Current Status of Lung Fluke Metacercarial Infection in Freshwater Crabs in the Kawane Area of Shizuoka Prefecture, Japan

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ABSTRACT. Feline cases of lung fluke infection were recently reported in the upper basin of the Oi River in Shizuoka Prefecture. The causative species of these cases were not identified, although a field survey conducted about 40 years ago in this area demonstrated the prevalence of *Paragonimus miyazakii* metacercariae in intermediate host crabs. To clarify the current status of lung fluke metacercarial infection in host crabs, we collected the Japanese freshwater crab or Sawagani, *Geothelphusa dehaani*, at 14 sampling sites in the Kawane area, which is located in the upper basin of the Oi River. Sawagani harboring *Paragonimus* metacercariae were collected at all sampling sites in this study with a total prevalence of 42% (281 of 677 crabs examined) and a range of 16% to 92%. The number of metacercariae per infected crab ranged from 1 to 19 with a mean of 2.2. Based on the morphological features of the metacercariae and adult worms recovered from rats that were experimentally infected with the metacercariae, the lung flukes examined were identified as *P. miyazakii* Kamo, Nishida, Hatsushika and Tomimura, 1961; ITS2 sequence data corroborate this conclusion. *P. miyazakii* is still widely prevalent in this area, implying that the recently reported feline paragonimiasis cases were likely caused by *P. miyazakii* infection.

KEY WORDS: Japanese freshwater crab, lung fluke, *Paragonimus miyazakii*, *Paragonimus skrjabini*, Sawagani.

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Lung flukes *Paragonimus westermani*, *Paragonimus miyazakii*, and *Paragonimus ohirai* occur in Japan; the first 2 species are zoonotic pathogens that cause pulmonary and sometimes extrapulmonary diseases [8]. These 2 lung fluke species share a major infection source, the Japanese freshwater crab or Sawagani (*sawa* means mountain stream and *gani* [or *kani*] means crab in Japanese), *Geothelphusa dehaani*. Sawagani has a geographical distribution that covers almost all of Japan except for Hokkaido and the southern islands of Okinawa Prefecture, and Sawagani harboring metacercariae of these 2 *Paragonimus* spp. have been identified in several areas throughout this geographical range [9]. Among the regions in Japan to which Sawagani is endemic, the Kawane area in the upper basin of the Oi River (now merged with Shimada City) of Shizuoka Prefecture received marked attention about 40 years ago when outbreaks of human paragonimiasis in Tokyo, Kanagawa and Yamanashi prefectures were traced to the consumption of raw Sawagani harvested in Shizuoka Prefecture, particularly the Kawane area [20]. Although the patient sputum and/or fecal samples were negative for lung fluke eggs, serological tests suggested infection with *P. miyazakii* [6, 20]. Extensive field surveys conducted throughout Shizuoka Prefecture revealed a high prevalence of *P. miyazakii* metacercariae in Sawagani

in regions along the Oi River, including in the Kawane area, but lower or zero prevalence in regions along rivers other than the Oi River [4]. Recently, paragonimiasis cases were reported in cats kept by villagers in the upper basin of the Oi River [11], but the causative species was not identified.

Here, we examined the current status of lung fluke metacercarial infection in Sawagani at 14 sampling sites in the Kawane area. These data will be useful for establishing countermeasures needed to control lung fluke infections not only in the Kawane area, but also in Tokyo, because Sawagani from several prefectures, including Shizuoka, are sold as foodstuffs at retail fish markets in Tokyo, and some were found to be positive for *P. westermani* and/or *P. miyazakii* metacercariae [17, 19].

MATERIALS AND METHODS

Isolation and examination of *Paragonimus* metacercariae: Between October 2010 and May 2012, we collected 677 Sawagani from 14 sampling sites in mountain streams in the Kawane area of Shizuoka Prefecture (Fig. 1). Stream names were assigned as sampling site names. Sawagani were examined one by one to gather accurate data on the prevalence and burden of *Paragonimus* metacercariae, as described previously [15]. Briefly, the internal organs including the gills, heart, midgut gland, and genital organs were excised from crabs after removing the carapace, compressed between 2 glass plates, and examined for the presence of metacercariae under a stereoscopic microscope. When *Paragonimus* metacercariae were identified, the glass plate covering the tissues was carefully removed and metacercariae in the compressed

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tissues were recovered using teasing needles or tweezers. Isolated metacercariae were placed on glass slides and pressed lightly under a coverslip for morphological observation and measurement.

Adult worms from test rats: Male Wistar rats ($n=2$, 5-week-old) were orally infected with 21 and 33 metacercariae from 2 different sampling sites. Necropsy was performed 69 and 81 days after infection, and worms were recovered from the lungs and pleural cavity by macroscopic examination. The recovered worms were compressed between 2 glass slides, fixed in 70% alcohol, stained with borax carmine,

and mounted with Canada balsam for morphological observations and measurements. Developmental stages of the recovered worms were determined according to the criterion of Shibahara [13].

DNA amplification and sequencing of the second internal transcribed spacer (ITS2) region of ribosomal DNA: The ITS2 region of 2 metacercariae from each sampling site was amplified by PCR and sequenced as described previously [16]. The primers used were 3S, 5'-GGTAC-CGGTGGATCACTCGGCTCGTG-3' (forward), and A28, 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (reverse). Sequences were aligned and compared using the GENETYX-Win software (Ver. 10, Genetyx Co., Tokyo, Japan).

RESULTS

Prevalence and burden of *Paragonimus metacercariae* in Sawagani: We collected 677 Sawagani from 14 sampling sites in the Kawane area (Fig. 1). At all examined sites, Sawagani harboring *Paragonimus metacercariae* were detected. The total prevalence of *Paragonimus metacercariae* over the entire Kawane area was 42% (281 of 677 crabs examined), ranging from 16% to 92% among sites (Table 1). A total of 631 metacercariae were collected from the 281 positive crabs, and the number collected from a single infected crab ranged from 1 to 19 (mean, 2.2 for infected crabs). Taken over all crabs collected, the mean number of metacercariae per individual was 0.93. Among sampling sites, the highest incidence (92%) of metacercarial infection and greatest mean metacercarial burden per infected crab (4.0) were found at Yano.

Morphology of metacercariae collected from Sawagani: *Paragonimus metacercariae* obtained from Sawagani were spherical with thin outer and thick inner walls (Fig. 2). The

Fig. 1. Study area and sites of freshwater crab sampling. (A) Map of central Japan showing the study area in Kawane, central Shizuoka Prefecture. (B) Detailed map of the study area, showing sampling sites in mountain streams at which freshwater crabs, *Geothelphusa dehaani*, were collected.

Table 1. Prevalence and burden of *Paragonimus metacercariae* in freshwater crabs collected from mountain streams in 14 locations in the Kawane area, Shizuoka Prefecture

Location	No. of crabs		% infected	Total no. of mc ^a detected	No. of mc per infected crab	
	Examined	Infected			Range	Average
Sakka	81	20	25	57	1–19	2.9
Minami	51	27	53	53	1–11	2.0
Tsuta	54	36	67	63	1–11	1.8
Kita	35	6	17	17	1–3	2.8
Horinouchi	69	11	16	16	1–2	1.5
Tera	55	33	60	60	1–10	1.8
Kurayama	32	11	34	34	1–4	3.1
Shimoho	48	26	54	54	1–10	2.1
Yano	25	23	92	92	1–10	4.0
Shindai	46	12	26	26	1–3	2.2
Kiriyama	61	33	54	58	1–11	1.8
Iwahana	54	21	39	39	1–4	1.9
Godo	25	8	32	32	1–3	4.0
Tozuru	41	14	34	30	1–7	2.1
Total	677	281	42	631	–	2.2

a) mc: metacercariae.

Fig. 2. Photomicrograph of a *Paragonimus* metacercaria. Note the very thick outermost membranous substance (arrow).

thickness of the outer cyst wall of 47 specimens from Yano ranged from 2.1 to 3.7 μm (mean, 2.6 μm); that of the inner cyst wall ranged from 10.0 to 22.9 μm (mean, 17.6 μm). Furthermore, the outer cyst of many specimens was tightly enveloped with a very thick membranous substance with a thickness ranging from 11.8 to 69.8 μm (mean, 30.6 μm). The diameter of the inner cyst after being freed from the outer cyst ranged from 398 to 515 μm (mean, 458 μm). Examination of 10 larvae showed that none had a stylet in the oral sucker.

Infection of rats with metacercariae and morphology of adult worms: Necropsy was performed on the two rats 69 and 81 days after inoculation with 21 and 33 *Paragonimus* metacercariae, respectively, collected from 2 different sampling sites in the Kawane area (Table 2). In Rat 1, 6 and 2 worms in the lungs and pleural cavity were identified as adults and pre-adults, respectively. Rat 2 had 10 worms in each of the lungs and pleural cavity of which 7 worms from the lungs and 2 worms from the pleural cavity were identified as adults. The remaining 11 worms were identified as pre-adults. The mean size of the 15 adult worms from the 2 rats that were mounted on glass slides was 5.8 $\times$ 2.7 mm, ranging from 4.4 to 6.6 mm in length and 2.2 to 3.3 mm in width. The ratio of body length to width ranged from 1.9 to 2.4 (mean, 2.1). The mean transverse diameter of the oral and ventral suckers was 540 and 680 μm , respectively.

A representative specimen is shown in Fig. 3, and the morphology of the adults is described as follows. The ventral sucker is always larger than the oral sucker and is situated slightly anterior to the middle of the body. The ovary is intricately branched, having a coral-like appearance (Fig. 3A). The uterus is situated opposite the ovary. The testes are situated on both sides of the posterior part of the body, and these are larger than the ovary. The cuticular spines are singly spaced (Fig. 3B).

ITS2 sequence analysis: The alignment of ribosomal DNA ITS2 sequences of 28 metacercariae (2 from each of 14 collection sites) was 361 bp in length and showed no variation in length or composition among the specimens. The nucleo-

Fig. 3. Photomicrograph of an adult worm. (A) Dorsal view of an adult worm isolated from the lung of a rat on necropsy 81 days after infection. Stained with borax carmine, showing the testes (T) and ovary (O). (B) Photomicrograph of cuticular spines. Note the singly spaced arrangement.

tide sequence was deposited in the DDBJ/EMBL/GenBank database under accession number AB733177.

Searches of nucleotide databases revealed that the ITS2

Table 2. Experimental infection of rats with *Paragonimus* metacercariae isolated from freshwater crabs

Rat No.	Source of mc ^{a),b)}	Dose of mc	Duration of infection (days)	Rate of mc recovered as (pre) adult ^{c)} worms (%)	No. of (pre)adult worms recovered		
					Total	Pleural cavity	Lungs
1	Tsuta	21	69	38	8	2	6
2	Yano	33	81	61	20	10	10

a) Locations in the Kawane area from where the infected freshwater crabs were collected.

b) mc: metacercariae.

c) (pre)adult: preadult and adult.

sequence determined in this study was identical to that of *P. miyazakii* isolated from Rokuroshi in Iwakuni City, Yamaguchi Prefecture (DDBJ/EMBL/GenBank accession number: AB713405), where Sawagani was first discovered to be the second intermediate host of *P. miyazakii* [5].

DISCUSSION

Three lung fluke species, *P. westermani*, *P. miyazakii* and *P. ohirai*, occur in Japan and can be differentiated by the morphological features observed in living metacercariae and mounted adult fluke specimens [7, 8]. The morphological features of the metacercariae and adult worms examined in this study are consistent only with those of *P. miyazakii* [7, 8] and, thus, we identified the specimens as *P. miyazakii*. The ITS2 sequence data corroborate this conclusion. Therefore, Sawagani infected with *P. miyazakii* are concluded to still be widely prevalent in the Kawane area of Shizuoka Prefecture as reported previously [4]. Thus, the recently reported feline paragonimiasis cases [11] were likely caused by *P. miyazakii* infection. Veterinarians and owners of carnivorous companion animals, particularly cats, kept in and around the Kawane area should be aware of the risk of *P. miyazakii* infection.

Paragonimus skrjabini infections in humans and animals have been reported in China, since this species was first described in 1959 [3]. *P. miyazakii* was designated as a new species 2 years after the designation of *P. skrjabini* [5]. *P. skrjabini* occurs in various parts of China as well as Thailand and northeastern India [8, 14], while *P. miyazakii* has been reported to occur exclusively throughout most of Japan [9]. Blair *et al.* [2] determined that *P. skrjabini* found in China is very closely related to *P. miyazakii* found in Japan based on identical ITS2 sequence in a recent phylogenetic study. A subsequent study by Blair *et al.* [1] comparing morphometric data and molecular phylogenetic findings based on mitochondrial DNA cytochrome *c* oxidase subunit I gene sequences showed that *P. skrjabini* from Fujian Province in Eastern China was very closely related to *P. miyazakii*. They proposed that these populations should be referred to as one and the same subspecies, *P. skrjabini miyazakii*, and that *P. skrjabini* populations from other parts of China should be referred to as a different subspecies, *P. skrjabini skrjabini*. Based on these findings, they concluded that *P. skrjabini* represents a species complex [1]. *P. miyazakii* or *P. skrjabini miyazakii* in Japan is a primary member of the *P. skrjabini* species complex, and it has been reported that this species/subspecies can be discriminated from *P. skrjabini*

populations in China, even those from Fujian Province, using morphometric parameters such as the ratio of the oral sucker diameter to body length [1]. Therefore, we retain the use of the original name, *P. miyazakii*, in the present study. In addition, recent scientific literature using the scientific name *P. miyazakii* has been published, implying that this name is widely accepted [12].

In Japan, paragonimiasis is still an important foodborne helminthic zoonosis; at least 30 human cases are reported annually [10]. To establish countermeasures for controlling lung fluke infection, we previously investigated the effect of heat on the infectivity of *P. westermani* metacercariae in Sawagani. Boiling Sawagani infected with *P. westermani* metacercariae at 55°C for 5 min eliminated the infectivity to test mice [18]. However, the conditions required to reduce or eliminate the infectivity of *P. miyazakii* metacercariae harbored in Sawagani have not yet been confirmed; we are currently conducting tests on Sawagani from the Kawane area. Therefore, currently, a health education campaign that emphasizes on the risks of *P. miyazakii* infection from consuming raw and undercooked Sawagani, including those sold at retail fish markets, is recommended as the most efficient countermeasure for preventing lung fluke infections.

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