 bp (*F. hepatica*) and one sequence of 946 bp (*F. gigantica*) were obtained (the two control sequences were not submitted to GenBank). The sequences were composed of the complete ITS-1 sequence of 422 bp, complete 5.8S sequence of 162 bp, and complete ITS-2 sequence of 362 or 361 bp. A comparison of the sample examined in the present study with those of *F. hepatica* and *F. gigantica* revealed that the patient's sample represented *F. hepatica* (Table 1).

For *pcox1* mtDNA region, no size variation among any of the amplicons examined was detected on the agarose gel. After trimming some base pairs at the



Figure 1. Flat, brownish, leaf-shaped adult specimen (1.0 cm × 0.8 cm) gathered from the patient.

beginning and end of the sequences, the sequence size for *pcox1* was 399 bp for the patient's sample and two control ones.

The specific PCR assays were then used to determine the specific identity of *Fasciola* spp. collected from the patient. On agarose gels, positive amplicons appeared as a single band of approximately 300 bp in size using corresponding specific primers, which allowed the identification of amplification against all *Fasciola* spp. The results showed that only specific primers for *F. hepatica* were able to amplify the young man's sample.

Topologies of the *pcox1* sequences inferred by different methods (NJ, MP, and ML) with different building strategies and/or different distance models were similar (Fig. 2). The phylogenetic tree consisted of two large clades: the first one contained the family Fasciolidae and Opisthorchiidae, and the other comprised Heterophyidae. Within the first clade, the patient's sample and two positive controls belonged to Fasciolidae; *C. sinensis* and *O. felineus* belonged to Opisthorchiidae. For the *Fasciolidae* cluster, the patient's sample and two positive controls were grouped, with a high bootstrap value (>50%) (Fig. 2). Within the second clade, *Schistosoma* samples were clustered together. This clustering was in agreement with the results of traditional classifications.

DISCUSSION

Human fascioliasis cases have markedly increased in the last decades. Given the global distribution and serious economic impact of fascioliasis (Mas-Coma *et al.*, 2005), precise identification of *Fasciola* spp. is crucial. However, the traditional morphological characterization is often laborious and time-consuming, and can generate discordant results, especially with the identification of eggs or juvenile stages (Mas-Coma *et al.*, 2014b). Recent PCR-based methods have greatly enhanced the ability to rapidly characterize the members of the genus *Fasciola* (Le *et al.*, 2012; Shafiei *et al.*, 2014; Shoriki *et al.*, 2015).

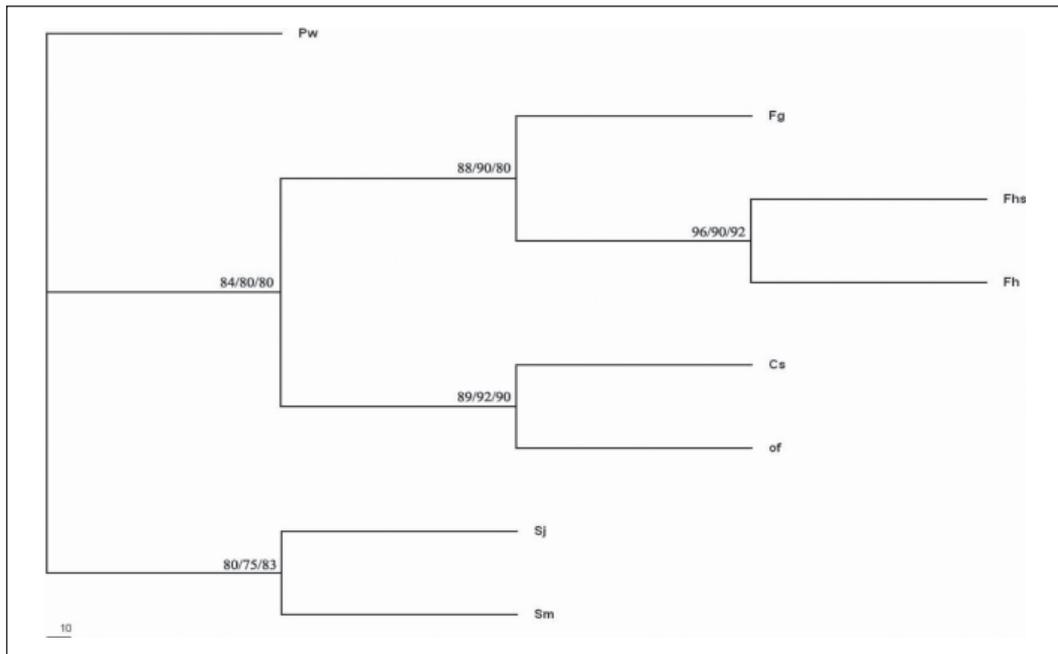


Figure 2. Phylogenetic relationship of *Fasciola hepatica* isolates from a young man in Guizhou, China, inferred by neighbor-joining analysis using the *pcox1* data, with *P. westermani* as the outgroup. Bootstrap values (in percentage) more than 50% from 1000 pseudo-replicates are shown for the neighbor-joining (the first value), maximum parsimony (the second value), and maximum likelihood analyses (the third value). Scale bar indicates an evolutionary distance of 10 substitutions per site in the sequence.

Previous phylogenetic analysis of isolates from different areas in China showed three distinct clades: *F. hepatica*, *F. gigantica*, and the “intermediate” type (Ai *et al.*, 2011). However, most of the human cases were just identified roughly by morphological differences (Fan *et al.*, 2006; Huang *et al.*, 2006; Huang *et al.*, 2009) owing to the deficiency of effective molecular techniques. *F. hepatica* is considered to be the major *Fasciola* species in humans in China with a total of 204 human cases documented, and 95.1% were attributed to *F. hepatica* (Xu *et al.*, 1999). According to the first national survey on parasitic diseases between 1986 and 1992, nine human cases with *F. gigantica* occurred in Hainan province, the southernmost province of China (Chen *et al.*, 1994). *F. gigantica* is currently reported from southwest China, including Guangxi, Guizhou, and Yunnan provinces (Song *et al.*, 2009; Shu *et al.*, 2012; Chen *et al.*, 2013). The only human fascioliasis case was characterized by molecular methods,

and the causative agent was *F. gigantica*, which was acquired from the first fascioliasis outbreak in Binchuan, Dali, and Yunnan province in 2013 (Chen *et al.*, 2013).

The patient in the present study had 5 years of experience working in Zhejiang province. The inquiry did not reveal the primary place of infection. However, the possible causes of infection were analyzed as follows:

1. Poor sanitary condition: The patient was born in Guizhou province, where the *Fasciola* spp. was prevalent in ruminant animals. The harmless treatment of animal feces was not effective in these areas because of the poor economic condition, leading to the easy spread of *Fasciola* eggs to the snails.
2. Practice of eating raw material: The patient claimed to consume raw aquatic plants, such as water bamboo, frequently. Metacercaria can be directly attached to the aquatic plants. Also, farmers fertilize

aquatic vegetables with the contaminated stool of cattle and other livestock, leading to the discharge of eggs into the water and their development into metacercaria. The direct consumption of raw or uncooked aquatic vegetables increases the risk of *Fasciola* infection in humans.

3. Accidental infection during swimming: The patient liked swimming in the lake in summer. Choking and drinking the metacercaria-contaminated water of the lake were unknown factors. Therefore, the epidemiology survey of *Fasciola* spp. in humans and animals in epidemic areas, healthy and food safety education promotion, expelling parasites from animals, and the harmless treatment of feces deserve the attention and collaboration from the government, medical departments, and veterinarian.

CONCLUSIONS

Human fascioliasis is indeed neglected in China. This zoonotic parasitic disease can be difficult to diagnose because of the atypical and severe clinical presentation. Problematic and delayed diagnosis is especially risky in nonendemic areas where clinicians are not familiar with this disease. The present case report provided detailed clinical information on this aspect, thus guiding the development of new diagnostic approaches for mitigating parasite transmission and virulence in humans.

Consent

Written informed consent was obtained from the patient for the publication of this case report.

Conflicts of interest

The authors declare no conflicts of interest with regards to this study or the manuscript prepared for publication.

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