Studies on modification of miracidium hatching technique (MHT) for preparation of single-genome DNA for use in population structure analysis of *Schistosoma japonicum* and development of ELISA for diagnosis of *S. mekongi* infection in humans

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日本住血吸虫の集団構造解析で使用するシングルゲノム DNA の調整を目的としたミラシジウムふ化法の改良およびメコン住血吸虫症の患者の診断を目的とした ELISA の開発に関する研究

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Abbreviations

DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assays
IPTG	Isopropyl-thio-β-D-galactoside
K	Kappa value
MDA	Mass drug administration
MHT	Miracidium hatching technique
NPV	Negative predictive value
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPV	Positive predictive value
rSjTPx-1	Recombinant antigen *Schistosoma mekongi* thioredoxin peroxidase 1
rSmTPx-1	Recombinant antigen *Schistosoma japonicum* thioredoxin peroxidase 1
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sm	*Schistosoma mekongi*
SmTPx-1	*Schistosoma mekongi* thioredoxin peroxidase 1

Sma	*Schistosoma mansoni*
SmaTPx-1	*Schistosoma mansoni* thioredoxin peroxidase 1
Sj	*Schistosoma japonicum*
SjTPx-1	*Schistosoma japonicum* thioredoxin peroxidase 1
Sj7TR	*Schistosoma japonicum* tandem repeat
SjPrx-4	*Schistosoma japonicum* peroxiredoxin-4
SjPCS	*Schistosoma japonicum* phytochelatin synthase
SjLP40	*Schistosoma japonicum*-like protein 40
SjSAP4	*Schistosoma japonicum* saposin-like protein 4
SjSAP5	*Schistosoma japonicum* saposin-like protein 5
Sj23LHD	*Schistosoma japonicum* 23 large hydrophilic domain
TPx	Thioredoxin peroxidase
T-PBS	Tween 20-phosphate-buffered saline
WHO	World Health Organization

Unit abbreviations

bp	: Base pair
°C	: degree Celsius
hr	: hour
kDa	: kilodalton
µg	: microgram
µl	: microliter
µm	: micrometer
µM	: micromolar
mg	: milligram
mL	: milliliter
mM	: millimolar
min	: minute
ng	: nanogram
%	: percentage
sec	: second

General introduction

1. Schistosomiasis

Schistosomiasis, also known as bilharzia, is a serious public health disease caused by several species of blood flukes belonging to the genus *Schistosoma.* It is endemic in tropical and sub-tropical countries including parts of South America, the Middle East, Sub-Saharan Africa, and Southeast Asia and affects more than 240 million people. This devastating parasitic disease ranks second only to malaria in terms of negative socioeconomic impact (Gundamaraju, 2014). Humans and other definitive hosts generally become infected with schistosomes following exposure in water contaminated with cercariae, which is an infective stage of the parasite. Activities such as routine agricultural work in rice paddies or continuous exposure of the skin in contaminated water increase the likelihood of infection (Evan Secor, 2014).

Human schistosomiasis is mainly caused by five species of schistosomes subdivided into urinary schistosomiasis caused by *Schistosoma haematobium* and intestinal schistosomiasis caused by *S. mansoni*, *S. intercalatum*, *S. japonicum*, and *S. mekongi* according to the habitat of the adult worms (Vonghachack et al., 2017). The symptoms of schistosomiasis are not caused by the adult worm. Instead, the host reacts to eggs deposited in the host tissues by causing granulomatous inflammation and fibrosis (Wu & Halim, 2000). The distribution of each blood fluke species depends on the availability of their respective intermediate snail hosts. Aquatic freshwater snails of the genus *Biomphalaria* serve as the intermediate host of *S. mansoni*, whereas *Bulinus* spp. is the intermediate host of *S. haematobium* and *S. intercalatum* (Ugbomoiko et al., 2022), and *Oncomelania* spp. is the intermediate host of *S. japonicum.* However, *S. mekongi*, a species closely related to *S. japonicum*, uses *Neotricula* spp. as its intermediate host (Attwood et al., 2008). *S. mansoni*

is mainly distributed in Africa, the Middle East, and South America (Colley et al., 2014). The distribution of *S. haematobium* is generally similar to that of *S. mansoni*, but this species is not endemic in South America. *S. intercalatum* is found in the Middle and West Africa.

Asian zoonotic schistosomiasis caused by *S. japonicum,* considered to be the most virulent species among the schistosome species that can infect humans based on the larger number of eggs laid by the female per day, is endemic in the Philippines, China, and parts of Indonesia (Hinz et al., 2017; Weerakoon et al., 2015). The severe symptoms of the disease caused by host reactions against eggs trapped in host tissues lead to inflammatory and obstructive pathologies (McManus et al., 2018). The closely related species *S. mekongi* is found in limited areas along the Mekong river particularly at the lower basin of Northern Cambodia and Southern Laos (Nickel et al., 2015). Similar to *S. japonicum*, *S. mekongi* is zoonotic and can infect a wide range of domestic animals. Furthermore, *S. mekongi* poses a risk to foreign visitors who might be infected by the parasite in its endemic areas, making it one of the significant concerns for travel medicine in Southeast Asia (Clerinx & Van Gompel, 2011).

2. *Schistosoma japonicum*

Schistosoma japonicum is a blood fluke that causes human schistosomiasis in China, the Philippines, and Indonesia, with 40 million people at risk of the infection in China alone (Shrivastava et al., 2005). Among schistosome species, *S. japonicum* is considered to be the most virulent species due to the larger number of eggs it can produce per day when compared to other schistosome species, thereby causing more damage and severe symptoms in the patients (He et al., 2001).

The life cycle of *S. japonicum* is complex and requires two hosts: an aquatic snail (*Oncomelania* sp.) as the intermediate host and the definitive mammalian host in which the parasite reaches sexual maturity and produces eggs. Asexual reproduction takes place in the snails through clonal replication, whereas sexual reproduction in the form of egg laying occurs in the mammalian host (Nelwan, 2019). Furthermore, the fact that the parasite can infect up to 40 different species of mammalian hosts, in which water buffaloes and dogs play an important role as reservoir hosts, significantly increases the chance of disease transmission to humans. The life cycle begins with an adult female worm shedding her eggs, which are excreted with feces of the definitive host into the external environment. Under optimal conditions, the eggs hatch and release free-swimming miracidia that penetrate and invade tissue of *Oncomelania hupensis* freshwater snails for infection (McManus et al., 2010). Then, the miracidia develop into sporocysts and, eventually, into cercariae (infective stage of the parasite). After that, cercariae are released from the *Oncomelania* snails as free-swimming larvae. Transmission takes place when the specific definitive hosts come in contact with cercariae-contaminated freshwater. Cercariae penetrate the skin of the definitive host and shed their forked tail to form the next stage of the life cycle, the schistosomulae. Schistosomulae migrate through the bloodstream via organs in the respiratory and circulatory systems and transform into adult dioecious worms. Adult male and female worms mate and form a pair in the liver and migrate into the superior mesenteric veins of the small intestines where the female worm lays her eggs (Fig. 1).

Diversities in the potential reservoir hosts involved in the life cycle provide substantial implications and complications for successful disease control and alter the dynamics of the infection (Wang et al., 2006). Therefore, knowledge regarding epidemiology and transmission dynamics among reservoir hosts is crucial for long-term prevention and effective control of the disease in humans

3. *Schistosoma mekongi*

Schistosoma mekongi causes schistosomiasis mekongi, which is prevalent in the communities along the Mekong River basin in Cambodia and Lao PDR (Muth et al., 2010). Over 120,000 people are estimated to be at risk of *S. mekongi* infection in the endemic areas (Urbani et al., 2002). This parasite species is closely related to *S. japonicum* based on evidence from morphological and molecular studies (McManus et al., 2009). The first human case of *S. mekongi* infection was described in 1957 from the Lao PDR and 10 years later from Cambodia (Dupont et al., 1957; Schneider et al., 1975). Earlier reports described the parasite infection as a *S. japonicum*-like infection due to similarities in the clinical symptoms and the presence of eggs in stool that are morphologically similar to those of *S. japonicum* until it became clear and was confirmed that *S. mekongi* was appropriately different from *S. japonicum* (Voge et al., 1978).

The life cycle of *S. mekongi* requires the gamma strain of the aquatic snail *Neotricula aperta,* which is a sole natural intermediate host commonly found along the Mekong River basin (Ohmae et al., 2004). Dogs and pigs play a role as reservoir hosts in the life cycle of the parasite species (Strandgaard et al., 2001). Schistosomiasis mekongi is endemic in limited areas, and therefore it was thought initially that disease control would not be difficult under the Mass Drug Administration (MDA) program with praziquantel, which is the cornerstone medication of the national control program. However, the transmission of schistosomiasis mekongi continues to this day.

For successful control of schistosomiasis, introduction of an accurate diagnostic method is crucial. The diagnosis of schistosomiasis mekongi at present depends entirely on the collection of stool to detect parasite eggs by a conventional parasitological diagnostic method, the Kato-Katz stool examination. Although this test is highly specific, it suffers decreased sensitivity due to the recent success of the MDA program. Therefore, a diagnosis

with high sensitivity and specificity that can replace the Kato-Katz stool examination is urgently needed to assess the epidemiology of schistosomiasis mekongi and to more successfully implement the MDA program.

4. Diagnosis of schistosomiasis

The diagnosis of intestinal schistosomiasis generally includes parasitological examination, molecular diagnosis, and serological diagnosis (Weerakoon et al., 2015). In *S. japonicum* and *S. mekongi* endemic areas, the diagnosis often depend on the Kato-Katz stool examination, in which the parasite egg is detected in stool specimens under microscopic observation (Doenhoff et al., 2004). This method can detect the parasite egg itself with high specificity and is simple as it requires no complex equipment other than a microscope. In addition, it is recommended by the World Health Organization (WHO) as the gold standard test for the diagnosis of schistosome infection. However, this technique has a problem due to its low diagnostic sensitivity, particularly in the areas with low prevalence and decreased intensity of the infection, which may lead to underestimation of disease prevalence (McManus et al., 2018). In addition, it is labor intensive and requires trained personnel who can identify the parasite egg. It is also not optimal for large-scale screening. The sensitivity of this technique can be improved by increasing the number of stool samples to be examined. However, it is labor intensive and may not be applicable in the field (Ajibola et al., 2018; Bärenbold et al., 2017). Several applications have been created to increase the sensitivity of Kato-Katz stool examination. The formalin-ether concentration and sedimentation technique, which can deal with a larger quantity of stool sample, and the miracidium hatching technique (MHT) using the positive phototropic behavior of the miracidium stage are useful for field diagnosis (Jurberg et al., 2008; Zhu et al., 2014).

Polymerase chain reaction (PCR) or PCR-based techniques (real-time quantitative PCR, nested PCR, and multiplex PCR) are widely used for the diagnosis of parasitic infections. They are known to have higher sensitivity and specificity compared to parasitological examinations (Weerakoon et al., 2015) and have been used in detecting *Schistosoma* spp. infection in their intermediate snail hosts and definitive mammalian hosts (Fung et al., 2012; Hamburger et al., 1987; Zhang et al., 2017). These molecular detection methods require expensive equipment and reagents. It is also recommended that the samples to be tested should be kept under a cold chain environment. Therfore, these methods are not suitable in field studies with large numbers of samples (Lier et al., 2009).

Serological diagnostic methods are the means of detecting antibody against the parasite antigen or the antigen itself in a serum sample of a patient. They are known to have higher sensitivity and to be less time-consuming compared to parasitological examinations. Several serological diagnostic methods have been evaluated for detecting *Schistosoma* spp. infection in humans and animals. They are the indirect hemagglutinatioin test, indirect immunofluorescence test, and enzyme-linked immumosorbent assay (ELISA). ELISA with a crude antigen such as soluble egg antigen (SEA) and the soluble adult worm antigen preparation (SWAP) have been used for the diagnosis of schistosomiasis by detectiing antibodies against them with high sensitivity and specificity. However, preparation of these antigens at a large scale under appropriate quality control for application in nationwide monitoring is difficult. In addition, these antigens have a major problem regarding cross-reactions with antibodies against other helminthic infections (Kirinoki et al., 2011). In contrast, several studies have proved the high sensitivity and specificity of ELISA with recombinant antigens in detecting *Schistosoma* spp. infection in humans and animals (Angeles et al., 2012a; Dang-Trinh et al., 2020; Hinz et al., 2017; McLaren et al., 1981).

5. Disease control

To date, no vaccine is available for schistosomiasis. Therefore, MDA with praziquantel has been the central strategy in the disease-endemic countries to control the morbidity of schistosomiasis (Doenhoff et al., 2004). The treatment kills the adult worms and eggs and prevents the disease from progressing into a severe chronic phase. After MDA with a single dose of praziquantel, the prevalence of schistosomiasis mekongi decreased to less than 5% based on Kato-Katz stool examinations (Khieu et al., 2019). However, the recent success of the MDA program has led to decreased sensitivity of the Kato-Katz method. Therefore, a diagnosis with high sensitivity and specificity that can replace the Kato-Katz stool examination is urgently needed to monitor the prevalence and assess the true status of schistosomiasis towards elimination of the disease with the MDA program.

6. Population genetic studies

Population genetics is the study of genetic variation and molecular evolution within and among populations. This approach provides information to understand epidemiology, the dynamics of disease transmission, and gene flow as it relates to disease control. Microsatellite markers are one of most popular molecular tools used to determine the genetic diversity of parasites among definitive hosts (Zane et al., 2002). Microsatellites or simple sequence repeats (SSRs) or short tandem repeats (STRs) are short repeated sequences of 1–6 base pairs. They are distributed in the whole genome in both noncoding and coding sequences with a high degree of polymorphism based on their variation in the number of repeats (Shrivastava et al., 2005). Furthermore, previous studies using microsatellite markers to assess and compare the genetic diversity and population structure of *Schistosoma* spp. among their reservoir hosts showed a high level of gene flow across parasite and reservoir hosts (Kebede et al., 2020; Rudge et al., 2008). This indicates that

reservoir hosts play an important role in transmission and maintenance of the infection. Therefore, microsatellite markers are now valuable for studying the population genetic structure of different species.

Studies on the population structure of *S. japonicum* and gene flow among the parasite isolates across definitive host species will provide useful information in understanding the genetic diversity of the parasite that can help in developing more effective MDA programs towards the elimination of schistosomiasis.

7. Aims of this study

For the control of this parasitic disease, comprehensive information including the life cycle of the parasite, epidemiology of the disease, and prevalence in definitive hosts is crucial. The general purpose of this study is to develop a means to obtain this information to promote disease control programs focused on the elimination of Asian zoonotic schistosomiasis from Southeast Asian countries such as the Philippines, Cambodia, and Lao PDR. The specific objectives are (1) to modify the MHT to recover miracidia samples for the single-genome DNA preparation to be used in a population genetics study; (2) to evaluate *S. japonicum* recombinant antigen with ELISA to detect *S. mekongi* infections in humans; and (3) to evaluate *S. mekongi* recombinant antigen with ELISA to detect *S. mekongi* infections in humans.

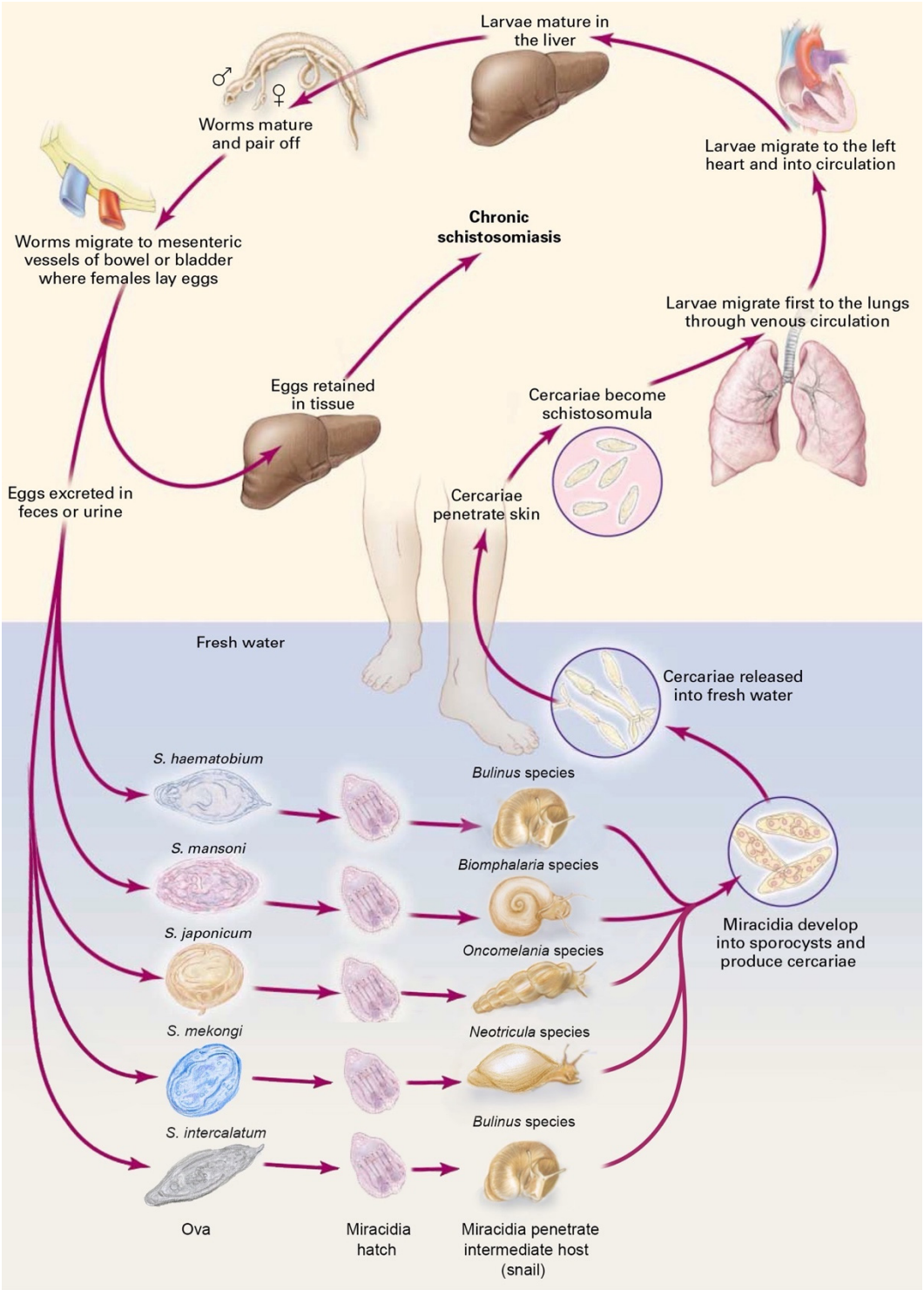

Fig.1 Life cycle of *Schistosoma* spp. (Weerakoon et al., 2015)

Chapter 1

A simple and efficient miracidium hatching technique for preparing a single-genome DNA sample of *Schistosoma japonicum*

1-1. Introduction

Schistosomiasis or bilharzia is one of the most important infectious parasitic diseases caused by trematodes of the genus *Schistosoma*, which are extensively spread in the tropical and subtropical areas and seriously endanger the health of both humans and animals. Schistosomiasis is found in 78 countries, and over 200 million people are affected by the disease, which causes over 200,000 deaths annually (WHO, 2021). The three main species known to infect humans, *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*, are distributed in Africa, the Middle East and Asian countries, respectively. *S. japonicum*, which causes Asian zoonotic schistosomiasis, infects more than 40 species of wild and domestic animals and is endemic in the Philippines and parts of China and Indonesia (He et al., 2001).

The disease in humans appears mainly in the chronic phase with periportal fibrosis and hepatosplenomegaly caused by deposition of eggs and subsequent granuloma formation in the liver and other organs where the eggs may be deposited (Costain et al., 2018). Among the parasite spices that can infect humans, *S. japonicum* is the most pathogenic as the daily production of eggs by adult worms living in the mesenteric vein is the largest in number and can reach approximately 1,000 eggs per female parasite (Cheever et al., 1994). About 50% of the eggs are excreted with stool into the

environment, whereas others can be trapped in organs such as the liver and in intestinal tissue. The life cycle of the parasite is complicated due to its zoonotic nature, in which water buffaloes and dogs play major roles as reservoir hosts. Information regarding the genetic diversity of *Schistosoma* will help to understand the epidemiology and transmission dynamics of the parasite among its humans and reservoir hosts in the field.

To analyze the genetic diversity of the parasite precisely with microsatellite markers, DNA samples derived from a single genome should be prepared. An adult worm and an egg are the parasite stages from which a single-genome DNA sample can be prepared. However, the collection of adult worms from definitive hosts directly in the field is not feasible because they live in the inferior mesenteric and superior hemorrhoidal vein (Ross et al., 2001). Experimental infection of laboratory animals with cercaria for preparation of the adult stage may pose loss of true genetic diversity under immunological selection due to an artificial infection (Shrivastava et al., 2005). Therefore, the egg is the most practical and reliable stage for single-genome DNA preparation despite the problem in breaking its hard shell using an original DNA extraction protocol. Miracidium is a larval stage of the parasite and may be useful for single-genome DNA preparation.

In the field, miracidia can hatch after eggs are released by adult worms in their definitive host with stool into fresh water, and this hatching process can be reproduced under experimental conditions to recover miracidia. Miracidium hatching technique (MHT) is one of the tools for schistosomiasis diagnosis (Jurberg et al., 2008). The original MHT requires specific equipment and is time consuming. In addition, contamination of miracidia with fecal bacteria may hamper the DNA sample to be used for subsequent application (Le Clec'h et al., 2018). Thus, in this study, the original MHT was modified to optimize this protocol to recover miracidia for preparation of

single-genome DNA that can be used for a DNA-based study such as a population genetics study with microsatellite markers.

1-2. Materials and methods

Parasites and animal infection

The *S. japonicum* Yamanashi strain was maintained using the snail intermediate host *Oncomelania hupensis nosophora*. The *O. h. nosophora* snails were infected with miracidia and were crushed 6 months later to collect the cercariae. Eleven 5-week-old female ICR mice (Clea Inc., Tokyo, Japan) were used for *S. japonicum* infection. The mice were percutaneously infected with 30 cercariae each, and fecal samples were collected after 6 weeks of infection. All animal experiments were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals established by Obihiro University of Agriculture and Veterinary Medicine and Dokkyo Medical University. This study was approved by the Committee for Animal Experimentation of Obihiro University of Agriculture and Veterinary Medicine (Approval no. 20-38) and Dokkyo Medical University (Approval nos. 29-52 and 0006).

Miracidium hatching technique (MHT)

One gram of fecal sample that had been collected from the parasite-infected mice was emulsified with 10 ml of dechlorinated tap water, filtered by stainless mesh (TESTING SIEVE: wire diameter of 71 μm and aperture of 106 μm; Tokyo Screen Co., Ltd., Tokyo, Japan), and kept for 20 min at room temperature (RT: 23–25°C) to deposit the eggs at the bottom of the container. After washing the eggs with 10 ml of dechlorinated tap water, they were aliquoted into each well of 96-well plastic ELISA plates with 350 μl of dechlorinated tap water (10 eggs per one well). According to the

original MHT protocol, one ELISA plate was kept on a bench in the laboratory and incubated under room light at RT for 24 hr to induce miracidium hatching (Sugiura et al., 1954; Yu et al., 2007). Each well with eggs was observed for miracidia hatching under stereo microscopy at 2, 4, 6 and 24 hr of incubation (room light condition). To evaluate the promotive effect of light from several different sources in the hatching process, the eggs were exposed to sunlight (natural light) and artificial lighting. An ELISA plate with eggs was kept beside a window inside a room to expose the eggs to sunlight (sunlight condition). Another plate was kept on the bench in a room (room lighting was on) under a 27-W fluorescent light at a distance of 40 cm (fluorescent light condition). An additional plate was kept on the bench in a room (room lighting was off) under a halogen light (OLYMPUS, LG-PS2, OLYMPUS Corp., Tokyo, Japan) at a distance of 30 cm (halogen light condition). The light intensity in lux for each condition was measured with a digital lux meter (AP-881D, Aoputtriver Technology Co. Ltd., Guangdong, China) and is summarized in Table 1. In addition, the plate was kept on the bench in a box (dark condition). Miracidia and unhatched eggs for these conditions were also counted under stereomicroscopy at 2, 4, 6 and 24 hr of incubation. A plate was kept under the dark condition for 5 days (see Results, *Miracidia hatching*). To evaluate the suppressive effect of a salinity condition on the hatching process, eggs were placed in a 0.85% NaCl solution and exposed to an artificial light under the fluorescent light condition for 2 hr. Miracidia and unhatched eggs were counted under stereomicroscopy after 2 hr of incubation (0.85% NaCl condition). All experiments were conducted at RT. The hatching processes of some eggs were video recorded, and individual miracidia were recovered with a micropipette into a plastic test tube with 1 μl of dechlorinated tap water. Single-genome DNA was extracted from these miracidia.

DNA extraction and microsatellite amplification

Genomic DNA was extracted from individual miracidia using a Stool Mini Kit and QIAamp DNA Micro kit (QIAGEN, Germantown, MD, USA). The DNA was quantified with NanoDrop™ (Thermo Fisher Scientific K. K., Tokyo, Japan) and stored at −30°C until use. To evaluate the DNA quality of a single miracidium, the 18S rRNA gene (Webster et al., 2006) was PCR amplified and sequenced.

Ten previously reported *S. japonicum* microsatellite markers including RRPS, M5A, TS2, MPA, 2AAA, J5, Sjp1, Sjp5, Sjp6 and Sjp9 were PCR amplified (Shrivastava et al., 2003; Yin et al., 2008). The amplifications were performed with a Veriti™ 96-well Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA) in 10 µl total reaction volume containing 5 µl 2X Gflex™ buffer, 0.2 µl of 10 µM forward, 0.2 µl of 10 µM reverse primer, 0.2 µl Tks Gflex DNA polymerase (Takara Bio Inc., Shiga, Japan) and 0.5 µl of the single miracidium DNA as DNA template. The PCR was run under the following condition: 94°C for 1 min, 35 cycles at 98°C for 10 sec, at 55°C for 15 sec and at 68°C for 30 sec. The amplicon was separated by gel electrophoresis and stained with ethidium bromine.

Statistical analysis

Data from MHT are presented as the mean ± standard deviation (SD). One-way ANOVA and Chi-square tests were used to analyze the data sets (Table 1), and a P-value of < 0.05 was considered statistically significant.

1-3. RESULTS

Hatching behaviour

The process of miracidium hatching was observed under stereomicroscopy and recorded with a video camera (Video 1). After the egg was exposed to the water under the fluorescent light condition, cilia covering the entire tegument began to move, and this step was easily observed. It took several minutes before the miracidium started this movement within the eggshell after the egg was exposed to water. After the eggshell ruptured, a miracidium within a sac emerged from the eggshell, escaped from the sac, and started to swim actively. The eggshell ruptured along the long axis of the egg. The hatching process was completed within a short time of less than 1 minute.

Miracidia hatching

The results of MHT conducted under the 4 lighting conditions (see Materials and Methods) are summarized in Table 2. The hatching rate at 24 hr of the experiment under the room lighting condition according to the original MHT protocol was 77.0% whereas that under the dark condition was 4.7% (Fig. 2). This result confirmed that the lighting condition was crucial for MHT. The MHT under the sunlight and fluorescent light conditions showed higher hatching rates than that under the room light condition in the original protocol (Fig. 2). The hatching process under the sunlight condition showed the highest hatching rate, with completion achieved within 6 hr of starting the experiment. Interestingly, eggs that had been kept under the dark condition for 5 days at RT could resume and complete the hatching process after being exposed to sunlight (Fig. 2). In the MHT with 0.85% NaCl, only two eggs hatched in the first 2 hr, and the hatching rate at 24 hr was only 1.9% (Fig. 3). This result confirmed that 0.85% NaCl solution could inhibit miracidium hatching.

Microsatellite amplification

Twenty samples of the single miracidium DNA were tested for microsatellite marker amplification. Their quality was initially evaluated by PCR amplification and sequencing of the 18S rRNA gene. The sequencing results confirmed that the miracidium sample was free from fecal bacteria and other organisms that may be contaminants through the MHT protocol (data not shown). The 10 markers were successfully amplified from all 20 DNA samples. Fig. 4 shows representative results of the amplification.

1-4. Discussion

In this study, we modified the original MHT protocol (Jurberg et al., 2008) to apply the technique for preparation of the parasite's single-genome DNA from miracidia collected using 96-well ELISA plates. The miracidium could hatch out from the egg, as shown in Video 1, in a similar manner as that previously observed by Jones *et al*., suggesting that the miracidia maintained their activity under the modified protocol (Jones et al., 2008). The microsatellite markers for use in population genetics studies could be successfully amplified by PCR from the single-miracidium DNA.

Several factors are considered to affect *S. japonicum* MHT, including temperature, lighting condition, salinity, and pH of the water; particularly, water temperature and lighting condition are both essential factors in hatching of the miracidia (Ito, 1955; Ye et al., 1997). Although a water temperature of 24–30°C was recommended for MHT, to our knowledge, the effects of different types of lighting and lighting intensity on the hatching process have not yet been compared (Ye et al., 1997). Previous studies have mostly focused on the effects of artificial lighting (fluorescent light), and only a few studies observed the effect of sunlight (Ye et al., 1997). The results in the present study

confirmed that lighting condition was crucial for MHT as only a few miracidia could hatch under the dark condition. However, earlier studies showed that there was no significant difference in hatching rates between light and dark conditions (Sugiura et al., 1954; Kassim and Gibertson, 1976; Xu and Dresden, 1990). This might be attributed to differences in experimental procedures or the schistosome strain used in each experiment. Of note, the eggs that had been kept at RT under the dark condition for 5 days could resume the hatching process with exposure to sunlight. Interestingly, the hatching rate observed after the eggs remained in the dark was comparable with that observed under sunlight condition. Miracidia hatching was resumed within a short time, indicating that the eggs maintained their hatchability under the dark condition and that this condition can be used for storing eggs before MHT.

To compare the effect of different types of lighting on the hatching rate, we exposed the eggs to sunlight, halogen light, and fluorescent light. The highest hatching rate was obtained with sunlight. Ultraviolet (wavelength less than 400 nm), visible (400–700 nm), and infrared (wavelengths greater than 700 nm) are the three main regions of the sunlight spectrum. The specific region of wavelength that affected miracidia hatching the most was not determined in this study although various studies have indicated that halogen and fluorescent light emit low doses of ultraviolet radiation (Klein et al., 2009). The effect of lighting intensity in the hatching rate of trematode eggs has been studied by several groups. Gold and Goldberg reported that there were no marked differences in the hatching rate of *Fasciola hepatica* eggs between various wavelengths of the visible light spectrum or different lighting intensities (Gold and Goldberg, 1976). However, Markum and Nollen reported that hatching of *Echinostoma caproni* eggs was delayed by 2 days under low lighting intensity (Markum and Nollen, 1996). In the present study, *S. japonicum* eggs kept under fluorescent lighting, which

provided a higher lighting intensity than that of room lighting in the original protocol, showed a higher hatching rate than that of the original protocol. This finding suggested that higher lighting intensity provided a promotive effect in the MHT of *S. japonicum*. Information regarding the specific wavelength region and optimal lighting intensity that best promote miracidia hatching may assist in the development of an automated MHT technology that can greatly help in the diagnosis and elimination of schistosomiasis. The World Health Organization has set a target of 2030 for the elimination of schistosomiasis as a public health problem (WHO, 2021).

Ito reported that NaCl concentration affected the hatchability of *S. japonicum* eggs and that the hatching rate dropped to approximately 2% of the original condition under fresh water when the NaCl concentration was increased to 1.0% (Ito, 1955). A similar finding was reported by Magath and Mathieson (Magath and Mathieson, 1946). The inhibitory effect of the salinity concentration for MHT was also confirmed in the present study, thus suggesting that eggs can be stored in a 0.85% NaCl solution under a dark condition before MHT is conducted in field studies.

The microsatellite fragments were amplified by PCR to confirm whether the quality of the single-genome DNA is appropriate for population genetics studies. The amplicons showed the expected size from 220 to 550 bp. Comparative studies between the genetic background of the parasite in each definitive host are important to understand the epidemiology and transmission dynamics of the parasite. Shrivastava *et al.* suggested that parasite DNA obtained from natural life cycle stages be used rather than that from experimental infection for studying the population genetics structure of *S. japonicum* and other multi-host pathogens (Shrivastava et al., 2005). The MHT protocol in the present study will help in the sampling procedure for population genetics

studies of *S. japonicum* by avoiding bias due to experimental host-induced selection and the bottleneck effect from the estimated population.

1-5. Summary

In summary, the MHT suggested in this study provides a more rapid and convenient protocol with relatively higher hatching rates than the original MHT. High-throughput DNA extraction processing can also be arranged by taking advantage of the setup using 96-well ELISA plates. The results confirmed that lightning is a crucial factor in the MHT, and sunlight provided the highest hatching rate. The amplification of microsatellite markers from the single-genome DNA prepared from the miracidia also confirmed that the quality of the DNA was acceptable for the conduction of population genetics studies, which are indispensable to understanding the epidemiology and transmission dynamics of *S. japonicum* in the regions where Asian zoonotic schistosomiasis is endemic.

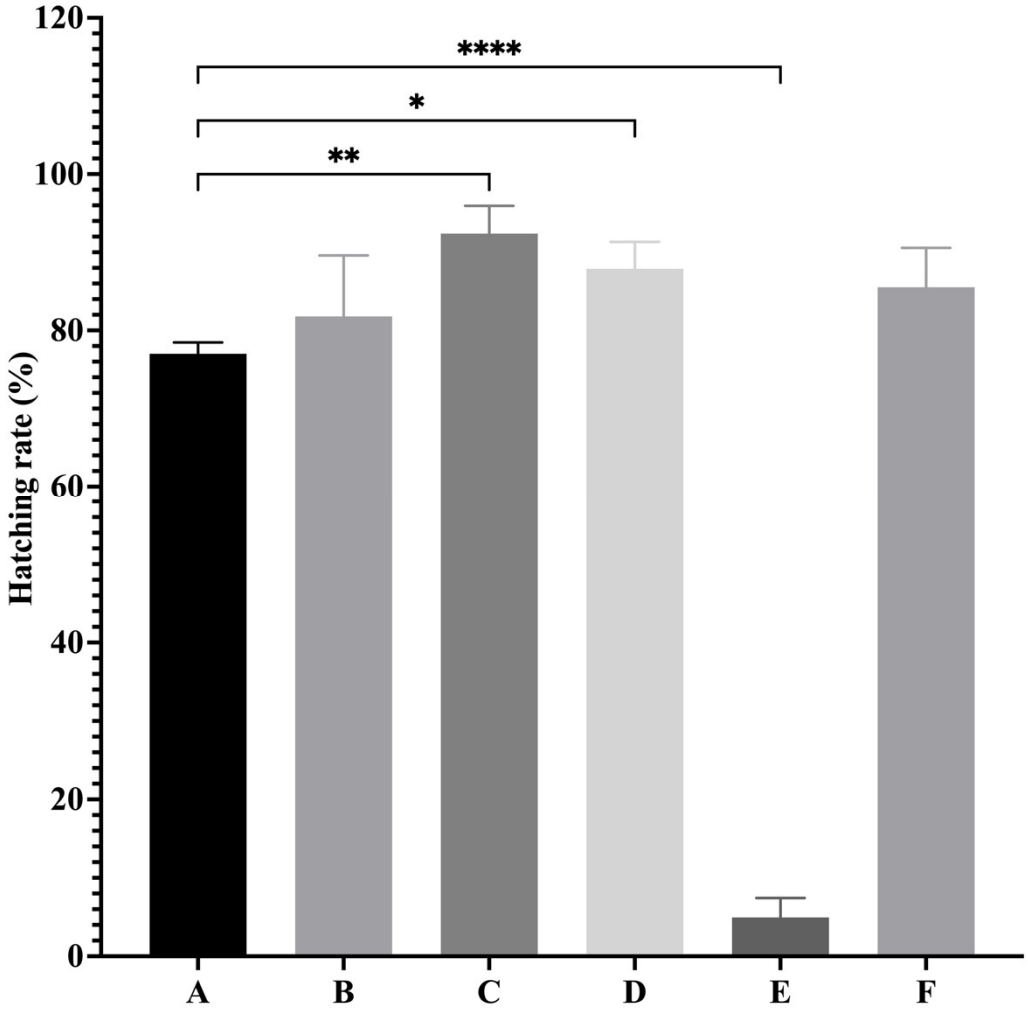

Fig. 2 Comparison of *S. japonicum* miracidium hatching rates at 24 hr under different lighting conditions. The rates are presented as the mean ± standard deviation (SD). One-way ANOVA was used to analyze the data sets. A post-hoc comparison of mean values between the conditions was performed using Dunnett's test, and a *P*-value of < 0.05 was considered statistically significant. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. A, room light condition (original protocol), B, halogen light condition, C, sunlight condition, D, fluorescent light condition, E, dark condition and F, eggs kept in the dark condition for 5 days before being exposed to sunlight.

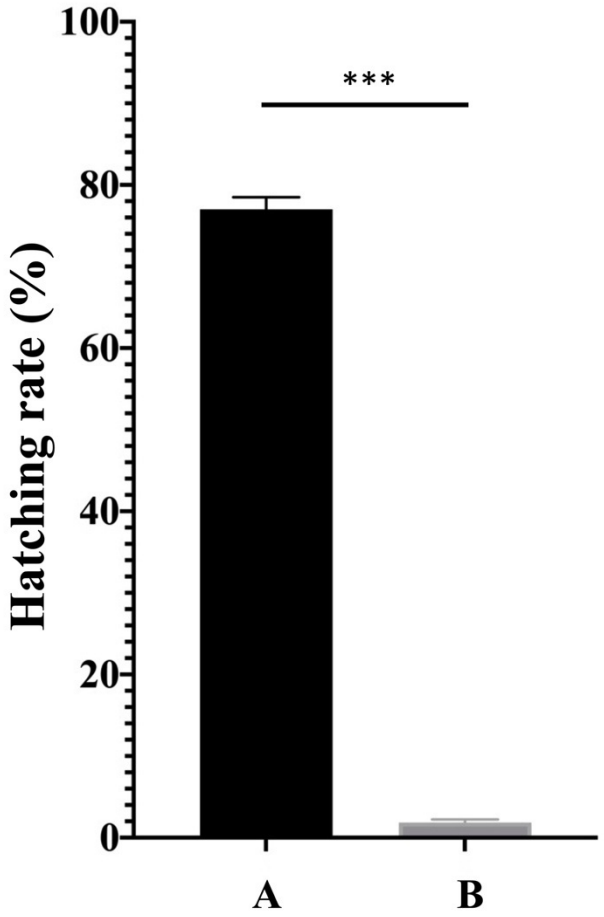

Fig. 3 Effect of the salinity condition on *S. japonicum* miracidia hatching. The data are presented as the mean ± SD. Asterisks indicate that the difference between the hatching rate under A = room light condition in tap water (original protocol) and that under B = room light condition in 0.85% NaCl is statistically significant at *** $P < 0.001$ (Chi-square test).

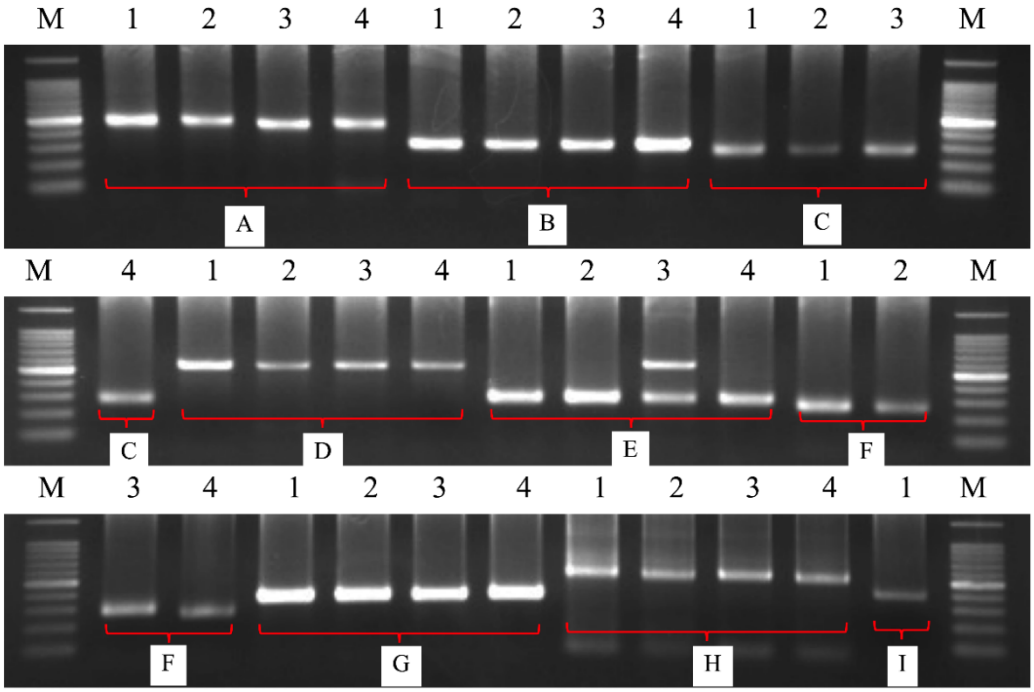

Fig. 4 Agarose gel electrophoresis illustrates representative PCR amplifications of the microsatellite marker genes from miracidium single-genome DNA. 1-4 = the single-genome DNA of miracidium. M = 100 bp marker, 1-4 = single miracidium DNA, A = microsatellite marker gene (MMG) amplified with primer set 2AAA (449-456 bp), B = MMG amplified with primer set M5A (312-344 bp), C = MMG amplified with primer set SjP1 (233-284 bp), D = MMG amplified with primer set MPA (524-548 bp), E = MMG amplified with primer set SjP9 (258-327 bp), F = MMG amplified with primer set SjP5 (224-272 bp), G = MMG amplified with primer set J5 (501-517 bp), and I = MMG amplified with primer set TS2 (360-385 bp). Nucleotide sequence of the amplicon was confirmed by direct sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Four samples were sequenced for each amplicon.

Table 1. Light intensity of each lighting condition

Condition	Light intensity (Lux)*
Room light (original protocol)	~414
Halogen light	~2040
Sunlight	~2800-3700
Fluorescent light	~1125

*The light intensity (Lux) for each condition was measured with a digital lux meter (AP-881D, Aoputtriver Technology Co. Ltd., Guangdong, China).

Table 2. Hatching rate of Schistosoma japonicum eggs under the various lighting conditions

C	Experiment I					Experiment II					Experiment III					MHR (%) ±SD	P-Value
	Experimental time (hours)*				HR***	Experimental time (hours)*				HR**	Experimental time (hours)*				HR***		
	2	4	6	24		2	4	6	24		2	4	6	24			
R	95/187	32/187	6/187	11/187	144/187	42/93	26/93	5/93	No	73/93	60/123	23/123	10/123	No	93/123	77±2.0	
	50.8	17.1	3.2	5.9	77	45.2	28	5.4		78.5	48.8	18.7	8.1		75.6		
H	90/104	1/104	No**	No	91/104	21/107	36/107	14/107	7/107	78/107	102/147	19/147	4/147	No	125/147	82.1±7.8	0.5751
	86.5	1			87.5	19.6	33.6	13.1	6.5	72.9	69.4	12.9	2.7		85		
S	42/85	29/85	9/85	No	80/85	62/120	23/120	21/120	No	106/120	110/135	18/135	No	No	128/135	92.4±3.6	0.0049
	49.4	34.1	10.5.		94.1	51.7	19.2	17.5		88.3	81.5	13.3			94.8		
F	131/143	No	No	No	131/143	56/110	21/110	16/110	3/110	96/110	74/138	26/138	14/138	3/138	117/138	88.0±3.4	0.043
	91.6				91.6	51	19.1	14.5	2.7	87.3	53.6	18.8	10.1	2.2	84.8		
D	No	No	No	3/131	3/131	No	No	No	7/97	7/97	No	No	No	6/112	6/112	4.7±2.4	<0.0001
				2.3	2.3				7.2	7.2				5.4	5.4		
D&S	106/123	4/123	1/123	No	111/123	62/87	8/87	5/87	No	75/87	76/106	6/106	3/106	No	85/106	85.8±5.0	0.1315
	86.2	3.3	0.2		90.2	71.3	9.2	5.7		86.2	71.7	5.7	2.8		80.2		

C: Light conditions, D: dark condition, D&S: eggs kept in the dark condition for 5 days before being exposed to sunlight, F: fluorescent light, H: halogen light, HR: hatching rate, MHR: mean hatching rate, R: room light, S: Sunlight.

*The hatching rates at every 2 hours until 6 hours of the experiment and at the end of the 18-hour period from 6-24 hours of the experiment are shown.

Upper row shows the number of eggs hatched in the time frame/total number of eggs used in the experiment. Lower row shows the hatching rate (%) in each time frame.

**No indicates that no hatching was observed in the time frame.

*** Upper row shows the number of eggs hatched over the 24 hours of the experiment/total number of egg used in the experiment. Lower row shows the hatching rate (%) over the 24 hours of the experiment

Chapter 2

Evaluation of *Schistosoma japonicum* recombinant antigens for the detection of *Schistosoma mekongi* human infection

2-1. Introduction

Schistosomiasis is parasitic disease affecting 78 counties worldwide with approximately 229 million people requiring preventive treatment (Chitsulo et al., 2004; Colley et al., 2014). This includes Asian schistosomiasis caused by the blood fluke *Schistosoma mekongi* which affects communities in Mekong River Basin in southern Lao People's Democratic Republic (Lao PDR) and northern Cambodia (Attwood et al., 2008; Muth et al., 2010; Vonghachack et al., 2017). Control strategies for schistosomiasis should include accurate, reliable, and inexpensive diagnostic methods that will monitor infection dynamics and treatment efficacy. However, technological gaps in the current diagnostic methods used by the endemic countries for *S. mekongi* infection impose significant limitations on epidemiological analysis and elimination programs.

Current parasitological methods like Kato-Katz have been producing unreliable results varying from one day to the next from the same patient. This significantly underestimantes the infection levels, particularly in low transmission setting as in the Cambodia and Lao PDR. As compared with fecal microscopic examination, serology provides a sensitivity tool for the diagnosis of schistosomiasis, especially for low-intensity infections (Dawson et al., 2013; McLaren et al., 1981; Weerakoon et al., 2015; Zhou et al., 2007). A sensitive and specific serological test might therefore be useful in assessing human infection im endemic areas. This test will also be useful in confirming the lack of

transmission in areas where disease elimination has been achieved. Serodiagnosis using sodium metaperiodate SMP-ELISA has shown high sensitivity and specificity for *S. mekongi* (Kirinoki et al., 2011), however, the production of SMP-ELISA is challenged by the unstable supply of *S. mekongi* in the pre-elimination setting; therefore, althernative serodiagnostic tools with similarly high sensitivity and specificty also should be exploreed.

In table 3 several schistosome-specific antigens have been evaluated for their diagnostic potential in *S. japonicum* humans and animals infection (McLaren et al., 1981; Angeles et al., 2011, 2012, 2020; Moendeg et al., 2015). The recombinant antigens including rSj7TR, rSjPCS (unpublish) , rSjPrx-4 and rSjChi3 (unpupblish) have high diagnostic performance for *S, japonicum* infection in previous studies (Angeles et al., 2012; Dang-Trinh et al., 2020). rSjPrx-4 was analyzed with results suggesting that this enzyme may play a role as an antioxidant of *S. japonicum* to deal with oxidative stress (Dang-Trinh et al., 2020). Also, serological analysis of rSjPrx-4 showed 83.3% sensitivity and 86.7% specificity. When combined with *S. japonicum* thioredoxin peroxidase-1 (rSjTPx-1) antigen, the sensitivity has improved to 90.0% (Dang-Trinh et al., 2020). Another enzyme, rSjPCS is an enzyme that catalyzes the biosynthesis of phytochelatin capable of scavening and detoxifying heavy metals (Grill et al., 1987). In *S. mansoni,* it was seen to be expressed in the eggs, schistosomula, and adult stages (Ray and Williams, 2011). rSjPCS was evaluated for the deteection of human schistosomiasis with sensitivity and specificity results of 73.3% and 83.3% respectively.

Tandem repeat proteins (TRPs) are often targets if humiral responses for helminthic parasites (Kim et al., 2001). One of the tandem repeats we have evaluated in our previous study is rSj7TR which showed 80.0% sensitivity and 93.3% specificty (Angeles et al., 2012). We have also evaluated its immunolocalization in different life stages of *S. japonicum* and our results showed that it was expressed in the eggs, schistosomules, and

juvenile adults (Angeles et al., 2013). On the other hand, rSjChi3 is a multiepitope protein constructed by combining the epitope sequences of rSjSAP4 (saposin), rSjTPx-1, rSj23LHD (large hydrophilic protein), and SjSAP5 (unpublished). Serological evaluation for human schistosomiasis of this chimeric protein showed 90% sensitivity and 93.3% specificity.

Although these are antigens found in *S. japonicum,* they also might have the potential to be useful in detecting *S. mekongi* infection. Due to these two species are closely related species according to previous studies in terms of molecular and morphological identification (Houston et al., 2004). This study, therefore, aims to evaluate these recombinant *S. japonicum* antigens for the detection of *S. mekongi* human infections as compared to the crude SjSEA using ELISA.

2-2. Materials and methods

Antigens

Soluble Egg Antigen (SjSEA)

SjSEA was prepared using the intestine of *S. japonicum* infected mice digested with 0.02% Pronase E (Actinase E in PBS). This was homogenized at 10,000 rpm for 1 min. twice and incubated at 37ºC for 2 hours with agitation. The homogenate was then filtered using a steel mesh and centrifuge at 13000 rpm for 5 mins at 4ºC. The residue was then dissolved in PBS, mixed, and centrifuged again. 10x volume of 0.05% Collagenase solution was added to the resulting pellet. The suspension was incubated at 37ºC for 1 ½ hour with agitation and then washed twice with PBS and centrifuged at 11000 rpm for 2 mins at 4ºC. Filtration was done to the solution to collect the schistosome eggs. The eggs were suspended in cold distilled water and then lyophilized. The lyophilized egg was homogenized in carbonate buffer solution, stored for 2 days at 4ºC with constant stirring.

The homogenate was centrifuged at 14000 rpm for 1 hour with the resulting supernatant being filtered through a 0.45 μm syringe filter. The antigen was kept at -80ºC until use.

Recombinant Antigens

Recombinant antigens from *S. japonicum* were prepared as previously described (Angeles et al., 2012). In brief, the genes inserted in the pET28 vector were transfected into *Escherichia coli* BL21 grown in SOB medium. The recombinant proteins were recovered using the Ni-NTA agarose, dialyzed, and eluted with 20 mM Tris, pH 8.0. The integrity and purity of the proteins were evaluated by 15% polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and subsequent Coomassie Brilliant Blue staining. The concentration of each expressed protein was measured using BCA Protein Assay.

Serum samples

Archived serum samples collected from Cambodia were used in the evaluation of SjSEA and the recombinant antigens. This includes negative samples collected in the non-endemic area of Phnom Penn (n=31) and *S. mekongi* egg-confirmed serum samples from an endemic area in Cambodia (n=28). To check cross-reaction, 21 *Opisthorchis viverrini* positive serum samples collected in Thailand were also evaluated. In addition, 15 positive and 16 negative samples for *S. japonicum* from the Philippines confirmed through stool microscopy were also tested for the cell-free expressed recombinant proteins. A panel of 31 human serum samples from U.S. volunteers (BioreclamationIVT, Baltimore, MD) was used to calculate the cut-off values as mean + 3SD.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was done as previously described in our published study (Angeles et al., 2011). In brief, horseradish peroxidase (HRP)-conjugated anti-human IgG goat serum was used for the secondary antibody, and 3,3',5,5'-tetramethylbenzidine will be used as the substrate for HRP. The wells of the microplates will be sensitized separately with the following antigen concentrations per well: SjSEA at 20 µg and 2 µg; SjTPx-1, Sj7TR, SjPCS, and SjPrx-4 at 200 ng; and SjChi3 at 200 ng and 20 ng. As for cell-free expressed proteins, 200 ng of GST-tagged rSjTPx-1 and 100 and 200 ng of HIS-tagged rSjTPx-1 were used for each well. Proteins were diluted with carbonate/bicarbonate buffer at pH 9.6. After blocking with 1% bovine serum albumin (BSA) in phosphate-buffered saline with 0.05% Tween 20 (T-PBS) (T-PBS-0.1%BSA), the antigen-coated well was filled with the serum. The test sera (0.1 ml) were diluted 1:400 with T-PBS-0.1%BSA and while the secondary antibody (0.1 ml) was diluted in 1:10,000. Color reaction was induced by adding the Peroxidase Substrate Solution (KPL, Gaithersburg, MD, USA) Optical density (OD) was measured at 450 nm using a microplate reader. All the tests were done in triplicates.

Statistical analysis

Diagnostic sensitivity and specificity of the recombinant antigens were calculated for the crude SjSEA and the recombinant antigens using the online software MedCalc (https://www.medcalc.org/calc/diagnostic_test.php).

2-3. Results

ELISA results showed that SjSEA and the recombinant antigens have high OD values with most of the *S. mekongi* positive samples (Fig. 5). However, non-endemic samples from Cambodia were also giving high OD values with the recombinant antigens.

Among the recombinant antigens, the highest number of positive results with the non-endemic negative samples were shown by SjChi3 at 200 ng concentration and the lowest with Sj7TR. The results from the recombinant antigen were surpassed by SjSEA at 2 μg concentration. Cross-reaction with *O. viverrini* was seen in SjSEA at 200 ng with 16 samples. This has greatly improved with 20 ng SjSEA showing no cross-reaction. Among the recombinant proteins, only SjChi3 showed no cross-reaction with *O. viverrini*.

Table 4 shows the sensitivity and specificity calculated for each antigen. SjSEA at 2 μg concentration has the highest diagnostic activity for the detection of *S. mekongi* with 96.4% sensitivity and 93.5% specificity.

2-4. Discussion

The successfully assembly of the *S. japonicum* genome data into a complete genome sequence has created opportunities for the advancement and enhancement of serological diagnostics utilizing recombinant antigen-ELISA from the excretory and secretory protein of *S. japonicum*. The use of recombinant proteins has been proven useful in the diagnosis of *S. japonicum* for humans and animals (Angeles et al., 2012, 2019, 2020) such as SjTPx-1, Sj7TR, SjPCS and SjChi3 (Angeles et al., 2011). However, the results of this study have shown otherwise. Seropositivity seen in negative samples from Cambodia and samples positive for *O. viverrini* suggests that the recombinant antigens in their present form could not be used in the region endemic for both *S. mekongi* and *O. viverrini.* Therefore, the development of improved antigens either from *S. japonicum* or *S. mekongi* should still be conducted.

Although tedious, the production of soluble egg antigen from *S. japonicum* is well-established because the parasite's life cycle can be maintained in the laboratory. Unfortunately, this was not done in *S. mekongi*. The results of this study, showing that

SjSEA is useful in the diagnosis of *S. mekongi* infection, could take advantage of the laboratory production of SjSEA through *Oncomelania* snail and animal infection. Production of large amounts of SjSEA for massive use in *S. mekongi* surveillance can be done in the laboratory by infecting rabbits with *S. japonicum* cercariae and purifying the schistosome eggs from their liver and intestine. Quality control can be done by evaluating the crude antigen solution through gel electrophoresis and preliminary ELISA run using standard serum samples. In conclusion, SjSEA at a low concentration of 2 μg should be used to improve the serodiagnostic capabilities for *S. mekongi* in endemic areas of Cambodia and Lao PDR.

2-5. Summary

Asian schistosomiasis caused by the blood fluke Schistosoma mekongi is endemic in Northern Cambodia and Southern Lao People's Democratic Republic. The disease is mainly diagnosed by stool microscopy. However, serodiagnosis like enzyme-linked immunosorbent assay (ELISA) with soluble egg antigen (SEA) has been shown to have better sensitivity compared to the stool examination, especially in the settings with low intensity of infection. To date, no recombinant antigen has been assessed using ELISA for the detection of S. mekongi infection due to the lack of genome information of this schistosome species. The present study, several recombinant *S. japonicum* antigens were evaluated for their potential for schistosomiasis mekongi diagnosis. ELISA results showed that S. japonicum SEA at low concentration showed better diagnostic performance than the recombinant antigens tested using the archived serum samples from Cambodia. However, further optimization on the recombinant antigens should be done in future studies to improve their diagnostic performance for S. mekongi detection.

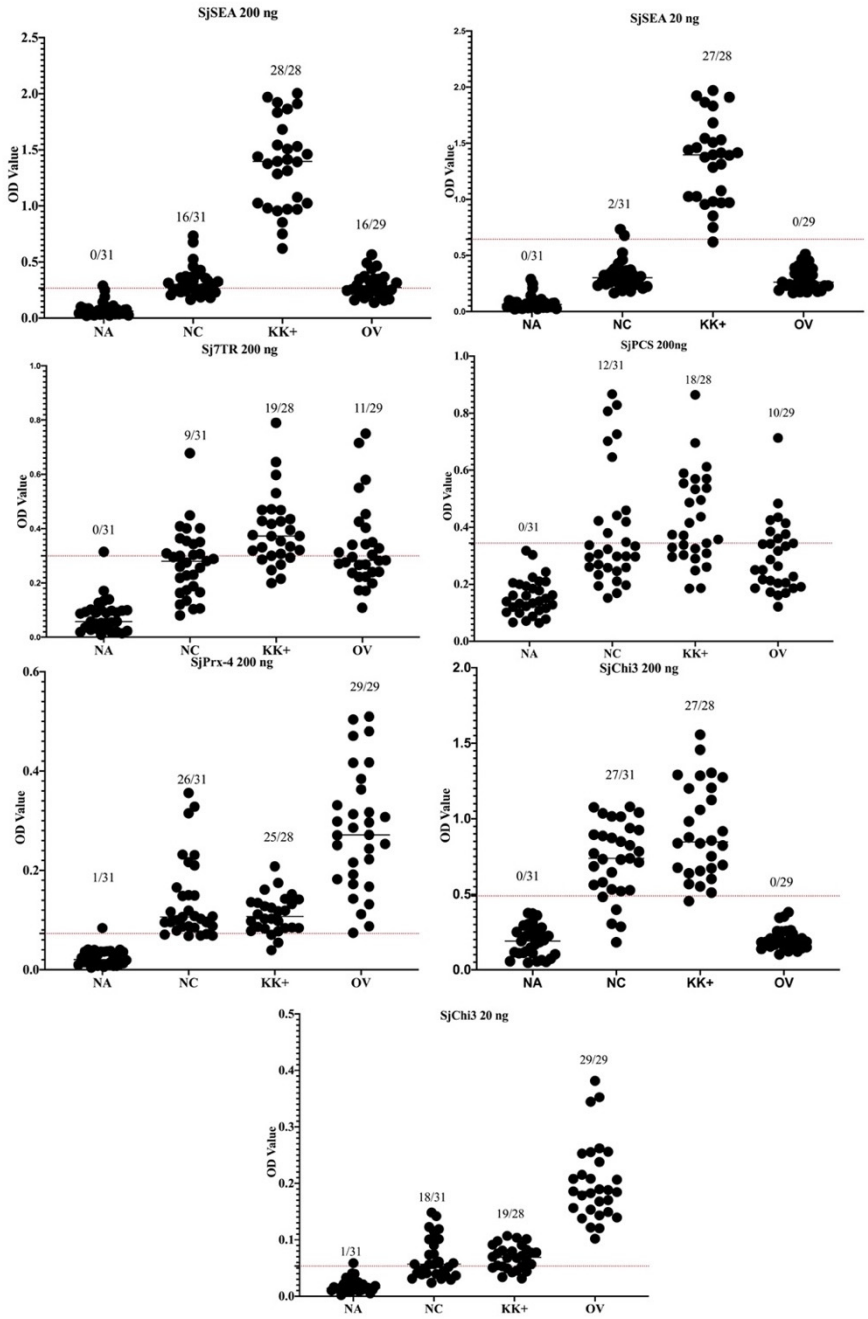

Fig. 5 ELISA Results of crude SjSEA and the recombinant antigens. Legend: NA, serum samples from non-endemic Americans used for the calculation of the cut-off values; NC, serum samples from the non-endemic area in Cambodia; KK+, serum samples from endemic areas in Cambodia confirmed as S. mekongi egg positive through Kato Katz technique; OV, serum samples from Thailand confirmed positive for *O. viverrini* using stool microscopy. Solid lines represent mean values; dotted lines represent the cut-off values.

Table 3. Diagnostic potentials of the recombinant antigens in the detection of *S. japonicum* human infection

Abbreviation	Sj Recombinant Proteins	Sensitivity	Specificity	Reference
rSj7TR	Tandem repeat protein	80.0%	93.3%	(12)
rSjPCS	Phytochelatin synthase	73.3%	83.3%	Unpublished
rSjPrx-4	Peroxiredoxin-4	83.3%	86.7%	(8)
rSjChi3	Chimeric protein consisting of selected peptides from SjSAP4, SjTPx-1, Sj23LHD, and SjSAP5	90%	93.3%	Unpublished

Table 4. Sensitivity and specificity of *S. japonicum* antigens in the detection of *S. mekongi* infection

Antigens	**Concentration**	**Sensitivity**	**Specificity**
SEA	200 ng	100%	48.4%
SEA	20 ng	96.4%	93.5%
rSj7TR	200 ng	67.9%	71.0%
rSjPCS	200 ng	82.1%	35.5%
rSjPrx-4	200 ng	89.3%	16.1%
rSjChi3	200 ng	89.3%	3%
rSjChi3	20 ng	67.9%	71.0%

Chapter 3

Cloning, expression, and evaluation of recombinant antigens for the *Schistosoma mekongi* serological diagnosis

3-1. Introduction

Schistosomiasis, also well-known as bilharzia, is a parasitic disease caused by blood-dwelling flukeworms or flatworms, belonging to the class Trematoda and genus *Schistosoma* (Gordon et al., 2019). This remains a serious public health concern in humans worldwide with more than 230 million people infected with *Schistosoma* spp. and 800 million individuals at risk of infection (Steinmann et al., 2006; Colley et al., 2014). Human schistosomiasis is mainly caused by *S. mansoni*, *S. haematobium*, *S. intercalatum*, *S. japonicum* and *S. mekongi* (McManus et al., 2018). *S. japonicum* and *S. mekongi* are the two species causing Asian zoonotic schistosomiasis. *S. japonicum* is endemic in the Philippines, the People's Republic of China and parts of Indonesia (He et al., 2001; Gordon et al., 2015). On the other hand, *S. mekongi* is found in Cambodia and Lao People's Democratic Republic (Lao PDR) (Gordon et al., 2019). It has been reported that these two species are closely related based on morphological and molecular studies (Houston et al., 2004).

Schistosomiasis mekongi was discovered in Lao PDR and Cambodia fifty years ago, and until now, the disease is still a public health concern for residents in the endemic area (Dupont et al., 1957; Schneider et al., 1975; Houston et al., 2004). The diagnosis of *S. mekongi* infection is based on a simple and low-cost Kato-Katz technique for detecting

schistosome eggs in stool samples which has been recommended as "gold standard" by the World Health Organization (WHO) (WHO, 2019). Kato-Katz technique shows high sensitivity in community settings having high schistosomiasis prevalence and it can estimate intensity of the infection as eggs per gram (EPG) (Bosch et al., 2021). However, this technique shows decreased sensitivity when it is used in endemic areas with low prevalence such as the foci in Cambodia and Lao PDR (Khieu et al., 2019). As such, this may lead to underestimation of the actual prevalence (Lin et al., 2008; Lier et al., 2009; Chen et al., 2021). In addition, Kato-Katz technique requires expertise on the egg's morphological identification under microscopic observation. This technique is also not suitable for large-scale surveillance since it is laborious and time-consuming (Zhang et al., 2009, 2015). Therefore, an appropriate diagnostic method which can replace Kato-Katz technique is needed for effective treatment and accurate prevalence survey of *S. mekongi* infection in Lao PDR and Cambodia.

It has been reported that enzyme-linked immunosorbent assay (ELISA) with recombinant antigen showed higher sensitivity than that of Kato-Katz technique for detecting human schistosomiasis (Moendeg et al., 2015; Belizario et al., 2016; Dang-Trinh et al., 2020). Several recombinant antigens have been evaluated for detecting *S. japonicum* infection in humans and animals in the locations where the disease has been nearly eliminated (Jin et al., 2010; Zhou et al., 2010; Angeles et al., 2012; Moendeg et al., 2015; Chen et al., 2021).

Thioredoxin peroxidase-1 which is expressed in tegument and as excretory/secretory products of adult worm and larva of *S. japonicum* (SjTPx-1) has been identified as a good antigen in ELISA for detecting human (Angeles et al., 2011; Macalanda et al., 2018) and animal schistosomiasis (Angeles et al., 2012, 2020). In this study, gene coding for TPx-1 of *S. mekongi* was cloned and recombinant protein was expressed. The

recombinant protein was evaluated for its diagnostic performance as antigen in ELISA for detecting *S. mekongi* infection in humans.

3-2. Materials and Methods

Ethics statement

This study was approved by the Committee for Research Ethics of Obihiro University of Agriculture and Veterinary Medicine (Approval no. 2020-01-2).

Human serum samples

Archived serum samples collected from schistosomiasis mekongi patients and individuals in the disease non-endemic area in Cambodia by National Center for Parasitology, Entomology and Malaria Control, Ministry of Health in Cambodia were used. Twenty-eight serum samples were collected from the patients whose infection were confirmed by Kato-Katz technique. Thirty negative serum samples were collected from individuals in Phnom Penn (Kirinoki et al., 2005). Thirty serum samples from healthy United States volunteers (BioreclamationIVT, Baltimore, MD, USA) were used to calculate cut-off values. In addition, 53 serum samples from patients infected with other parasites were also used to evaluate cross-reaction of them with the recombinant antigens in ELISA including 48 serum samples from Thai patients infected with *Opisthorchis viverrini* and 5 serum samples from Japanese patients infected with *Paragonimus westermani*.

TPx-1 Sequence

So far, the whole genome information of *S. mekongi* has not yet been available. Sequence information of the gene coding for TPx-1 of *S. mekongi* (*SmTPx-1*) was retrieved

from *S. mekongi* genome project which is currently under way in collaboration with Research Center for Zoonosis Control (CZC), Hokkaido University and Veterinary School of Rakunogakuen University. (Unpublished data). The coding sequence of TPx-1 of *S. japonicum* (*SjTPx-1*) was obtained from GenBank accession on. AB126036.2).

Recombinant protein preparation

Recombinant protein of SjTPx-1was expressed as previously described (Angeles et al, 2011). For expression of recombinant protein of SmTPx-1, total RNA was isolated from 10 pairs of *S. mekongi* adult worms (Shimada et al, 2007) using TRIzol reagent (Invitrogen, San Diego, USA) following the manufacturer's protocol. cDNA was synthesized using the Ready-To-Go T-Primed First Strand Kit (Amersham Biosciences, UK) with oligo (dT) primer. The double stranded full-length coding sequence was amplified by PCR. The Primers were designed according to *SmTPx-1* with *Bam*HI and *Xho*I restriction enzyme sites (underlined) as follows; the forward (5'-GC GGA TCC ATG GTA CTT CC-3') and reverse primes (5'-GC CTC GAG TTA GTG ATT AGT TTT AAT TC-3'), respectively. PCR product was cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.). The identity of the cloned sequence with the sequence in the draft genome was confirmed by sequencing using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The coding sequence was inserted into pET28 vector (Novagen, Madison, WI, US A) and the plasmid was transfected into *Escherichia coli* Rosetta (DE3) (Novagen) and was grown in LB medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 50 μg/ml of kanamycin. Induction of the expression of the recombinant proteins with 0.5 mM isopropyl-thio-β-D-galactoside (IPTG) was done in the SOB medium (BD, Sparks, MD, USA) and then maintained for 3 hours. Using nickel nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany), the recombinant protein was recovered and purified, followed

by elution and dialysis with 20 mM Tris, pH 8.0. The quality of the protein was then evaluated using 12% polyacrylamide gel electrophoresis (SDS-PAGE) while the quantity was measured using bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific Inc, Rockford, IL, US A). The recombinant proteins were stored in aliquots at -80°C until used.

Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect ELISA was performed as described in our previous studies (Angeles et al., 2011; Dang-Trinh et al., 2020). In brief, 96-well polystyrene plate (Thermo Fisher Scientific) was coated with 200 ng in 100 µl/well of recombinant antigens diluted with carbonate/bicarbonate buffer (pH 9.6) at 4°C for overnight. Each well was washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (T-PBS) and blocking was done for 30 min at room temperature with 130 µl/well of T-PBS containing 0.1% bovine serum albumin. One hundred µl of serum sample which had been diluted at 1:400 in blocking buffer was added into each well in triplicate and incubated at 37°C for 1 hour. After washing the wells with T-PBS for three times, 100 µl of horseradish peroxidase-conjugated anti-human IgG goat serum (Proteintech Group, Inc., Rosemont, IL, USA) as the secondary antibody which had been dilutied at 1:10,000 with blocking buffer was added into each well. The plate was incubated at 37°C for 1 hour. After washing the wells with T-PBS for three times, color reaction was induced by adding the Peroxidase Substrate Solution (KPL, Gaithersburg, MD, USA) and the optical density (OD) was measured at 450 nm using Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific).

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the recombinant antigens were calculated using online software MedCalc

(https://www.medcalc.org/calc/diagnostic_test.php). An Agreement between the ELISA and Kato-Katz technique for detecting the patients was estimated as Kappa value (Viera et. al., 2005).

3-3. Results

Cloning and sequencing of *SmTPx-1*

The coding sequence of SmTPx-1 (*SmTPx-1*) was successfully amplified and sequenced. 555 bp of the sequence coded a protein comprised of 184 amino acids. *SmTPx-1* showed 94.8% identity with the coding sequence of SjTPx-1 (GenBank accession no. AB126036.2). *SmTPx-1* showed 81.8% identity with the coding sequence of *S. mansoni* TPx-1 (GenBank accession no. AF121199.1) (Fig. 6(.

ELISA

ELISA results showed that rSmTPx-1 antigen detected 25 out of 28 patient's serum-samples as positive and confirmed 20 out of 30 serum samples from individuals in the disease non-endemic area as negative. On the other hand, rSjTPx-1 antigen detected 20 out of 28 patient's serum-samples as positive (Fig. 7). In comparison with rSjTPx-1 antigen, rSmTPx-1 antigen showed higher sensitivity, specificity PPV and NPV (89.3% vs. 71.4%, 97.3% vs. 66.7%, 92.6% vs. 66.7% and 90.3% vs. 71.4%, respectively) as shown in Table 1. rSmTPx-1 antigen showed cross-reactions with 18 out of 48 samples positive for *O. viverrni* infection, while rSjTPx-1 antigen showed the cross-reactions with the 22 samples (Fig. 8). Both recombinant antigens did not show cross-reaction with samples positive for *P. westermani* infection (Fig. 8). The Kappa value calculated between ELISA with rSmTPx-1 antigen and Kato-Katz was 0.82 and it was higher than that calculated between ELISA with rSjTPx-1 antigen and Kato-Katz (0.38) (Table 1). The result confirmed good

agreement between ELISA with rSmTPx-1 antigen and Kato-Katz. for detecting the patients with *S. mekongi* infection. The results suggested that the ELISA can detect active infection.

3-4. Discussion

Several recombinant antigens have been evaluated for their diagnostic potential for human and animal schistosomiasis (Jin et al., 2010; Angeles et al., 2011; Lv et al., 2016). Recombinant antigens of the proteins expressed on the tegument of *S. mansoni* such as rSM200 had been successfully applied for serological diagnosis of the schistosomiasis in human with high sensitivity and specificity (Grenfell et al., 2013). Another recombinant antigen, rRP26 had also been used in ELISA for detecting *S. mansoni* infection in human with low cross-reactivity against other parasitic infections (Makarova et al., 2005). Several recombinant antigens have been evaluated for their potential in detecting acute and chronic *S. japonicum* infections (Zhou et al., 2010). In contrast, very few studies have been reported so far on serological diagnosis of *S. mekongi* infection with recombinant antigens. Sangfuang et al (2016) evaluated recombinant antigen of *S. mekongi* cathepsin B (rSmekcatB) for detecting the infection in mice. rSmekcatB showed 91.7% sensitivity and 100% specificity against the mice infection, however the antigen was not evaluated for its potential against *S. mekongi* infections in human.

TPxs are member of peroxidase important for antioxidant defense in schistosome parasites (Kwatia et al., 2000). TPxs help parasite to protect oxidative stress caused by a variety of peroxides and alkyl hydroperoxides in cells (Hong et al., 2013). Four types of TPx have been successfully cloned and characterized in *S. japonicum* including SjTPx-1, SjTPx-2, SjTPx-3 and SjPrx-4 (Kumagai et al., 2006; Dang-Trinh et al., 2020). Three TPxs including SmTPx-1, SmTPx-2 and SmTPx-3 have also been cloned and characterized in *S.*

mansoni (Kwatia et al., 2000; Sayed et al., 2006). Among TPxs of the parasites, TPx-1 of *S. japonicum* has been reported to be a potential antigen with high sensitivity and specificity in ELISA (Jin et al., 2010; Angeles et al., 2012; Macalanda et al., 2018). TPx-1 is expressed mainly in tegument of adult and larval worms of the parasite and that direct exposure to the host immune system makes the protein as a good antigen for serological diagnosis (Fonseca et al., 2012). In the present study, TPx-1 of *S. mekongi* was cloned and the recombinant protein was expressed for evaluation of its diagnostic potential as antigen in ELISA. Amino acid sequence of *S. mekongi* TPx-1 showed high identity of 93.5% with that of *S. japonicum* TPx-1, suggesting close relation between the two species according to the previous studies (Hofstetter et al., 1981; Upatham et al., 1987; Houston et al., 2004; Hirose et al., 2007).

rSmTPx-1 antigen showed good diagnostic performance in ELISA with high sensitivity, specificity, PPV, NPV and Kappa value against Kato-Katz technique (Table 5). To date, there were only a few studies on ELISA for the diagnosis of *S. mekongi* infection (Hinz et al., 2017). Soluble egg antigen of *S. mekongi* (SmekSEA) has been used in ELISA for diagnosis of the patients with 100% sensitivity and specificity (Kirinoki et al., 2011). However, SmekSEA showed cross-reactivity against other parasitic infections including ascariasis, echinostomiasis, opisthorchiasis and hookworm infection (Zhu et al., 2005; Kirinoki et al., 2011). Nickel et al. (2015) reported that soluble adult worm antigen (SWAP) prepared from *S. mansoni* showed 94.5% sensitivity in ELISA for diagnosis of *S. mekongi* infection in human. However, preparation of crude antigens such as SEA and SWAP is problematic due to observed low yields and poor quality control (Tanaka et al., 2021). In addition, wide and high cross reactions against other trematode infections were of concern when they are used in prevalence survey (Angeles et al., 2011). In this study, rSmTPx-1 antigen showed no cross reaction with serum samples from paragonimiasis patients. However, it showed 38% cross reactions with serum samples from opisthorchiasis patients

(Fig. 2). It is recommended that the amino acid sequence between SmTPx-1 and *O. viverrini* TPx-1should be compared in order to modify rSmTPx-1 by removing the peptide sequence with high identity between these parasite species which may trigger the cross reaction.

3-5. Summary

In the present study, *SmTPx-1*, the gene coding for *S. mekongi* TPx-1 was cloned and the recombinant protein, rSmTPx-1 was successfully expressed. rSmTPx-1 antigen showed higher sensitivity and specificity as compared to those of rSjTPx-1 antigen in ELISA with a panel of serum samples. A good agreement between ELISA with rSmTPx-1 antigen and Kato-Katz suggested that the ELISA can detect an active infection which requires treatment with praziquantel. The results suggested that rSmTPx-1 can be a potential antigen in ELISA for diagnosis of schistosomiasis mekongi in the regions where the disease is still endemic.

Fig. 6 Alignment of nucleotide and amino acid sequences of *Schistosoma mekongi* TPx-1 with *S. japaonicum* TPx-1 (AB126036.2) and *S. mansoni* TPx-1 (AF121199.1). The alignment was obtained by ClustalW. The identical nucleotides and residues between the sequences are boxed.

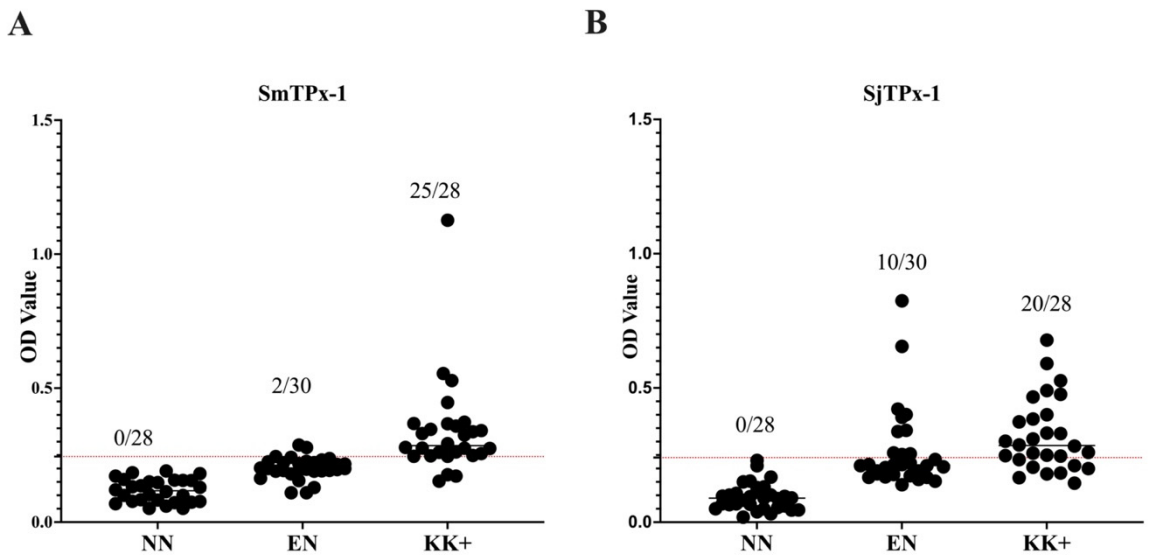

Fig. 7 Results of ELISA with rSmTPx-1 antigen (rSmTPx-1 ELISA) (A) and rSjTPx-1 ELISA (B). Panel of serum samples tested included non-endemic negative serum samples from USA volunteer (NN), endemic negative serum samples collected from individuals in Phnom Pehn (EN) and serum samples collected from patients confirmed positive by Kato-Katz technique (KK+). The cut-off OD values were calculated from the values of 28 NN as mean + 3SD and were presented by the red dotted lines. Numbers in the figures indicate number of samples with OD value higher than the cut-off values (positive samples)/number of samples tested.

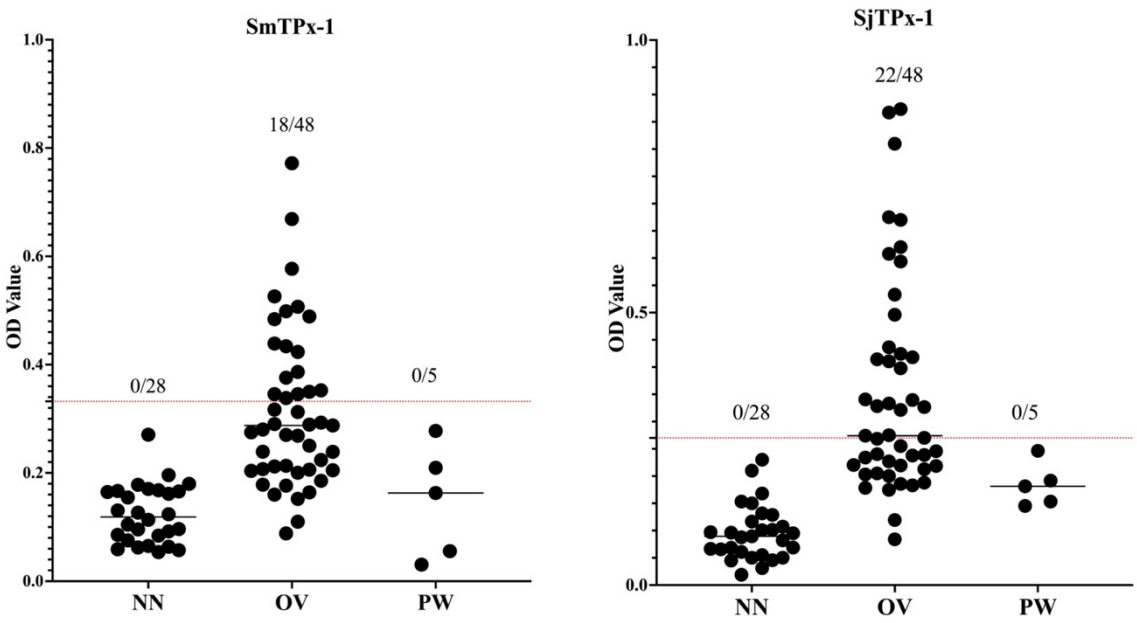

Fig. 8 Cross-reactions observed in rSmTPx-1 ELISA (A) and rSjTPx-1 ELISA (B) with non-endemic negative serum samples from USA volunteer (NN), serum samples obtained from patients infected with *Opisthorchis viverrini* (OV) and serum samples obtained from patients infected with *Paragonimus westermani* (PW). The cut-off OD values were calculated from the values of 28 NN as mean + 3SD and were presented by the red dotted lines. Numbers in the figures indicate number of samples with OD value higher than the cut-off values (positive samples)/number of samples tested.

Table 5. Statistical analysis of ELISA results with rSmTPx-1 and rSjTPx-1.

Antigen	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	K[a]
SmTPx-1	89.3	93.3	92.6	90.3	0.82
SjTPx-1	71.4	66.7	66.7	71.4	0.38

a: Kappa values were estimated against Kato-Katz technique.

General Discussion

Asian zoonotic schistosomiasis is caused by *S. japonicum* and *S. mekongi* infections. The WHO has targets to accomplish the elimination of this disease by 2025. The keys to achieving effective control and elimination of schistosomiasis are better understanding of the genetic diversity of schistosomes among definitive hosts and precise prevalence monitoring based on an accurate diagnosis of *Schistosoma* spp. infection. Thus, this study aims to modify the previous MHT to prepare single-genome DNA from the field samples of *S. japonicum* to be applied to a population genetics study with microsatellite markers and to develop recombinant antigen-ELISA to be applied for prevalence monitoring of *S. mekongi* infection in humans.

For the study of population genetic structure, unbiased sample collection is crucial for estimation of population indices. Utilization of adult worms from natural infections should be the means of sampling. However, field collection of adult schistosome worms from definitive hosts in the field is not feasible because they reside within the mesenteric veins of the host during infection. In addition, utilizing adult worms from experimentally infected laboratory animals may lead to the loss of true genetic diversity due to the immunological selection from the host (Shrivastava et al., 2005). Therefore, the miracidium larval stage of the parasite from a natural definitive host may be suitable for the preparation of single-genome DNA for microsatellite studies. Thus, the first chapter of this study aims to modify the MHT so as to simplify its setup in the field to recover miracidium samples for preparation of single-genome DNA. Several factors are considered to affect the *S. japonicum* MHT, including temperature, lighting conditions, salinity, and pH of the water. To our knowledge, the effects of different types of lighting and lighting intensity on the hatching process have not yet been compared (Ito, 1955; Kassim & Gibertson, 1976). In this chapter, modifications of an original MHT protocol (Jurberg et al., 2008) were made

to apply the technique for preparation of the single-genome DNA from miracidia under several conditions. The results of this study confirmed that lighting condition was important for miracidium hatching, with a high rate of miracidium hatching observed under the light condition, whereas only a few miracidia could hatch under the dark condition. However, earlier studies showed that there was no significant difference in hatching rates between light and dark conditions (Kassim & Gibertson, 1976; Sugiura et al., 1954; Xu & Dresden, 1990). This might be attributed to differences in experimental procedures or the schistosome strain used in each experiment. Interestingly, the egg samples that were kept under the dark condition for 5 days remained hatchable and were triggered to hatch upon exposure to light. These results indicated that the eggs maintained their viability under the dark condition. Thus, this condition can be used for storing eggs before hatching.

Studies on the effect of different types of light on the ability of miracidium to hatch were performed that included sunlight, fluorescent light, and halogen light conditions. The results showed for the first time, to our knowledge, that the sunlight condition was an effective illumination source for stimulating a greater rate of hatching in a shorter amount of time. The creation of an automated MHT that can significantly aid in the diagnosis and elimination of schistosomiasis may benefit from knowing the precise wavelength area in sunlight that best encourages miracidium hatching.

Microsatellite fragments were amplified by PCR to confirm whether the quality of single-genome DNA is appropriate for population genetics studies. Comparative studies between the genetic background of the parasite in each definitive host are important to understand epidemiology and transmission dynamics. The MHT protocol in this study will help in the sampling procedure for population genetics studies of *S. japonicum* by avoiding bias due to experimental host-induced selection and the bottleneck effect from the estimated population in the future.

S. japonicum genome data has been successfully assembled into a whole genome sequence, which has opened up possibilities for the further development and improvement of serological diagnosis using recombinant antigen-ELISA from excretory and secretory protein of *S. japonicum* (Angeles et al., 2020; Dang-Trinh et al., 2020; Feng et al., 2017; Jin et al., 2010; Macalanda et al., 2018). In contrast, there are only few studies on recombinant antigens for schistosomiasis mekongi diagnosis due to the lack of genome data on the parasite. Although these are antigens found in *S. japonicum,* they might also have the potential to be used in detecting *S. mekongi* infection due to these two species being closely related in terms of molecular and morphological identification according to previous studies (Houston et al., 2004). Therefore, the objective of the second chapter aimed to evaluate recombinant antigen-based serology from *S. japonicum* for the diagnosis of *S. mekongi* human infections. The results of this study showed that SjSEA crude antigen at a low concentration of 2 ng can be useful in the diagnosis of *S. mekongi* infection, thus making the laboratory production of SjSEA through animal infection more efficient. However, concerns regarding the cross-reactivity of crude antigens with other parasite species exist especially with trematode species prevalent in schistosomiasis mekongi endemic areas (Angeles et al., 2011; Kirinoki et al., 2011). Moreover, large-scale preparation of these antigens has proved to be difficult in terms of quality and quantity control. Although the recombinant antigen results were relatively inferior compared to the diagnostic sensitivity of SjSEA crude antigen at low concentrations, utilizing recombinant antigens for *S. mekongi* detection may still be more advantageous than crude antigen in terms of quality, quantity, and ease of production.

Schistosomiasis diagnosis in Lao PDR and Cambodia is mainly based on the Kato-Katz stool examination, which has high specificity and can detect current infection. However, its sensitivity is decreased when used under decreased prevalence and low

intensity of infection (Belizario et al., 2016; Knopp et al., 2009). The status of schistosomiasis mekongi in Lao PDR and Cambodia after the MDA programs showed that the prevalence of infection was decreased to less than 5% based on microscopy examination. This could affect the Kato-Katz technique because it might have low sensitivity for the detection of the parasite's egg. Thus, development of a diagnostic tool for high sensitivity, specificity, and accuracy is urgently needed for monitoring national surveillance after a MDA program. In areas where the disease has almost been completely eliminated, application of recombinant antigen-ELISA showed significant sensitivity and specificity in the diagnosis of schistosomiasis in humans (Dang-Trinh et al., 2020). The recombinant antigen SjTPx-1 showed adequate potential as a diagnostic antigen for detecting antibody by ELISA in humans and animals in previous studies (Angeles et al., 2011; Angeles et al., 2012b, 2020). Thus, the objective of this chapter aimed to express a recombinant antigen of *S. mekongi* TPx-1 (rSmTPx-1) and evaluate the antigen for detecting *S. mekongi* infection in humans by ELISA. The results of this study showed that recombinant antigen SmTPx-1 had better performance in ELISA with high sensitivity, specificity, PPV, and NPV. The ELISA showed a higher Kappa value against the Kato-Katz technique as compared to ELISA with recombinant antigen of *S. japonicum* TPx-1 (rSjTPx-1). TPx-1 is a member of the peroxidase protein family, which is important for antioxidant defense in schistosome species (Kwatia et al., 2000). It is expressed mainly in the teguments of adult worms and larval stages directly exposed to the host immune system. These factors make TPx-1 a good candidate antigen for the serological diagnosis of schistosomiasis.

The cross-reactivity of these recombinant antigens with samples collected from the patients with other helminthic infections was also an important factor. Thus, the recombinant antigens were tested with serum samples from patients infected with *O. viverrini*. The results showed that rSmTPx-1 had lower cross-reactivity with the samples

as compared to rSjTPx-1. I would recommend that the amino acid sequence between SmTPx-1 and *O. viverrini* TPx-1 be compared to modify rSmTPx-1 by removing the peptide sequence with high identity between these parasite species, which may trigger the cross reaction.

Taking advantage of rSmTPx-1 with ELISA for schistosomiasis detection will help assist in determining the actual status of infection and in assessing the effectiveness of the MDA programs, which may eventually lead to the elimination of Asian zoonotic schistosomiasis from Lao PDR and Cambodia. In addition, other recombinant antigens that have been reported as good antigens for detecting *S. japonicum* infection should be cloned, expressed, and evaluated for their diagnostic potential to detect *S. mekongi* infection in humans and reservoir animals such as dogs and pigs. Utilizing multiple antigens, such as cocktail antigens, could possibly improve the sensitivity and specificity of the ELISA (Dang-Trinh et al., 2020; Moendeg et al., 2015). Taking all of these results together, a common and reliable diagnostic method for detecting infections in multiple host species should be developed in a future study.

General summary

Schistosomiasis is a disease caused by blood flukes belonging to the genus *Schistosoma*. These parasites are endemic in 78 countries throughout the world wherein more than 240 million people suffer from the infection among over 700 million people at risk. Asian zoonotic schistosomiasis is caused by *S. japonicum* and *S. mekongi* infections. Their zoonotic nature leads to enhanced disease transmission, making schistosomiasis management challenging. Understanding the epidemiology and dynamics of the parasite's transmission between humans and reservoir animal hosts in the field will be aided by knowledge of the genetic diversity of *S. japonicum*. In addition, there is no vaccine available for schistosomiasis, and thus, disease control is mainly based on MDA with praziquantel. Diagnostic tools with high sensitivity and specificity are needed to accurately assess the status of the endemicity within a community and to monitor the efficacy of the MDA program. The general aim of this study is to develop a means to obtain this information to promote MDA towards elimination of Asian zoonotic schistosomiasis.

In Chapter 1, the previous MHT protocol was modified to apply the technique for preparation of *S. japonicum* single-genome DNA. The information regarding the genetic diversity of *S. japonicum* is indispensable to obtaining an accurate understanding of the epidemiology of schistosomiasis and the transmission dynamics of schistosomes among their definitive hosts. DNA samples originating from a single genome should be prepared to accurately examine the parasite's genetic diversity. The miracidium, a larval stage of the parasite, can be useful material for single-genome DNA preparation. In this study, the previous protocol for the MHT was modified with 96-well plastic ELISA plates to individually collect a miracidium for single-genome DNA preparation. In addition, the effects of light conditions on hatching rates were evaluated. The results showed that the

highest hatching rate was observed under the sunlight condition (92.4%), followed by fluorescent light (88.0%). The lowest hatching rate was recorded under the dark condition (4.7%). These results indicated for the first time, to our knowledge, that sunlight was the most efficient light source for the MHT. Furthermore, the study confirmed that a 0.85% NaCl solution and the dark condition prevented miracidium hatching and could be used to store the eggs until the MHT is conducted. In addition, successful amplification of 18S rRNA gene and microsatellite markers from DNA isolated from a single miracidium also confirmed the quality of the single-genome DNA for subsequent application.

In Chapter 2, recombinant antigen-based serology from *S. japonicum* was evaluated for the diagnosis of *S. mekongi* human infections. To date, no recombinant antigen has been assessed using ELISA for the detection of *S. mekongi* infection due to the lack of genome information of this parasite species. In this study, several recombinant *S. japonicum* antigens that had been developed previously were evaluated for the detection of *S. mekongi* infection. ELISA results showed that *S. japonicum* SEA at low concentration showed better diagnostic performance than the recombinant antigens tested using the archived serum samples from Cambodia. Because recombinant antigen has several advantages over crude antigen in the application with ELISA, further optimization of the recombinant antigens should be done in future studies to improve their diagnostic performance for detecting *S. mekongi* infection in humans.

In Chapter 3, recombinant antigen of *S. mekongi* TPx-1 (rSmTPx-1) was expressed, and the antigen was evaluated for its performance in detecting *S. mekongi* infection in humans by ELISA. Decades of use of numerous control measures, which include MDA using praziquantel, have resulted in a decrease in the prevalence of schistosomiasis mekongi. This, however, has led to a decrease in sensitivity of the Kato-Katz stool examination, which is considered the gold standard in the diagnosis of schistosomiasis

mekongi. In this study, a gene coding for *S. mekongi* TPx-1 was cloned, and the recombinant antigen (rSmTPx-1) was expressed to develop a serological assay with high sensitivity and specificity that could replace the Kato-Katz technique. Diagnostic performance of rSmTPx-1 as an antigen in ELISA for detecting human schistosomiasis was compared with that of the recombinant antigen of *S. japonicum* TPx-1 (rSjTPx-1) using a panel of serum samples collected from endemic foci in Cambodia. The sensitivity and specificity of rSmTPx-1 in ELISA were 89.3% and 93.3%, respectively, whereas those of rSjTPx-1 were 71.4% and 66.7%, respectively. In addition, the higher Kappa value of 0.82, which was calculated between ELISA with rSmTPx-1 and Kato-Katz, confirmed better agreement between them than between ELISA with rSjTPx-1 and Kato-Katz (Kappa value 0.38). These results suggested that ELISA with rSmTPx-1 could be a potential diagnostic method for detecting an active human *S. mekongi* infection.

In conclusion, this study has proposed two useful tools for disease surveillance and assessment of the efficacy of an MDA program that can lead to the elimination of schistosomiasis. The modified MHT can be used in field studies for recovering miracidium to prepare single-genome DNA for population genetic studies in the future. Application of additional recombinant *S. mekongi* antigens with ELISA might have a potential role in developing a reliable and accurate diagnostic test for detecting *S. mekongi* infections in humans and reservoir animals in the future.

和文要約

住血吸虫症は，*Schistosoma* 属の吸虫によって引き起こされる寄生虫病で，世界 78 ヶ国で流行が認められる。世界人口の 7 億人以上がこの病気への感染の危険の下で日々の生活を営み，また，そのうちの 2 億 4,000 万人以上がこの寄生虫病の症状に苦しんでいる。アジアに認められる人獣共通性の住血吸虫症（アジア型住血吸虫症）は，日本住血吸虫（*S. japonicum*）及びメコン住血吸虫（*S. mekongi*）の感染によって引き起こされ，ヒトと保虫宿主動物の間でも感染が成立することから，病気の伝搬が加速され，対策がより難しくなっている。日本住血吸虫の遺伝的多様性に関する知見は，野外での寄生虫ライフサイクルにおけるヒトと保虫宿主の相互関係や，寄生虫病の疫学を理解する上で重要な情報になる。一方，住血吸虫症に対するワクチンは未だ開発されていないことから，第一選択薬 Praziquantel（PZQ）を用いた流行地住民への集団投薬（mass drug administration: MDA）が，住血吸虫症の流行が認められる国と地域での主要な寄生虫病対策になっている。MDA を成功裏に推進するためには，流行地住民における住血吸虫症の流行状態を正確に把握し，また MDA の効果を査定するための，高感度・高精度の診断法の導入が必要になる。そこでこの研究では，アジア型住血吸虫症の排除に向けた取り組みに必須となる情報を得るための手法として，寄生虫の集団構造解析に使用するシングルゲノム DNA の調整を目的としたミラシジウムふ化法の改良及び，メコン住血吸虫症の患者の診断を目的とした ELISA の開発を行った。

第 1 章では，日本住血吸虫の集団構造解析で使用するシングルゲノム DNA の調整を目的としたミラシジウムふ化法（MHT）の改良を行った。寄生虫の遺伝

団的多様性に関する情報は，住血吸虫症の疫学及び，保虫宿主嗜好性など寄生虫のライフサイクルを理解するのに不可欠な情報になる。寄生虫の遺伝的多様性を正確に調べるには単一のゲノムから調整した DNA を準備する必要があり，虫卵内に形成される幼虫ミラシジウムが有用な材料になる。そこで，シングルゲノム DNA 調製用のミラシジウムを効率的に収集するため，大型の専用フラスコを用いる既存のラシジウムふ化法を，96 穴プラスチック ELISA プレートでの簡易法に改良した。更に，ミラシジウムのふ化率に与える光照射の影響についても検討を加えた。その結果，日光（太陽光）下でミラシジウムのふ化を誘導した時に最も高い孵化率（92.4%）が，蛍光灯下で誘導した時に次に高いふ化率（88.0%）が，また，虫卵を暗所に置いた時に最も低いふ化率（4.7%）が得られた。これらから，通常は蛍光灯下行う MHT を日光下で行っても，効率的にミラシジウムのふ化が誘導できることが初めて明らかになった。また，虫卵を 0.85% NaCl 溶液中で暗所に置くとミラシジウムのふ化が抑えられることも確認され，この条件が虫卵の保存に有用なことも解った。シングルゲノム DNA を鋳型にした PCR では，18S rRNA 遺伝子及びマイクロサテライトメーカー遺伝子が増幅され，MHT の改良法が寄生虫の集団構造解析の研究にも十分応用可能であることが確認できた。

第 2 章では，日本住血吸虫由来の抗原を応用して，メコン住血吸虫症を診断する ELISA の開発を行った。メコン住血吸虫症では，メコン住血吸虫のゲノム情報の不足から，未だ組換体抗原を応用した ELISA が開発出来ていない。そこで，これまでに開発された日本住血吸虫由来の組換体抗原及び虫卵由来の可溶性粗抗原（SEA）の，メコン住血吸虫症を診断する ELISA における有用性を評価した。カンボジア人患者及び虫卵検査（Kato-Katz 法）陰性の流行地住民から採取した

血清のパネルを用いて検討した結果，低濃度 SEA を抗原とする ELISA に，組換体抗原を用いる ELISA を上回る優れた診断性能が認められた。一方，組換体抗原を用いる ELISA は，抗原の大量供給と品質管理が容易で，大規模調査への応用も可能になる。ELISA 用組換体抗原の改良と開発が待たれるところである。

第 3 章では，メコン住血吸虫由来の組換体抗原を作製して，メコン住血吸虫症の診断を目的とした ELISA に応用した。数十年に及ぶ MDA の結果，メコン住血吸虫症流行地では，この寄生虫病の有病率が減少すると同時に感染の強度も低下したことで，Kato-Katz 法の感度が低下している。そこで，Kato-Katz 法に替わる高感度及び高特異性の ELISA を開発するため，メコン住血吸虫のチオレドキシンペルオキシダーゼ-1（SmTPx-1）遺伝子を単離して，組換体タンパク質（rSmTPx-1）を大腸菌で作製した。カンボジア人患者及び Kato-Katz 法陰性の流行地住民から採取した血清のパネルを用いて検討した結果，rSmTPx-1 抗原を用いた ELISA の感度と特異性は，それぞれ，89.3%と 93.3%であった。一方，日本住血吸虫由来の組換体タンパク質（rSjTPx-1）を抗原として用いた ELISA の感度と特異性は，それぞれ，71.4%と 66.7%であった。rSmTPx-1 抗原を用いた ELISA と Kato-Katz 法間のカッパ係数（κ 係数）は 0.82 で，両者には高い相関が認められた。一方，rSjTPx-1 抗原を用いた ELISA と Kato-Katz 法間の κ 係数は 0.38 であった。このことから，rSmTPx-1 抗原を用いた ELISA では，虫卵を排泄する Active な患者の検出が可能なことが示唆された。

本研究では，住血吸虫症の排除に向けた，疾病流行状況の監視と MDA 効果の査定に有用な二種類の手法を提案することが出来た。改良型の MHT を野外材料からのシングルゲノム DNA の調整に応用することで，寄生虫のライフサイクル解

明に有用な，遺伝団的多様性に関する情報の入手が可能になる。また，rSmTPx-1 以外の組換体抗原を ELISA に応用することで，メコン住血吸虫症の患者と保虫宿主動物での寄生虫感染を検出可能な，高感度及び高特異性の血清診断法の開発が可能になる。

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