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▣ BRIEF COMMUNICATION

Human Infections with Liver and Minute Intestinal Flukes in Guangxi, China: Analysis by DNA Sequencing, Ultrasonography, and Immunoaffinity Chromatography

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Abstract: The prevalence of liver and intestinal fluke infections was determined by surveying inhabitants of Hengxuan, Fusui, and Shanglin villages which were known to be endemic for liver flukes in Guangxi, China in May 2010. A total of 718 people were examined for helminth eggs by the Kato-Katz thick smear technique, ultrasonography, immunoaffinity chromatography, and DNA sequencing. The overall egg positive rate was found to be 59.6% (28.0-70.6%) that included mixed infections with liver and intestinal flukes. Cases showing higher than 20,000 eggs per gram of feces (EPG) were detected between 1.3% and 16.2%. Ultrasonographic findings exhibited overall 28.2% (72 of 255 cases) dilatation rate of the intrahepatic bile duct. *Clonorchis sinensis* infection was detected serologically in 88.3% (38 of 43 cases) among *C. sinensis* egg positive subjects by the immunoaffinity chromatography using a specific antigen for *C. sinensis*. For differential diagnosis of the liver and intestinal flukes, more precise PCR and nucleotide sequencing for copro-DNA were performed for 46 egg positive cases. Mixed infections with *C. sinensis* and *Metagonimus yokogawai* were detected in 8 of 46 egg positive cases, whereas 29 specimens were positive for *Haplorchis taichui*. Ultrasonographic findings and immunoaffinity chromatography results showed usefulness, even in a limited way, in figuring out of the liver fluke endemicity.

Key words: *Clonorchis sinensis, Metagonimus yokogawai, Haplorchis taichui*, minute intestinal flukes, fecal DNA, immunoaffinity chromatography, China

Fish-borne trematodiasis is an infection characterized by liver or minute intestinal flukes (MIF) that can cause chronic diseases and result in a neglected public health issue in Southeast Asia. Specifically, the liver fluke *Opisthorchis viverrini* is a common source of infection in Cambodia, Laos, Thailand, and Vietnam, whereas *Clonorchis sinensis* is distributed mainly in China, Korea, Taiwan, and Vietnam [1]. The liver fluke is most common in regions of Anhui, Guangdong, Guangxi, Hainan, Helongjiang, Jilin, and Sichuan in China, with Guangdong and Guangxi being the most prevalent areas of these trematodes. The prevalence of *C. sinensis* infection was found to be 0.4% in a nationwide survey in China between 1988 and 1992 [2]. Previous studies showed that intestinal flukes detected in China consisted of 8 species of the Heterophyidae and 5 species of the Echinostomatidae. Metacercariae found in fish from Guangxi consisted of 5 species (*C. sinensis*, *Metagonimus yokogawai, Haplorchis taichui, Haplorchis pumilio,* and *Centrocestus formosanus*) [3]. Among them, *H. taichui* and *H. pumilio* were the most common MIF. These opisthorchiid liver flukes and heterophyid intestinal flukes are highly prevalent in other Southeast Asian countries. However, the source of infections (freshwater fish species) is similar and also the egg shape is similar. Discrimination of egg types in these countries is difficult when conventional microscopic stool examination is used.

Over the last decade, morphologic diagnosis has been developed to distinguish the egg shape of haplorchiinae and opisthorchiid flukes [4], but this method is not applicable for precise differentiation of the fluke species. More recently, PCR assays have been developed to improve the specificity and sensitivity of discriminating between fish-borne trematodes [5-7]. In the present study, mixed infections of *C. sinensis* and *H. taichui* from fecal samples in Guangxi, China were examined us-

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ing species-specific PCR and sequence analysis of mitochondrial *cox1* gene. The serologic method, immunoaffinity chromatography, was used in combination with ultrasonography to figure out the endemicity of the fluke infections.

A total of 718 fecal specimens were collected from Gudongchun (n=160), Liaopingchun (n=143), Chunghechun (n=67), Changsachun (n=75), Buguchun (n=122), and Weihanchun (n=151) in Guangxi, China in May 2010 (Fig. 1). The eggs were examined by the Kato-Katz thick smear technique, in which the number of eggs counted in the entire field of 41.7 mg of compressed fecal smear is multiplied by 24 to obtain the number of eggs per gram of feces (EPG). For PCR analysis, fecal samples with enough amount for the test were collected from 46 individuals residing in 5 of the 6 villages.

Genomic DNA was extracted from the eggs using the QIAamp Stool Kit (Qiagen, Valencia, California, USA). Five pieces of small feces (200 mg) per 1 specimen (total 1 g) were mixed with 200 µl of buffer (ASL, Qiagen) and then incubated for 5 min at 95℃.

PCR was performed in a 25 µl reaction volume, containing 15 µl distilled water, 10 units of Expand High Fidelity Taq polymerase (Roche, Mannheim, Germany), 25 mM $MgCl_2$, 2.5 mM dNTP, 10 *p*mol of each primer, and 50 ng of the genomic DNA. The PCR protocol consisted of 35 cycles of predenaturation (5 min at 94℃), denaturation (30 sec at 94℃), annealing (40 sec at 45℃), and extension (60 sec at 72℃), followed by 1 cycle of 5 min at 72℃. The PCR primers were JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′ and JB4.5 (5′-TAA AGA AAG AAC ATA ATG AAA-3′) for *cox1*, which yielded a 349 bp product. The second PCR reaction and sequencing primers were CsF (5′-AGR TTT GCY GAT TYG TTG AAG TTG-3′) and CsR (5′-RCA AGT AAA WGG MAC WTT ACA ATA CTC-3′) for *C. sinensis*, and HtF (5′-GTG ACT ATG GTT ATA GGA GTC-3′) and HtR (5′-GAA GAC AAC ACA ATC CCG GTC-3′) for *H. taichui*, and MyF (5′-GCA TAT ATG CAT GAC TCT G-3′) and MyR (5′-CAT TAT GGA GGC CGA TAA G-3′) for *M. yokogawai*. The purified PCR amplified fragments of the *cox1* gene were then directly sequenced. The cycle sequencing from both ends of the fragments was performed using a Big-Dye Terminator Cycle sequencing reaction Kit (Applied Biosystem Co., Grand Island, New York, USA), and the reaction products were electrophoresed on an automated DNA sequencer (model 3730KL, Applied Biosystem). The sequences were assembled and aligned using the CLUSTAL X [8] and MEGA 4.0 [9]. The molecular identification of the liver fluke and MIFs was based on similarity of nucleotide sequences to the mitochondrial *cox1* gene of *C. sinensis* (GenBank No. AF217089) and *H. taichui* (GenBank No. EF055885).

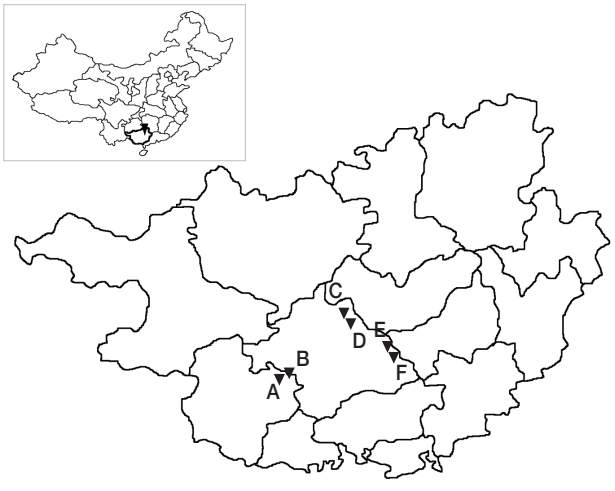

Fig. 1. Surveyed areas for liver and minute intestinal flukes in Guangxi (arrow), China. A: Chunghechun, Fusui, B: Changsachun, Fusui, C: Weihanchun, Shanglin, D: Buguchun, Shanglin, E: Gudongchun, Hengxuan, F: Liaopingchun, Hengxuan.

Sonographic examinations were performed on random subjects who voluntarily visited local health center for examination of the liver using a Voluson e ultrasound scanner (GE Medical Systems, Zipf, Austria). The sonographic findings were obtained which determines diffuse dilatations of the intrahepatic bile duct, extrahepatic bile duct, and pancreatic duct, periductal echogenicity, stones in the bile ducts, and distention of the gallbladder [10]: dilatation of 1 or more peripheral bile ducts was regarded as the minimal positive criterion.

The immunoaffinity chromatography was performed on the subjects who were examined by ultrasonography and agreed on the test using a NanoSign Cs Antibody (Bioland, Cheongwon, Chungcheongbuk-do, Korea) containing 0.34 µg antigen of *C. sinensis* to detect sera with liver and intestinal flukes. Bioland NanoSign Cs Ab is a chromatographic immunoassay kit for rapid and qualitative detection of anti-*C. sinensis* antibodies in serum and plasma from humans. The highly purified *C. sinensis* antigens are immobilized on nitrocellulose papers and conjugated to 40 nm of colloidal gold particles. This conjugate was placed on a polyester or glass pad as the conjugate pad. When the sample was dropped into the sample well on the device, the solubilized conjugate migrated with the sample by passive diffusion, and both the conjugate and sample came into contact with the *C. sinensis* antigen that was immobilized

onto a nitrocellulose membrane. If the sample contained antibodies against *C. sinensis*, the result was visible within 10 min in the form of a red line that develops on the membrane in the device. The antigen and antibody-binding solution continues to migrate to encounter a control reagent that binds a control conjugate, thereby producing a second red line according to the product demonstration of Bioland, Cheongwon, Korea.

The overall egg positive rate by stool examination was 59.6% (428/718) for *C. sinensis* and MIF. Each prevalence was revealed as 69.3% (111/160) in Gudongchun, 70.6% (101/143) in Liaopingchun, 64.1% (43/67) in Chunghechun, 28.0% (21/75) in Changsachun, 45.9% (56/122) in Buguchun, and 63.5% (96/151) in Weihanchun. Cases of heavy infection (EPG ≥ 20,000) in Gudongchun were detected as much as in 16.2% (26/160), Liapingchun 18.1% (26/143), Chunghechun 5.9% (4/67), Buguchun 3.2% (4/122), and Weihanchun 1.3% (2/151). Ultrasonograpic findings exhibited an average 28.2% (72/255) positive rate which varied from 0 to 49.2% according to different villages. The positivity of ultrasonographic (USG) findings tended to coincide with an increase or decrease of EPG (≥ 20,000) with the exception of Buguchun that showed a low EPG and a high USG positive values. The seropositive rate of *C. sinensis* infection was 88.3% (38/43), examined on *C. sinensis* egg positive cases by the immunoaffinity chromatography using a specific antigen for *C. sinensis*. Most of the villages showed positivity of higher than 85.7% with the exception of Weihanchun village which revealed 55.5%. A total of 46 small trematode egg positive cases were identified as *C. sinensis*, of which 29 specimens were also positive for *H. taichui* by mitochondrial *cox1* gene sequences (Table 1). Mixed infections of *C. sinensis* and *M. yokogawai* were detected in 8 of 46 egg positive cases. Most of the DNA-analyzed specimens revealed *C. sinensis* sequences with the exception of a single infection case of *H. taichui* in Chunghechun. In summary, mixed infections of *C. sinensis*, *M. yokogawai*, and *H. taichui* were detected in fecal samples, while *H. pumilio* and *H. yokogawai* were not detected among these egg positive cases in Guangxi by PCR and nucleotide sequence analysis of the mitochondrial *cox1* gene.

Guangxi, located in the southern part of China, has many minority peoples of different ethnic origins. The prevalence of *C. sinensis* infection in this province has been previously reported as much higher than that of the national average [2]. A previous study on second intermediate hosts of *C. sinensis* reported that only 3 of 31 fish species were positive for *C. sinensis*, while the remaining species were identified having metacercariae of MIF [3]. Intestinal flukes reported were *M. yokogawai*, *H. taichui*, *H. pumilio*, and *Centrocestus formosanus*, with the metacercariae of *M. yokogawai* being the most common species in fish in Guangxi. In the present study, the prevalence of the liver and intestinal flukes by egg microscopy was higher than that of the previous report. This may have been due to the fact that the villages that were previously recognized as endemic for trematode infections were chosen selectively. However, in reality, many (n = 29) of the reported cases were mixed infections with the liver fluke and MIF but not the cases of the *C. sinensis* alone.

Sonography is considered a helpful tool for diagnosing clonorchiasis, particularly in individuals with moderate or heavy infections. Sonographic findings of these subjects usually show low (n = 19) or moderate (n = 10) pathologic changes, which may be due to light infections with *C. sinensis* or mixed infections with intestinal flukes. Those patients diagnosed as *C. si-*

Table 1. The comparative results of ultrasonography, stool examination, immunoaffinity chromatography, and DNA sequencing for detection of liver and minute intestinal fluke (MIF) infections in Guangxi, China (2010)

Villages	Stool exam. (%)	EPG 20,000 or higher (%)	USG[a] (%)	Rapid test[b] (%)	Cox1 sequence finding
Gudongchun, Hengxuan	111/160 (69.3)	26/160 (16.2)	15/70 (21.4)	-[c]	-
Liaopingchun, Hengxuan	101/143 (70.6)	26/143 (18.1)	35/71 (49.2)	14/14 (100)	15 (Cs+[d]Ht: n = 11; Cs+My+Ht: n = 1; Cs: n = 3)
Chunghechun, Fusui	43/67 (64.1)	4/67 (5.9)	0/21 (0)	10/10 (100)	11(Cs+My: n = 4; Cs+Ht: n = 5; Ht: n = 1; Cs: n = 1)
Changsachun, Fusui	21/75 (28.0)	0	0/21 (0)	6/7 (85.7)	8 (Cs+Ht: n = 7; Cs: n = 1)
Buguchun, Shanglin	56/122 (45.9)	4/122 (3.2)	22/46 (47.8)	3/3 (100)	5 (Cs+Ht: n = 4; Cs: n = 1)
Weihanchun, Shanglin	96/151 (63.5)	2/151 (1.3)	0/26 (0)	5/9 (55.5)	9 (Cs+My: n = 2; Cs+My+Ht: n = 1; Cs: n = 6)
Total	428/718 (59.6)	62/718 (8.6)	72/255 (28.2)	38/43 (88.3)	46

[a]Ultrasonography; percentage of positive cases that exhibit dilatation of 1 or more peripheral bile ducts in number.
[b]Test against *C. sinensis* egg positive cases using chromatographic immunoassay kit for detection of anti-*C. sinensis* antibodies in serum.
[c]Test not done.
[d]Mixed infections with Cs (*Clonorchis sinensis*), My (*Metagonimus yokogawai*), or Ht (*Haplorchis taichui*).

nensis infections by microscopic examinations turned out to be mixed infections with *C. sinensis* and *H. taichui* by the nucleotide sequence analysis of mitochondrial *cox1* gene. In this regard, ultrasonography seemed to be valuable for figuring out the liver fluke endemicity and not for precise individual diagnosis. Buguchun was not well understood for inconsistency between the data of EPG and USG; however, there may be a possibility for major distribution of moderate infections of *C. sinensis* in this village. Immunoaffinity chromatography showed high sensitivity of 88.3% (38/43) to detect *C. sinensis* infections but light or mixed infection cases with MIF in Weihanchun village may be the case of relatively low positivity (55.5%). According to the results, sonography and immunoaffinity chromatography showed low positivity in light infection cases of *C. sinensis* or mixed infections with intestinal flukes. In contrary, the PCR test developed in this study was capable of amplifying *C. sinensis* and MIF directly from eggs in feces for distinguishing each other. In addition, this test has the advantage of being capable of amplifying MIF species and identifying their nucleotide sequences even without the availability of adult worms.

Conclusively, the results from this study demonstrated that PCR approach can accurately detect mixed *C. sinensis*, *M. yokogawai*, and *H. taichui* infections in endemic areas. For MIF, like *H. pumilio* and *C. formosanus*, further molecular studies are needed to figure out clearer picture of fluke endemicity in this area of China.

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