Field survey focused on Opisthorchis viverrini infections in five provinces of Cambodia

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Field survey focused on *Opisthorchis viverrini* infection in five provinces of Cambodia

カンボジア 5 県におけるタイ肝吸虫症を焦点としたフィールド研究

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Field survey focused on *Opisthorchis viverrini* infection in five provinces of Cambodia

Contents

Title:

Field survey focused on *Opisthorchis viverrini* infection in five provinces of Cambodia

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Key words: *Opisthorchis viverrini*; endemic infection; Cambodia

1. Introduction

Opisthorchis viverrini is one of the most important food-borne liver flukes in Southeast Asia. It is endemic in Thailand and Lao People's Democratic Republic (Lao PDR) and constitutes a major public health problem in the entire Mekong Basin [1–13]. Although Cambodia is located in the Mekong Basin, previous reports only referred to the country as a whole and did not break down infection rates by province or district [1,4–10]. Before the present research started in 2006, seven manuscripts on *O. viverrini* infection were found in the Pub Med/1980–2005 database, and only one published in 2002 was a parasitological survey focusing on Cambodia. A Korean team using an unspecified technique reported that the *Opisthorchis* spp. infection rate was 4.0% in primary schools in Kampong Cham [14]. Thus, *O. viverrini* infection was suspected to be a prevalent yet neglected tropical disease in Cambodia.

When we conducted research in 2005 on anemia in pregnant women in Takaev province, the results suggested that some areas of Cambodia had a high prevalence of *O. viverrini* infection. The National Center for Malaria Control, Parasitology and Entomology (CNM), Ministry of Health (MOH), Cambodia, became interested in cooperating with an epidemiological survey. We prepared the first research plan in February 2006 and began the survey in March. Stool samples were examined using the Kato-Katz technique in two villages in the Prey Kabas district, Takaev province, in the first field research focusing on *O. viverrini* infection in Cambodia, which is continuing. This paper is a summary of the results obtained from March 2006 to August 2012.

1.1 Purpose of the research

The investigations focused on confirming that *O. viverrini* infection is endemic in several provinces in Cambodia.

2. Materials and methods

2.1. *Study areas*

This study was conducted in 55 villages in five provinces located in central and south Cambodia from March 2006 to August 2012 (Table 1, Fig. 1). The five provinces are located in the Mekong River basin and tributary areas. The target villages were located near streams, lakes, or ponds formed after flooding in the rainy season. The survey began in Takaev province, where two villages in which high endemic rates of opisthorchiasis were found in March 2006. It continued in Kandal province to the north of Takaev, then moved to adjoining Prey Veng and Kampong Cham provinces, and finally to Kampong Thom bordering on Kampong Cham (Fig. 1).

2.2. *Methods*

The survey included the collection of stool specimens for examination along with intermediate host surveillance; in addition, interviews were conducted on basic living conditions, and the environmental conditions of villages thought to be related to *O. viverrini* infection were noted.

2.2.1. Stool sample examinations using the Kato-Katz technique

Single stool samples from one man and one woman over 20 years of age were collected from each target family within the surveyed villages each morning and then immediately examined by technicians from the CNM. The Kato-Katz thick-smear technique was used to detect helminth eggs. Initially, all target village households were surveyed, but in 2010 the protocol was changed to quasi-random sampling in order to cover more areas of Kampong Cham and Kampong Thom provinces.

2.2.2. Detection of *O. viverrini* DNA from stool samples using PCR

After identifying villages in which *O. viverrini* infection was endemic in Kampong Cham and Kampong Thom provinces, five villages that had not yet undergone deworming with praziquantel administration were selected. After the initial Kato-Katz screening, 33 villagers whose egg per gram (EPG) values of *O. viverrini* were high agreed to provide additional samples. Approximately 3 ml of feces was placed in a test tube containing 6 ml of ethanol (70–80% density) and mixed thoroughly [15,16]. Nested PCR was performed at Dokkyo Medical University in Japan with the primers for *O. viverrini* according to the method of Lovis et al. [17].

2.2.3. Surveillance of intermediate hosts

Snails and fish known to be the first and second intermediate hosts of *O. viverrini* were examined in infection-endemic villages to confirm the existence of vectors. Village leaders, residents, and fishermen cooperated to provide information concerning types of fish normally eaten raw and to collect cyprinid fish and *Bithynia* spp. snails from all endemic areas. Those samples came from ponds, small lakes, rice fields, and streams near the villages. All the fish were photographed, and some were fixed in formalin for morphological classification by a specialist. After removal of the intestines, the fish samples were cooled on ice, transferred to the laboratory of the CNM in Phnom Penh or Dokkyo Medical University in Japan, and then examined.

Snail shells were crushed, and the presence of cercariae was determined using a stereoscopic microscope. Cercariae of *O. viverrini* from snails were identified morphologically using a biological microscope. The fins, scales, and/or muscles of fish were observed using a stereoscopic microscope. Muscles were examined with the compressing and/or digestion methods. Suspected metacercariae were collected and identified morphologically using a biological microscope [1]. Some metacercariae were used for experimental infection in hamsters. Fourteen male Syrian hamsters were infected with 10–60 metacercariae by intragastric inoculation. Two to 3 months after infection, the animals were killed, and *O. viverrini* worms were collected from the bile ducts. In addition to morphological identification, PCR was also performed using ethanol-fixed cercariae and metacercariae specimens based on the method of Lovis et al. [17].

2.2.4. Adult worm collection

Adult worms were collected from patients in one Kandal province village that had a high egg-positive rate. Seven patients who had high egg density but no other health problems were chosen to participate after giving informed consent. These patients received a single dose of praziquantel 30 mg/kg, which is half the dose regularly administered by the CNM, early in the morning before breakfast to acquire as many complete adult worms as possible. After 3–4 h, magnesium sulfate ($MgSO_4$) 10–15 g (10 g for patients weighing <50 kg and 15 g for those weighing >50 kg) was administered with as much water as possible under the supervision of a Cambodian physician. After 1–2 h, defecation occurred, and the total feces were collected. One month later, the patients began to receive regular praziquantel treatment from local healthcare workers. The adult worms obtained were fixed in 10% formalin and transported to Japan, where they were stained with carmine solution and mounted on glass slides for examination.

2.2.5. Investigation of factors affecting opisthorchiasis endemicity

Village residents participating in this study were registered by name and age and questioned on their consumption of raw freshwater fish and whether the residents' homes had an enclosed toilet. The types of fish eaten and how the dishes containing raw fish were prepared were noted.

Observations of environmental conditions focused on fishing spots, housing, and the general condition of villages. Some areas, especially infection-endemic villages, were visited in both the rainy and dry seasons. CNM staff carried out most of the interviews, supplemented by a Khmer-speaking Japanese researcher.

2.2.6. Ethical considerations

This epidemiological study was performed in strict accordance with all relevant guidelines; researchers visited target homes to obtain informed consent from residents for survey participation. The research plan, including informed consent, was approved by the National Ethics Committees, Cambodian MOH, and Dokkyo Medical University (permit no. 23007).

Animal experiments with hamsters performed in this study were conducted according to the ethical guidelines for the use of animal samples of the Animal Care and Use Committee, Dokkyo Medical University (permit nos. 0523 and 0688), in accordance with the Guidelines for the Care and Use of Laboratory Animals, Dokkyo Medical University, the Law Concerning Kind Treatment and Management of Animals (law no. 221), and the Japanese Government Notification on Feeding and Safe-keeping of Laboratory Animals (no. 6).

3. Results

3.1. *Stool examinations*

3.1.1. Results of stool sample examinations using the Kato-Katz technique

A total of 16,082 stool samples were examined from 55 villages, of which 1,232 were egg positive (Table 1). In 15 villages that had a greater than 10% egg-positive rate, eggs were found in 998 stool samples. The egg-positive rate was 27.8% in 3,585 samples examined from those 15 villages (Table 2). Samples may have included the eggs of several small trematodes, because it is difficult to distinguish the eggs of *O. viverrini* from those of *Haplorchis* spp., for example, on smears. These 15 endemic villages are located in 10 communes of seven districts in the four provinces of Takaev, Kandal, Kampong Cham, and Kampong Thom (Table 2).

The Kato-Katz technique showed that the egg-positive rate among residents in these villages was greater than 10%, ranging from 10.7% to 65.1% (Table 2). The egg-positive and fish consumption rates in Kandal and Takaev provinces are shown on the map in Fig.2A, and there were also several infection-endemic villages in the Sour Kong and Reay Pai communes of Kampong Cham province. Egg-positive and fish consumption rates there are shown on the map in Fig. 2B.

Data from one infection-endemic village in Prey Kabas district, Takaev province, were analyzed to determine the egg-positive rate by age (Table 3). Adults were selected as the target age-group, although a few samples from individuals less than 20 years of age were included. The results of the chi-square test showed that although the infection rate in those older than 20 years greater than 20% and those in their 40s had the highest infection rate of 34.7%, it did not reach the level of statistical significance.

Table 4 shows the egg-positive rate in an infection-endemic village by EPG value and gender. Around half of both male and female residents of the village were selected for examination, following the same selection method used in most villages. Gender did not significantly affect egg positivity in the results of the chi-square test. The Kato-Katz technique was used for calculating EPG values in all villages (egg numbers on one smear × 24). In the same village for which the egg-positive rate is shown in Table 4, more than 90% of the feces samples collected had an EPG value of less than 500. A few feces samples had very high EPG values of more than 10,000 in other infection-endemic villages, although they comprised less than 5% of total samples.

3.1.2. Other helminthic infections in the survey area

Eggs of other helminths were also found in feces samples in the target villages. The results from two infection-endemic villages are shown in Table 5. There was a high prevalence of hookworm in most villages. Other studies also found that hookworms are prevalent parasites in Cambodia [15,18,19].

3.2. *Detection of* O. viverrini *DNA from stool samples using PCR*

Thirty of 33 samples were positive for *O. viverrini* DNA in PCR analysis. Residents of two villages in Kampong Thom and three villages in Kampong Cham were found to be infected with *O. viverrini*.

3.3. *Survey of intermediate hosts in endemic areas*

Among the numerous snails collected in infection-endemic areas, many were identified as *Bithynia* spp. morphologically. Many species of freshwater fish were also found, including those in the Cyprinid family. Some fish samples were examined individually, while others were pooled.

3.3.1. First intermediate hosts and cercariae

Numerous snails were found in Takaev, Kandal, and Kampong Cham provinces, including *B. siamensis siamensis*. Some were infected with *O. viverrini* cercariae and the cercariae of other species. A study in Kampong Cham in November 2011 showed that one *O. viverrini* cercaria was identified using PCR among 406 *Bithynia* spp. samples studied; the positive rate was thus 0.25%. Other species of cercariae were also detected in those 406 snails.

3.3.2. Second intermediate hosts and metacercariae

Many cyprinid fish are consumed raw in endemic villages in four of the provinces studied and some were found to be infected by *O. viverrini* metacercariae. Metacercariae of *O. viverrini* were identified by morphological observation, animal experiments, or PCR in six species of fish in 2012 in the target areas: *Osteochilus hasseltii*, *Puntius brevis*, *Cyclocheilichthys repasson*, *Hampala dispar*, *Labeo chrysophekadion*, and *Labiobarbus siamensis* (Table 6). Adult worms of *O. viverrini* were also obtained from hamsters experimentally infected with metacercariae from *C. repasson* and *H. dispar* or *H. macrolepidota*.

3.3.3. Location of *O. viverrini* infection-endemic villages and fishing spots inhabited by intermediate hosts

The four villages where *O. viverrini* infection was endemic in Takaev and Kandal provinces were located along tributaries of the Bassak River, one of the main tributaries of the Mekong. The names of most of these tributaries, except for the Bassak River, are not found on maps, and villagers simply call them "*Tonle*" in Khmer, meaning "the River." In the rainy season, the tributaries flood and form a large lake (around N 11°50' and E 104° 56' on Google Earth) where freshwater fish are caught for raw consumption by residents of these four villages. This fishing spot forms annually from floodwater from tributaries of the Bassak River. Some ponds remain year round, while most disappear in the dry season. Environmental conditions therefore undergo marked seasonal variation.

O. viverrini infection-endemic villages in Kandal province are all located 2–8 km from the main stream of the Bassak River. However, residents generally catch freshwater fish for raw consumption from lakes and/or the rainy season or year-round ponds that form near their villages instead of from the Bassak. In addition, villages in Kampong Thom province are not near the Mekong River or the major tributaries of the Bassak or Tonle Sap River. Two *O. viverrini* infection-endemic villages found in 2012 were located about 80 km from the Mekong and about 45 km from the Tonle Sap, although there were some streams and ponds near these villages where residents caught freshwater fish.

3.4. *Adult worms of* O. viverrini *from feces of patients in an endemic village in Kandal*

The complete bodies of 16 adult worms were gathered from feces samples from three persons who showed high egg-positive rates in one of the *O. viverrini* infection-endemic villages in Kandal province. These were determined morphologically to be *O. viverrini*, confirming the existence of opisthorchiasis in this village.

3.5. *Living conditions and customs related to the* O. viverrini *infection cycle*

3.5.1. Raw fish consumption

Approximately 70% to 100% of residents of *O. viverrini* infection-endemic areas eat raw freshwater fish. "*Plia trai chau*" is the most common dish (Fig. 3A). The same dish is called by different names in other villages, but the recipes are nearly identical. Other dishes containing raw fish are "*mam*," "*ph'ouk*," and "*plohok*" (Fig. 3B). "*Plia trai chau*" is very similar to the raw fish dish called "*koi pla*," and "*plohok*" is similar to *"pla ra,"* which are consumed in Thailand and Lao PDR [10,13]. Cyprinid fish, mainly of the same species, are used for these dishes; "*plia trai chau*" is prepared from fresh fish caught near residents' homes especially during the flood season when fish are found and caught everywhere near the homes of residents.

The main reason for consuming raw fish is not a lack of fuel for cooking, but because people enjoy eating the traditional food. This is a long-standing custom dating back to the pre-Pol Pot period, when many people starved due to famine.

3.5.2. Covered toilets

The number of residents making use of covered toilets ranged from 0% to about 30% in most target villages. The usual practice is to defecate in a rice field or bush near homes, easily contaminating water sources by eggs in feces and facilitating the infection of snails.

4. Discussion

4.1. *Endemicity of* O. viverrini *in four provinces studied*

The eggs, cercariae, metacercariae, and adult worms found in endemic areas were morphologically typical of *O. viverrini*. The eggs, cercariae, and metacercariae were identified as *O. viverrini* using PCR and were shown to infect experimental hamsters.

In five endemic villages in Kampong Cham and Kampong Thom, stool samples of some residents included *O. viverrini* eggs as confirmed in PCR analysis. In four endemic villages in Takaev and Kandal provinces, residents use same fishing spots where both cercariae and metacercariae from intermediate hosts were found. Adult worms were found in villagers who had high egg-positive rates from one of these villages. The infection cycle of *O. viverrini* was therefore established in the area.

In another six endemic villages located in Kampong Cham province, cercariae and/or metacercariae were found in intermediate hosts in all except for one fishing spots, although it is likely that intermediate hosts occurred there as well. To confirm this, more research is needed to establish the infection cycle. In Prey Veng province, only metacercariae were found in fish. Residents of some villages typically consume raw freshwater fish, although examinations of stool samples from two villages were negative using the Kato-Katz method.

4.2 *Difficulty in determining* O. viverrini *infection-endemic areas for research in Cambodia*

It is difficult to pinpoint *O. viverrini*-endemic areas in Cambodia by conducting field surveys. For example, after locating two endemic villages in Takaev province, 28 villages in 13 communes located downstream along the same river basin of several large tributaries of the Bassak River and small streams from those tributaries were investigated. Although numerous residents consumed raw fish in those downstream villages, *O. viverrini* infection was not endemic. An egg-positive rate of 0% was found in 24 of those villages and of only 4.8% and 5.5% in another two. In addition, even in villages where homes were built along rivers 3–5 m wide and residents consumed raw freshwater fish caught from them, *O. viverrini* infection was not endemic (Fig. 2A). In Kandal province, there were only two *O. viverrini* infection-endemic villages where residents caught freshwater fish using the same fishing spots as the endemic villages in neighboring Takaev province. In addition, even in villages where homes were near the Bassak River and residents consumed raw freshwater fish caught from the river, the egg-positive rates were 1.2% and 2.8%. *O. viverrini* infection was not endemic in these villages near the Bassak (Fig. 2A). However, those villages were located along the Bassak River or its tributaries within a 5-km radius from endemic villages in Takaev and Kandal provinces (Fig. 2A).

The results in Kampong Chan province (Fig. 2B) showed that endemic villages and none endemic villages coexisted within a distance of only 9 km, where the raw freshwater fish consumption rates were very high. For example, village X had a 27.9% egg-positive rate and 100% fish consumption and was located next to village Y with a 0% egg-positive rate and 96.9% fish consumption and also next to village Z with a 5.7% egg-positive rate and 96.4% fish consumption. If villages with 0% or 5.7% egg-positive rates had been selected for stool sample examination, the results could be misinterpreted as indicating that these areas were not *O. viverrini* infection endemic, although highly endemic villages (with infection rates of 27.9% or 29.7%) were located adjacent to those with lower rates.

Therefore, when assessing the data, the following assumptions should be made: when fecal specimens from selected individuals in a village in one commune or district are examined, the average egg-positive rate might be not very high. If villages in which the egg-positive rate was high were included, that information could easily be masked in the statistical analysis. This may be why only a few *O. viverrini* infection-endemic areas have been found in Cambodia so far. More careful planning may be necessary when conducting surveys in Cambodia compared with Thailand or Lao PDR to avoid overlooking endemic areas. Environmental factors in many areas also contribute to the difficulties in confirming *O. viverrini* infection-endemic areas.

4.3. *Comparison of results from Cambodia and from Thailand or Lao PDR*

To the best of our knowledge, no previously published report on opisthorchiasis in Thailand and Lao PDR described details of prevalence and location similar to those described here. In Thailand, Kaewpitoon et al. summarized the prevalence of *O. viverrini* infection at the regional and provincial levels [3,6]. Sriamporn et al. reported that the crude proportion of *O. viverrini* infection in Khon Kaen province was 2.1–70.8% (1990–2001) at the district level [20]. Tesana also described it at the regional level [21]. Since research on *O. viverrini* infection in Cambodia has started only recently, the prevalence at the regional, provincial, and district levels are unknown. It is therefore difficult to compare our results with those reported in Thailand.

Somphou et al. reported that *O. viverrini* infection rates in 13 villages in one district in Lao PDR ranged from 14.3% to 79.9% [22]. The villages were located about 8–12 km from each other. Kobayashi et al. found positive rates of 52.9% and 55.0% in two rural communities and of 60.7% in an urban community in Lao PDR [23]. Forrer et al. conducted an epidemiological survey in 51villages in Champasack province, southern Lao PDR close to Cambodia; the average prevalence rate in the province was 61.1% (range 0–95.1%). Almost three-quarters of villages surveyed (37/51) had prevalence rates above 50%. [11]. Although the percentage of rate of *O. viverrini* infection endemic villages was higher than that in Cambodia found in the present study, the results of Champasack province included the prevalence rates of 0% and over 90%. Endemic and none endemic villages are therefore present in a single province. It is possible that the situation is similar in Lao PDR and Cambodia, but further investigations are needed to confirm this.

4.4. *Need for additional surveys of* O. viverrini *infection in Cambodia*

O. viverrini infection research has been carried out in several Cambodian provinces [18,19,24,25]. The CNM conducted a food-borne trematode survey in five provinces during 2006–2009, supported by the Asian Development Bank, and found egg-positive rates ranging from 0% to 16.0%, whereas only one of the survey areas in Takaev province had a 16.0% rate. An endemic area was also found in Kratie province [19]. Thus, there is evidence of *O. viverrini* infection in very limited areas, and the status of infection in

Cambodia remains unclear.

4.5. *Preventive measures against* O. viverrini *infection*

Most residents in *O. viverrini* infection-endemic areas habitually consume raw freshwater fish. This is a long-held traditional custom that will not be easy to change. After the present survey, feedback meetings were conducted in endemic villages that included health education on *O. viverrini* infection. During those meetings, villagers, CNM staff, and the research team held joint discussions, and while many understood the problem and *O. viverrini* infection cycle, solutions appeared difficult.

5. Conclusions

There are endemic areas of *O. viverrini* infection in several Cambodian provinces, although the data presented here are only partial results for the country as a whole. It is therefore necessary to conduct a nationwide baseline survey. Our study indicated that complex environmental factors might be involved in the distribution of *O. viverrini* infection-endemic areas. Many problems remain to be resolved regarding the status of *O. viverrini* infection in Cambodia.

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References

[1] WHO. Control of foodborne trematode infections. WHO Technical Report Series, 849; 1995.

[2] Jongsuksuntigul P, Imsomboon T. Opisthorchiasis control in Thailand. Acta Trop 2003;88:229–32.

[3] Kaewpitoon N, Kaewpitoon SJ, Pengsaa P. Opisthorchiasis in Thailand: Review and current status. World J Gastroenterol 2008;14:2297–302.

[4] Andrews RH, Sithithaworn P, Petney TN. *Opisthorchis viverrini*: an underestimated parasite in world health. Trends Parasitol 2008;24:497–501.

[5] Kaewkes S. Taxonomy and biology of liver flukes. Acta Trop 2003;88:177–86.

[6] Kaewpitoon N, Kaewpitoon SJ, Pengsaa P, Sripa B. *Opisthorchis viverrini*: the carcinogenic human liver fluke. World J Gastroenterol 2008;14:666–74.

[7] Suttiprapa S, Mulvenna J, Huong NT, Pearson MS, Brindley PJ, Laha T, et al. Ov-APR-1, an aspartic protease from the carcinogenic liver fluke, *Opisthorchis viverrini*: functional expression, immunolocalization and subsite specificity. Int J Biochem Cell Biol 2009;41:1148–56.

[8] Sripa B, Kaewkes S, Intapan PM, Maleewong W, Brindley PJ. Food-borne trematodiases in Southeast Asia: epidemiology, pathology, clinical manifestation and control. Adv Parasitol 2010;72:305–50.

[9] Janwan P, Intapan PM, Thanchomnang T, Lulitanond V, Anamnart W, Maleewong W.

Rapid detection of *Opisthorchis viverrini* and *Strongyloides stercoralis* in human fecal samples using a duplex real-time PCR and melting curve analysis. Parasitol Res 2011;109:1593–601.

[10] Grundy-Warr C, Andrews RH, Sithithaworn P, Petney TN, Sripa B, Laithavewat L, et al. Raw attitudes, wetland cultures, life-cycles: socio-cultural dynamics relating to *Opisthorchis viverrini* in the Mekong Basin. Parasitol Int 2012;61:65–70.

[11] Forrer A, Sayasone S, Vounatsou P, Vonghachack Y, Bouakhasith D, Vogt S, et al. Spatial distribution of, and risk factors for, *Opisthorchis viverrini* infection in southern Lao PDR. PLoS Negl Trop Dis 2012;6:e1481. Epub 2012 Feb 14.

[12] Sripa B, Brindley PJ, Mulvenna J, Laha T, Smout MJ, Mairiang E, et al. The tumorigenic liver fluke *Opisthorchis viverrini*—multiple pathways to cancer. Trends Parasitol 2012;28:395–407.

[13] Sithithaworn P, Andrews RH, Nguyen VD, Wongsaroj T. The current status of opisthorchiasis and clonorchiasis in the Mekong Basin. Parasitol Int 2012;61:10–6.

[14] Lee KJ, Bae YT, Kim DH, Deung YK, Ryang YS, Kim HJ, et al. Status of intestinal parasite infections among primary school children in Kampongcham, Cambodia. Korean J Parasitol 2002;40:153–5.

[15] Sato M, Pongvongsa T, Sanguankiat S, Yoonuan T, Dekumyoy P, Kalambaheti T, et al. Copro-DNA diagnosis of *Opisthorchis viverrini* and *Haplorchis taichui* infection in an endemic area of Lao PDR. Southeast Asian J Trop Med Publ Health 2010;41:28–35.

[16] Sato M, Thaenkham U, Dekumyoy P, Waikagul J. Discrimination of *O. viverrini*, *C. sinensis*, *H. pumilio* and *H. taichui* using nuclear DNA-based PCR targeting ribosomal DNA ITS regions. Acta Trop 2009;109:81–3. Epub 2008 Oct 4.

[17] Lovis L, Mak TK, Phongluxa K, Soukhathammavong P, Sayasone S, Akkhavong K, et al. PCR diagnosis of *Opisthorchis viverrini* and *Haplorchis taichui* infections in a Lao community in an area of endemicity and comparison of diagnostic methods for parasitological field surveys. J Clin Microbiol 2009;47:1517–23.

[18] Sohn WM, Yong TS, Eom KS, Pyo KH, Lee MY, Lim H, et al. Prevalence of *Opisthorchis viverrini* infection in humans and fish in Kratie Province, Cambodia. Acta Trop 2012;124:215–20.

[19] National Centre for Parasitology, Entomology and Malaria Control (CNM). Control of neglected tropical diseases in Cambodia. Integrated national plan of action focused on diseases controlled and eliminated by preventive chemotherapy, 2012–2015. Phnom Penh: CNM; 2011.

[20] Sriamporn S, Pisani P, Pipitgool V, Suwanrungruang K, Kamsa-ard S, Parking MD. Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. Tropical Med Int Health 2004;9:588–94.

[21] Tesana S. Opisthorchiasis in Thailand. In: Naoki A, Jong-Yil C, Yukifumi N, Yuzo T, editors. Food-borne helminthiasis in Asia. Asian parasitology, vol. 1. Chiba, Japan: AAA Committee・Federation of Asian Parasitologists; 2005, p. 113–24.

[22] Sayasone S, Odermatt P, Phoumindr N, Vongsaravane X, Sensombath V, Phetsouvanh R, et al. Epidemiology of *Opisthorchis viverrini* in a rural district of southern Lao PDR. Trans R Soc Trop Med Hyg 2007;101:40–7.

[23] Kobayashi J, Vannachone B, Sato Y, Manivong K, Nambanya S, Inthakone S. An epidemiological study on *Opisthorchis viverrini* infection in Lao villages. Southeast Asian J Trop Med Publ Health 2000;31:128–32.

[24] Touch S, Komalamisra C, Radomyos P, Waikagul J. Discovery of *Opisthorchis viverrini* metacercariae in freshwater fish in southern Cambodia. Acta Trop 2009;111:108–13. Epub 2009 Mar 21.

[25] Yong TS, Shin EH, Chai JY, Sohn WM, Eom KS, Lee DM, et al. High prevalence of *Opisthorchis viverrini* infection in a riparian population in Takaev Province, Cambodia. Korean J Parasitol 2012;50:173–6.

Figures and Tables

Fig.1 Map of surveyed areas in Cambodia. Target provinces and endemic districts are shown.

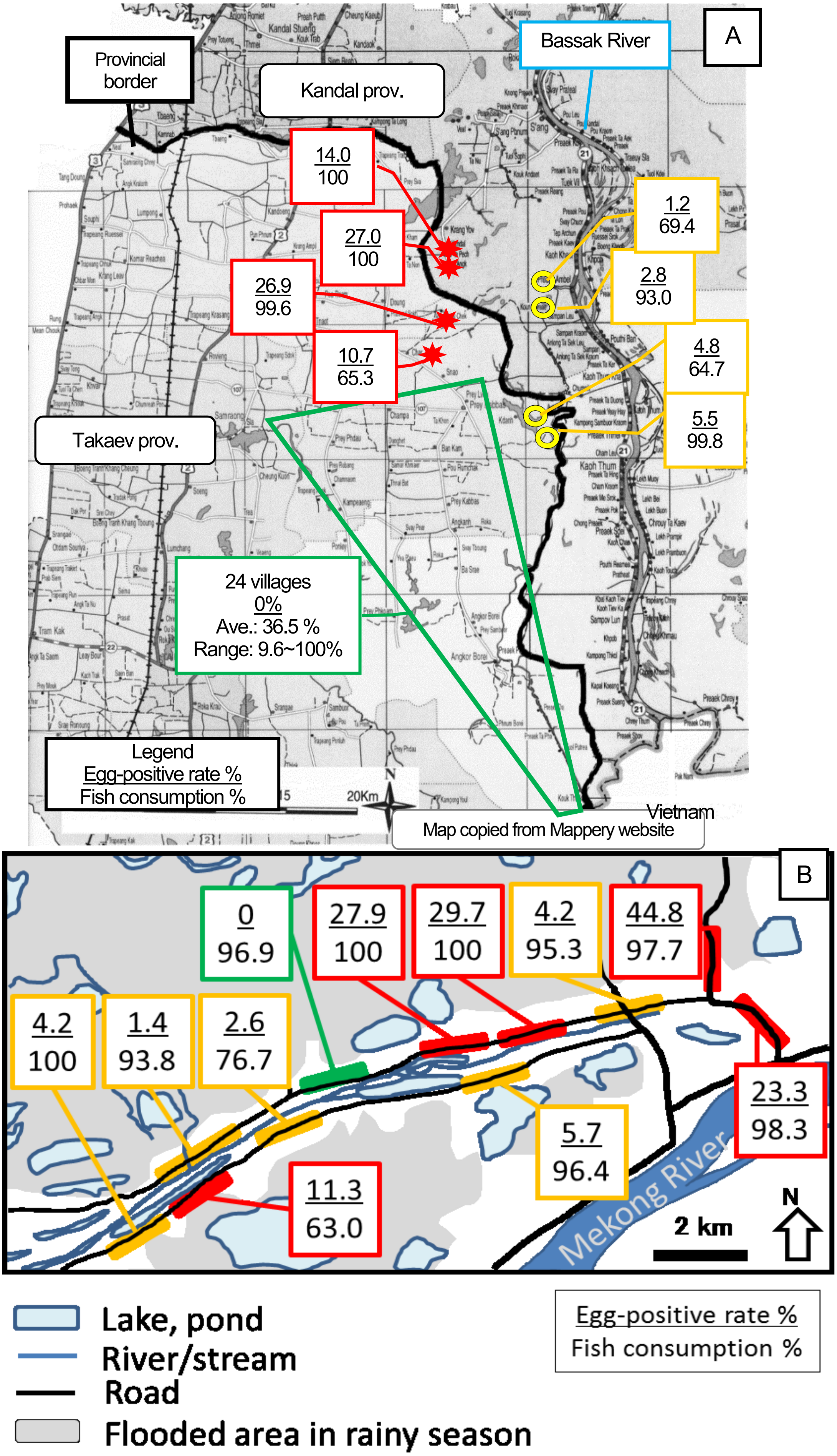

Fig.2 Egg-positive rates and location of villages in A)Kandal and Takaev provinces, along the banks of the Bassak River, and B) Sour Kong and Riay Pai communes, Kang Meas district, Kampong Cham province. Red,>10%; Yellow,<10%; Green, 0%

Fig.3. Typical dishes made from raw fresh water fish in Cambodia. A, *Plia trai chau* prepared by a family in Takaev province. B, *Plohok* stored in the home of residents for 6 months. C, D, Preparation method of *plia trai chau.* Minched freshwater fish, herbs such as basil and green tamarind, and various seasonings such as soy sauce and lime juice are mixed. For *plia trai chau*,fish less than about 10cm in length are used.

Table 1. Surveillance times and areas.

Time[a]	Province	District	No. of communes	No. of villages	No. of *O. viverrini* positive villages (>10%)		No. of stool samples examined (positive)	
Mar 2006	Takeo	Prey Kabas	1	2	2	(2)	807	(352)
Nov 2006–Dec 2007	Takeo	Prey Kabas	7	16	2	(0)	10,422	(21)
	Takeo	Angkor Borei	6	12	0	(0)		(0)
Apr–May 2008	Kandal	S'ang	1	2	2	(2)	797	(161)
	Kandal	Preaek Ambel	1	2	2	(0)	615	(11)
Aug–Nov 2010	Kampong Cham	Kampong Cham	1	1	1	(1)	379	(108)
	Kampong Cham	Kang Meas	2	11	10	(5)	1,358	(322)
	Prey Veng	Ba Phnom	1	1	0	(0)	643	(0)
Jun–Jul 2012	Kampong Cham	Kang Meas	1	1	1	(1)	105	(22)
	Kampong Cham	Kampong Siem	1	1	1	(1)	126	(82)
	Kampong Cham	Srei Santhor	2	3	2	(1)	427	(53)
	Kampong Thom	Baray	1	1	1	(1)	183	(89)
	Kampong Thom	Santuk	1	1	1	(1)	104	(11)
	Prey Veng	Ba Phnom	1	1	0	(0)	116	(0)
Total no.	5 provinces	11 districts	27	55	26	(15)	16,082	(1,232)

[a]Intermediate host surveillance was conducted in endemic areas during additional time periods.

Table 2. Results of stool sample examination in endemic areas.

Province	District	Commune	Village record	Target family	No. of stool specimens	Egg-positive rate (%)[a]	Raw fish consumption (%)[b]
Takeo	Prey Kabas	Char	Ch1	All	535	26.9	99.6
			Ch2	All	272	10.7	65.3
Kandal	S'ang	Krang Yov	KY1	All	415	14.0	100
			KY2	All	382	27.0	100
Kampong Cham	Kang Meas	Sour Kong	SK1	All	420	44.8	97.7
			SK2	All	305	23.3	98.3
		Reay Pay	RP1	Quasi-rando	74	29.7	100
			RP2	Quasi-rando	68	27.9	100
			RP3	Quasi-rando	71	11.3	63.0
		Preaek Krabau	PK1	Quasi-rando	105	21.0	77.0
	Kampong Cham	Sambuor Meas	SM1	All	360	29.2	81.1
	Kampong Siem	Srak	Sr1	Quasi-rando	126	65.1	93.5
	Srey Sentho	Banteay	Bt1	Quasi-rando	165	28.5	89.1
Kampong Thom	Baray	Tanaot Chum	TC1	Quasi-rando	183	48.6	75.9
	Santuk	Ti Pou	TP1	Quasi-rando	104	10.6	67.4
4 provinces	8 districts	10 communes	15 villages	-	3,585	27.8	-

[a]Egg-positive rate includes eggs of *O. viverrini* and other small trematode eggs. Rates >10% are listed here.
[b]Percentage of residents who habitually consume raw freshwater fish.

Table 3. Egg positive rate in feces by age: one of the examples in endemic villages (by Kato-Katz technique).

Age (y)	Samples		Ov positive[a]	
	No.	(%)	No.	(%)
<10	3	(0.6)	0	
<20	7	(1.3)	0	
<30	124	(23.2)	28	(22.6)
<40	137	(25.6)	39	(28.5)
<50	98	(18.3)	34	(34.7)
<60	77	(14.4)	22	(28.6)
<70	40	(7.5)	10	(25.0)
>70	18	(3.4)	4	(22.2)
NA	31	(5.8)	7	(22.6)
Total	535	(100)	144	(26.9)

Table 4. EPG and egg-positive rate in feces by gender in one endemic village (by Kato-Katz technique).

	Male		Female		NA[a]		Total	
EPG	No.	(%)	No.	(%)	No.	(%)	No.	(%)
<100	39	(27.1)	39	(27.1)	1	(0.7)	79	(54.9)
<500	25	(17.4)	27	(18.8)	0	(0)	52	(36.1)
<1000	4	(2.8)	3	(2.1)	2	(1.4)	9	(6.3)
<2000	0	(0)	2	(1.4)	0	(0)	2	(1.4)
>2000	1	(0.7)	1	(0.7)	0	(0)	2	(1.4)
Total	69	(47.9)	72	(50.0)	3	(2.1)	144	(100)

[a]Gender was not specified in the questionnaire.

Table 5. Helminths found in fecal samples in two villages in the endemic area (by Kato-Katz technique).

	Village A		Village B	
	Total no.	(%)	Total no.	(%)
No. of samples examined	535	(86.3)	272	(82.4)
No. of egg-positive parasites	268	(50.1)	84	(30.9)
2 or 3 types of worm	72	(13.5)	13	(4.8)
Helminth				
Ascaris lumbricoides	3	(0.6)	2	(0.7)
Trichuris trichiura	4	(0.7)	1	(0.4)
Hookworm	187	(35.0)	63	(23.2)
Opisthorchis viverrini	144	(26.9)	29	(10.7)
Vampirolepis nana	4	(0.7)	2	(0.7)
Taenia sp.	0	(0)	1	(0.4)
Enterobius vermicularis	1	(0.2)	0	(0)

Table 6. List of second intermediate hosts infected by metacercariae of *O. viverrini*.

Scientific name	Local name	Hamsters infected	PCR	Province
Osteochilus hasseltii	Kros		+	Kampong Cham
Puntius brevis	Sroka Kdam		+	
Cyclocheilichthys repasson	Sroka Kdam	+	+	
Hampala dispar	Khman [a]	+	+	Takaev
	Khman		+	Kampong Cham
Labeo chrysophekadion	Krek		+	
Labiobarbus siamensis	Khnang Veng		+	
	Real		+	
	Kros/Arch kok		+	Prey Veng

[a] *H. dispar* or *H. macrolepidota*.

Appendix

- Additional figures
- Abstract and highlights of the article in the journal of " Parasitology International"

Fig. AP1

O. viverrini egg and adult worm from feces of patients. Panel A depicts a morphologically typical egg from a patient in Takaev province. Panel B depicts an adult worm from a patient in Kandal province.

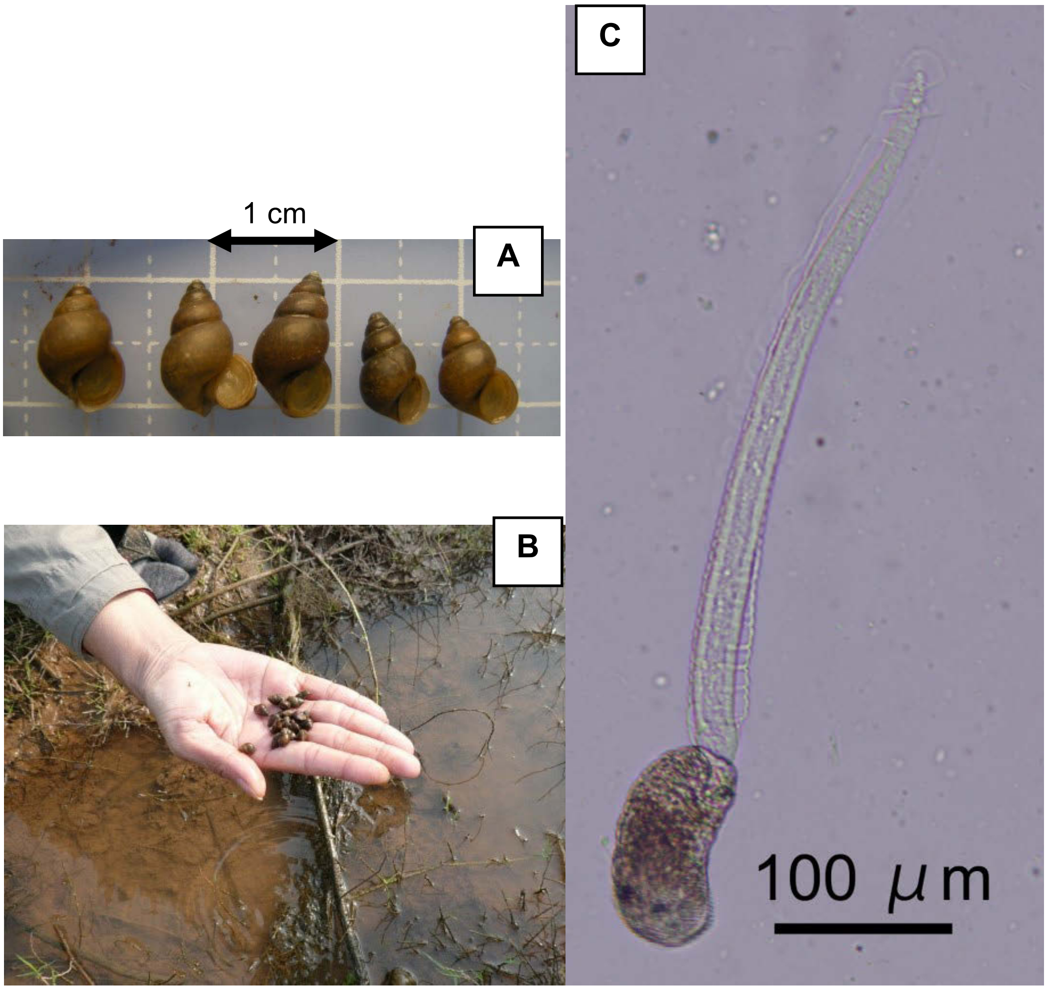

Fig. AP2

Panel A: First intermediate host, *Bithynia siamensis siamensis,* collected in Kampong Cham province. Panel B: Snails depicted in Panel A collected from a pond near the endemic village. Panel C: Cercaria from these snails.

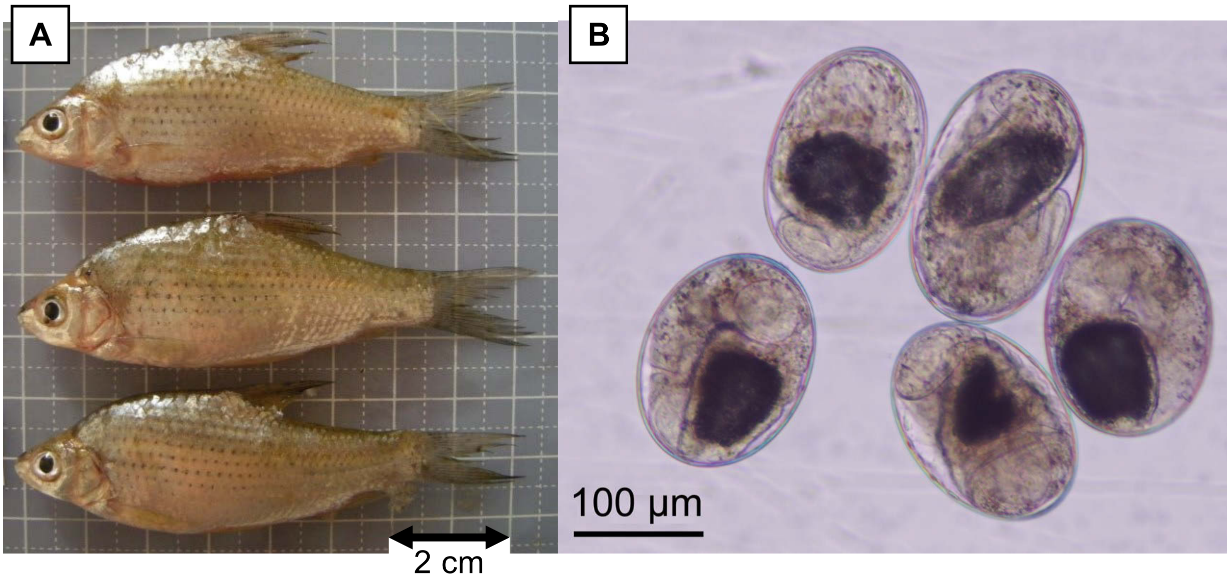

Fig. AP3

Second intermediate hosts and metacercariae from epidemic areas.

Panel A: *Cyclocheilichthys repasson*. Panel B: Metacercariae collected from these fish have typical morphology.

Abstract

Background: Opisthorchiasis is endemic in Thailand and Lao People's Democratic Republic and constitutes a major public health problem throughout the Mekong Basin. Although Cambodia is located in the Mekong Basin, the status of *O. viverrini* infection in that country was not previously clarified. This research was conducted to document the extent and distribution of *O. viverrini* infection in Cambodia.

Methods: Surveillance was conducted in 55 villages in five Cambodian provinces. Research tools included stool examination using the Kato-Katz thick-smear technique, identification of intermediate hosts, and interviews covering factors related to *O. viverrini* infection. Some larvae and egg-positive stool samples were examined using PCR to detect *O. viverrini* DNA.

Results: A total of 16,082 stool samples from the 55 villages were examined, of which 1,232 were egg positive. In 15 villages with egg-positive rates of greater than 10%, eggs were found in 998 of 3,585 stool samples, for an egg-positive rate of 27.8%. PCR analysis showed that 30 of 33 samples were positive for *O. viverrini* DNA from five villages in Kampong Cham and Kampong Thom provinces. The first intermediate host *Bithynia siamensis siamensis* was identified in the target areas of Takaev, Kandal, and Kampong Cham provinces. Cercariae were identified morphologically as *O. viverrini* and some were confirmed using PCR. Metacercariae of *O. viverrini* were identified by morphologic observations, animal experiments, or PCR in six species of fish in the target areas.

Discussion and Conclusions: Four Cambodian provinces were identified as endemic areas of *O. viverrini* infection. Careful planning is necessary for effective field surveys, because complex environmental factors are involved in the distribution of *O. viverrini* infection-endemic areas in Cambodia. Many problems remain to be resolved regarding the status of *O. viverrini* infection in Cambodia, and a nationwide baseline survey is necessary.

Highlights:

1. *Opisthorchis viverrini* infection was endemic in four provinces of Cambodia.
2. Life cycle components of the *O. viverrini* infection cycle were identified in endemic areas.
3. It is difficult to pinpoint endemic areas due to the environmental factors involved.