brucei SDH4 needs to be tested in future studies. It is also possible that trypanosomatid-specific subunits could be assembled as a jigsaw puzzle-like membrane anchor.

Spectroscopic Properties of T. cruzi Complex II—Pyridine ferrohemochrome analysis showed that *T. cruzi* Complex II binds a stoichiometric amount of protoheme IX (0.85 heme/monomer of enzyme) indicating that monomer enzyme complex contains one heme. At room temperature, the air-oxidized and fully reduced forms of the purified enzyme showed peaks at 413 and 426, 527, and 561 nm, respectively (Fig. 6). Peak positions are similar to those reported for Complex II from *E. coli* (40), adult *A. suum* (41), and bovine (42, 43), where heme is ligated via histidine in the second helices of SDH3 and SDH4. Although heme has an important role in the assembly of Complex II, it is not essential for the reduction of ubiquinones (43, 44).

Enzymatic Properties of T. cruzi Complex II—We examined SQR activity of the purified enzyme and found the difference in apparent K_m values between Q_1 (33.9 ± 3.6 μM) and Q_2 (18.8 ± 6.4 μM) (Fig. 7), indicating that the 6-polyprenyl group of ubiquinone contributes to the binding affinity. The apparent V_{max} value of the *T. cruzi* Complex II was rather constant, 11.9 ± 2.2 for Q_1 and 11.5 ± 0.4 Q_2 units/mg proteins, respectively, and one-fourth of those reported for bovine and *E. coli* enzymes (45, 46). This is not surprising because *T. cruzi* complex II has about 2–3 times more proteins than the other enzymes. K_m values for ubiquinone and succinate (18.8 ± 6.4 μM (Q_2) and 1.48 ± 0.17 mM, respectively) were higher than 0.3 and 130 μM, respectively, of bovine enzyme (45), and 2 and 277 μM, respectively, of the *E. coli* enzyme (46, 47). Notably, the K_m value for succinate was comparable with 610 μM in adult *A. suum* (10), which expresses the stage-specific Complex II as quinol:fumarate reductase under hypoxic habitats in host organisms.

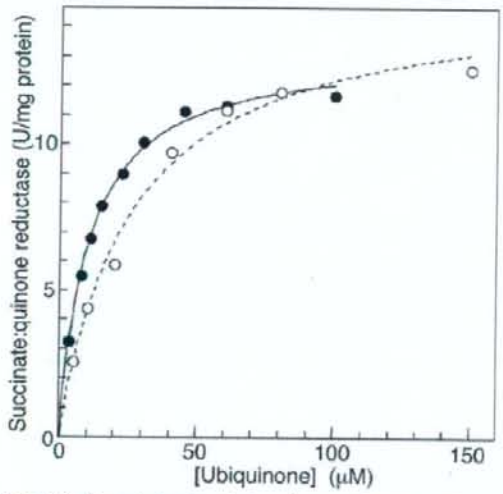

FIGURE 7. **Kinetic analysis of succinate-quinone reductase activity.** Succinate:ubiquinone reductase activity of the purified Complex II was determined with Q_1 (○) and Q_2 (●) at a protein concentration of 1.25 μg/ml in the presence of 10 mM sodium succinate. Data were fitted with the Michaelis-Menten equation using KaleidaGraph, and apparent K_m and V_{max} values were 30.3 ± 4.3 μM and 14.0 ± 1.2 units/mg protein, respectively, for Q_1 and 12.4 ± 0.7 μM and 11.9 ± 0.3 units/mg protein, respectively, for Q_2.

Then we examined effects of inhibitors for binding sites of quinones and dicarboxylates on SQR activity. Atpenin A5, a potent inhibitor for Complex II, inhibited the *T. cruzi* enzyme with the IC_{50} value of 6.4 ± 2.4 μM, which is 3 orders of magnitude higher than that of bovine Complex II (4 nM) (48). Furthermore, carboxin, 2-theonyltrifluoroacetone, plumbagin, and 2-heptyl-4-hydroxyquinoline *N*-oxide were ineffective (100 μM $<IC_{50}$). Structural divergence in trypanosomatid SDH3 and SDH4 could be the cause for lower binding affinities for both quinones and inhibitors. In addition, we found for the dicarboxylate-binding site that the IC_{50} value for malonate (40 μM) was much higher than the K_i value for bovine Complex II (1.3 μM) (45).

Structure of Trypanosomatid Complex II—To the best of our knowledge, this is the first report on the isolation of protist Complex II. *T. cruzi* Complex II has unusual subunit organization with six each of hydrophilic and hydrophobic subunits. Such a supramolecular structure and heterodimeric SDH2 ($SDH2_N$ and $SDH2_C$) are conserved in the Trypanosomatida. Furthermore, SDH1, $SDH2_N$, $SDH2_C$, SDH3, SDH4, and SDH8–SDH10 can be identified in the ongoing genome projects on the evolutionary relatives, the photosynthetic free-living *Euglena gracilis*, and the nonphotosynthetic euglenoid *Astasia longa* in the Euglenida. Thus a part of these features are common in the Euglenozoa, a divergent lineage of eukaryotes (Fig. 8).

Accumulation of noncatalytic subunits through expanding the protein interaction network could be a driving force for protein evolution. Structural and catalytic features are unique, and thus this enzyme could be a potential target for novel chemotherapeutic agents for trypanosomiasis and leishmaniasis.

Conclusion—The parasitic protist *T. brucei* is a gold mine where unprecedented biological phenomena like RNA editing and trans-splicing in mitochondria were originally discovered. It was found recently in *Diplonema papillatum*, a free-living evolutionary cousin,

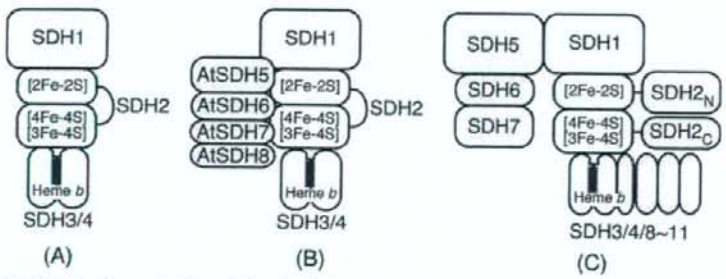

FIGURE 8. **Subunit organization of Complex II.** *A*, common four-subunit Complex II (*e.g.* mammals, *E. coli*); *B*, eight-subunit Complex II in plants (*e.g. A. thaliana*); and *C*, 12-subunit Complex II in the Trypanosomatida. Noncatalytic subunits and domains are shown in *yellow* and heme in *red*.

that mature mRNA for cytochrome *c* oxidase CoxI was assembled from nine gene fragments by a jigsaw puzzle mechanism (49). From a characterization of Complex II from *T. cruzi*, we revealed a novel supramolecular organization, which is conserved in the Trypanosomatida.

Parasites have exploited unique energy metabolic pathways as adaptations to their natural habitats within their hosts (50, 51). In fact, the respiratory systems of parasites typically show greater diversity in electron transfer pathways than those of host animals. As shown in this study, such is also the case with Complex II, which is a well known marker enzyme of mitochondria. Studies on the role of supramolecular Complex II in adaptation of trypanosomatids is now underway in our laboratory.

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肝胆道系酵素の測定は，住血吸虫症の診断に役立つのか

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Key Words：肝胆道系酵素，住血吸虫症，肝線維化

はじめに

現在わが国で行われる健康診断では，肝機能検査として，aspartate aminotransferase（AST，GOT），alanine aminotransferase（ALT，GPT），alkaline phospahatase（ALP），gamma-glutamyl transpeptidase（γ－GTP，GGT）といった肝胆道系酵素が計測されることが多い。また，これらの検査項目は，渡航者の帰国後健診や在日外国人を対象とした健診にも含まれるのが一般的である。

肝機能障害を起こす日本住血吸虫症やマンソン住血吸虫症では，従来，急激に発症する例や肝肥大をきたすような例を中心に，ASTやALP，γ－GTPなどが上昇することが多いと報告されてきた[1,2]。しかし，最近の国内の住血吸虫症報告例では，無症状で経過し画像検査などで偶然発見されるような例も多い[3]。世界的にみても，対策の一環としてプラジカンテルによる集団治療が積極的に行われるようになって以来，多くの浸淫地で，住血吸虫によるmorbidityは改善しており，肝胆道系酵素の上昇を示すような例は，以前に比して減少していると思われる。そこで，住血吸虫症診断における肝胆道系酵素計測の意義を日本住血吸虫症浸淫地で集団的治療が本格的に行われた前後で比較し，あわせて実験的・文献的考察も行った。

対象・方法

フィリピン，レイテ島の日本住血吸虫症浸淫地では，1990年代中半からプラジカンテルによる集団的治療が本格化した。そこで，1991年8月および2000年8月に，レイテ島，パロのSchistosomiasis Research Hospitalを受診し，糞便検査によって診断

Diagnostic Value of Serum Enzyme Tests in Schistosomiasis

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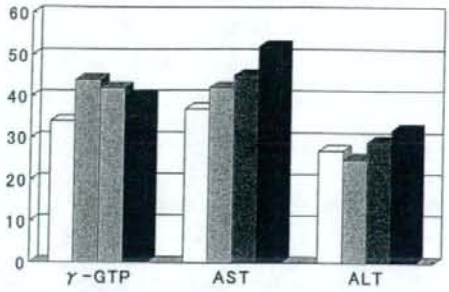

図1a　日本住血吸虫感染者における血清中のγ-GTP，AST，ALTと肝線維化
（1991年の調査結果）

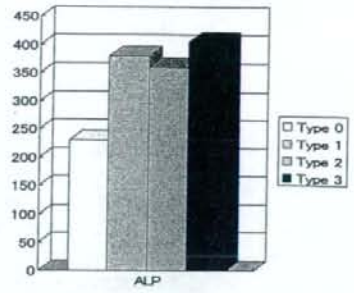

図1b　日本住血吸虫感染者における血清中のALPと肝線維化
（1991年の調査結果）

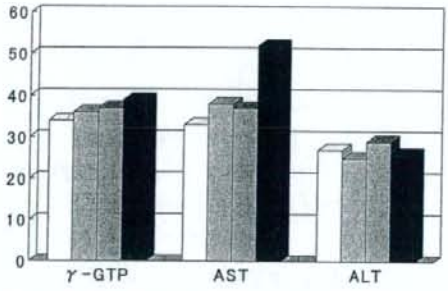

図2a　日本住血吸虫感染者における血清中のγ-GTP，AST，ALTと肝線維化
（2000年の調査結果）

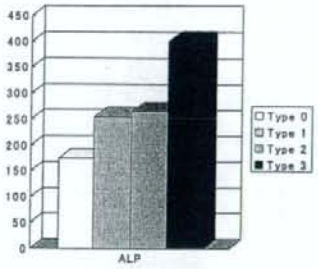

図2b　日本住血吸虫感染者における血清中のALPと肝線維化（2000年の調査結果）

された18歳以上の日本住血吸虫感染者を対象として比較した。書面で承諾を得た後，採取された血液を用いて肝胆道系酵素の変動を調べるとともに，腹部超音波検査もあわせて行い，肝線維化の進行と肝胆道系酵素の変動の関係について検討した。対象者は，1991年118人，2000年131人で，男女比は，両期間ともおよそ2：1となった。また，HBs抗原陽性者とHCV抗体陽性者，問診で過度のアルコール摂取歴が疑われた例は，対象から除外された。肝線維化に関しては，腹部超音波検査の結果により，ほとんど線維化所見がみられないType 0：Normal patternから，進行した不可逆的線維化とされるType 3：Network pattern（網目状パターン）まで，4段階に分類された[4]。

結　果

図1と2には，おのおのの肝胆道系酵素について，1991年，2000年の検査結果の平均値を肝臓の超音波パターン毎に示した。1991年，2000年とも，日本住血吸虫感染者で，γ－GTP（正常域：10～50 IU/l）やALT（正常域：6～43 IU/l）の上昇を示した例は，ほとんどみられなかった（図1a，2a）。一方，AST（正常域：11～40 IU/l）とALP（正常域：80～260 IU/l）は，1991年の時点では，上昇を示した例が多く，特に超音波検査で進行した肝線維化と判断される例ほど，異常値となることが多かった（図1a，b）。ところが，2000年に行った調査では，超音波検査でType 3：Network patternを示すような，進行した肝線維化と診断された例でのみ，ASTやALPの上昇がみられた（図2a，b）。

表1　住血吸虫感染における肝胆道系酵素の意義（1980年以降の主な調査結果）

	対　象	結　果	調　査　者	調査・報告年度
マンソン住血吸虫症	浸淫地住民	感染者では，AST，ALPが高値を示す例が多い。	Mansour MM, *et al.*	Trans R Soc Trop Med Hyg. 1981 Trans R Soc Trop Med Hyg. 1997
	浸淫地住民	299人の感染者中，約30%でALPや胆汁酸が高値。	田邊将信 他	
	浸淫地住民	肝線維化が進んだ例やC型肝炎合併例で，AST，ALT，ALP，GGTが高値。	Fahim FA, *et al.*	Dis Markers, 2000
	渡航時日本人感染者	抗体陽性者5例中，異常値を示した例は0。	前田拓哉 他	2008年度日本寄生虫学会臨床検討会
日本住血吸虫症	浸淫地住民（病院受診者）	何人かの異常値を示す例がみられるが，10年間でかなり減少。	大前比呂思 他	(1991年調査) (2000年調査)
	浸淫地住民（住民検診）	ミンドロ島で174人の感染者のAST及びALTを調べ，異常者は0。	千種雄一 他	2002年度日本寄生虫学会 (2001年調査)
メコン住血吸虫症	浸淫地住民（住民検診）ただし全員が有症状者	AST異常：7/32名 ALT異常：1/32名	大竹英博 他	2000年度日本熱帯医学会 (1999年調査)

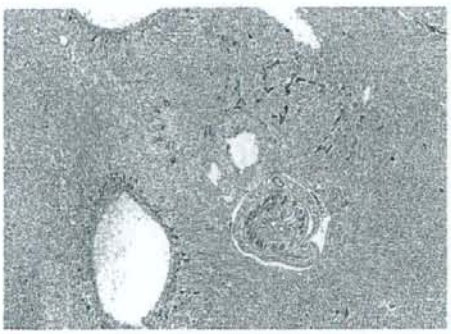

図3　日本血吸虫感染9週後のマウス肝臓病理組織標本（マッソン・トリクローム染色）

考　察

マウスに日本住血吸虫を感染させると，感染9週を過ぎたあたりから，門脈域を中心とした肝線維化（Periportal liver fibrosis）がはっきりしてくる（図3）。この線維化は虫卵性肉芽腫の周囲に顕著で，抱合した雌雄成虫に周囲では目立たない。また，図3の写真でも，肝内門脈に隣接する肝内胆管の周囲に線維化病変が及んでいるが，このような病変によって生じる慢性的胆管炎の結果，肝胆道系酵素が上昇すると言われている。

住血吸虫感染者で肝胆道系酵素を計測した1980年以降の主な報告例をまとめてみると，結果は，調査時期や対象者の違いによって様々である（表1）。総じて1980年代，1990年代前半の調査では，マンソン・日本住血吸虫症とも，肝胆道系酵素（特にASTとALP）が上昇していたとの報告が多い[2),4)]。一方，1990年代後半以降の調査になると，ウイルス肝炎と合併した住血吸虫症では，様々な肝胆道系酵素の上昇が確認されるが，ウイルス肝炎を合併していない例では，肝胆道系酵素の異常を示すことは少ない[5)]。また，病院受診者を対象とした今回の調査によると，肝線維化の進んだ例では，ウイルス肝炎を合併していなくてもASTの上昇を示したが，検診を受診した無症状者を主な対象としたフィリピン，ミンドロ島の日本住血吸虫浸淫地での調査では，174人の感染者のうち，ASTやALTの異常を

示した例は全く認められなかった（表1）[6]。

また，わが国におけるマンソン住血吸虫症や日本住血吸虫症の輸入例でも，最近は肝胆道系酵素の上昇を示す報告は少ない[3]。最近の集団治療を中心とした対策によるmorbidity改善により，住血吸虫浸淫地で，肝胆道系酵素の異常を示す感染例が減少した結果が反映されたことによると思われる。一方，日本人で海外渡航時の住血吸虫感染が疑われた例でも，最近の報告では，好酸球増多や肝胆道系酵素上昇を示す例はほとんどいない[7]。もっとも，初感染後に肝肥大を示す例や典型的な症状（Katayama fever）を示す例では，最近の報告でも従来と同様，好酸球増多と並んで肝胆道系酵素の上昇が報告されている[2][8][9]。住血吸虫症浸淫地で初感染する場合も，最近は大量の住血吸虫セルカリアに同時に暴露される可能性が，以前に比して相対的に減少しており，結果として，渡航者の初感染で典型的症状を示す例が減少しているのかもしれない。

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The evaluation of control measures against *Schistosoma mekongi* in Cambodia by a mathematical model

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ABSTRACT

We constructed a mathematical model for the transmission of *Schistosoma mekongi* in Cambodia. The simulation of the model will be instrumental in planning schistosomiasis control measures. The model includes two definitive hosts, humans and dogs, as animal reservoirs. Dogs are recognized to play an important role in schistosomiasis transmission in Cambodia. For the purpose of dealing with age-specific prevalence and intensity of infection, the human population was classified into eight age categories in the model. To describe the seasonal fluctuation of the intermediate host population of *S. mekongi*, the "Post-Spate Survival" hypothesis was adopted for the population dynamics of *Neotricula aperta* present in the Mekong River. We carried out simulations to evaluate the effect of universal treatment (UT) and targeted mass treatment (TT) with praziquantel on the reduction in prevalence of *S. mekongi*. The simulations indicated that biyearly UT for 8 years or yearly TT for 5 years after three courses of yearly UT could reduce the prevalence to below 5% when a UT or TT coverage of 85% of inhabitants was achieved. The simulation suggested that the suppression of *S. mekongi* in Cambodia would be possible by UT or TT with a high coverage rate.

1. Introduction

Schistosomiasis mekongi is prevalent in the Mekong River basin from the Khong district in southern Laos to Kratie province in northern Cambodia. The total population at risk for schistosomiasis mekongi is estimated as 60,000 in Laos and 80,000 in Cambodia [1].

Schistosoma mekongi can be parasitic in various mammalian hosts such as humans, dogs, and pigs [2]. *Neotricula aperta*, an aquatic snail, is known to be the intermediate host of *S. mekongi* [3]. It was observed that the water level of the Mekong River fluctuates seasonally; the period of low water lasts from February to May, while that of high water lasts from June to January. The transmission of *S. mekongi* from snails to humans occurs during the low water period because water contact of humans is practicable [1].

In Cambodia, a control program of annual mass drug administration was initiated by the Ministry of Health, Cambodia and Médicins Sans Frontières in 1995 (present program conductor: National Center for Parasitology, Entomology and Malaria Control) [4]. Sasakawa Memorial Health Foundation (SMHF) joined the cooperative program in 1997, and mainly took charge of examination of animal reservoirs, serodiagnostic surveys, and evaluation of morbidity using ultrasound. The control programs in Cambodia are considered to be successful because of the low level of detection of egg positive cases in recent years, although there remains a high positive rate by ELISA in several villages where *S. mekongi* is endemic [5]. In Laos, the average prevalence of schistosomiasis mekongi among the villages decreased to less than 1% after six courses of mass treatment with praziquantel during a 10-year control program, which resulted in a cessation of the control program in 1999 [6]. Thereafter, the resurgence of schistosomiasis in the Khong district of Laos was confirmed by epidemiological surveys by WHO in 2003 [7], and it was revealed that the prevalence was restored to 20–50% in the same area [8]. The situation of re-emergence of *S. mekongi* in Laos indicates the necessity for the continuation of both surveillance and control programs, which are required in order to adopt more cost-effective measures, in Cambodia despite the low rate infection of *S. mekongi* [4].

A mathematical model is useful to predict of the effect of various control measures on suppression of infectious diseases. Macdonald [9] first proposed a mathematical model for the transmission of schistosomiasis, and thereafter a number of mathematical models for schistosomiasis transmission have been published [10–14]. Chan and Bundy [15] constructed an age-structured model for *Schistosoma*

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mansoni transmission to predict the prevalence and morbidity for the long-term consequences of drug treatment. Ishikawa et al. [16] developed a model of *Schistosoma japonicum* transmission that took account of a seasonal variation of snail density to predict the effect of control measures against *S. japonicum* in the Philippines. We previously proposed a mathematical model for the transmission of *S. mekongi* in Cambodia that was described by a system of partial differential equations of time and age, which was aimed at estimating the coverage rate and range of ages in targeted mass treatment to interrupt schistosomiasis transmission [17].

In this study, we constructed a mathematical model for *S. mekongi* transmission to evaluate the effect of control measures in Chatnaol village in Cambodia. We incorporated the fluctuation of water level in the Mekong River, dynamics of the intermediate snail host population, and the contribution of an animal reservoir, dogs, to the prevalence of *S. mekongi* into the model. We applied the dynamics of the intermediate snail host based on the Post Spate Survival hypothesis [18]. In the model, snails that survive during the high water period of the Mekong River start to lay eggs from January, and afterwards an abundance of new-born snails appear in the low water period in April–May, when the transmission of *S. mekongi* occurs mainly. In Cambodia, dogs are known to play an important role as an animal reservoir in *S. mekongi* transmission [19]. Therefore, there were two kinds of definitive hosts in the model, humans and dogs. The parameter values in the model were estimated by field data or experimental data. The human population in the model was divided into 8 age categories because the prevalence and the intensity of infection are strongly dependent on age.

We focused on simulations of the transition in the prevalence of *S. mekongi* in a village together with the execution of control measures for humans. An application of molluscicide against *S. mekongi* appeared to be ineffective in the Mekong River basin [6]. The simulation results showed that a biyearly universal treatment or a yearly targeted mass treatment for children 5–19 years old with a 85% coverage rate, which was more effective than a yearly universal treatment with a 70% coverage rate, could sustain a low prevalence in humans after three courses of yearly universal treatment. Health intervention for 8 years, which is presumed to reduce both a probability of water contact and an amount of fecal output of humans to 50%, would make the prevalence of *S. mekongi* in both humans and dogs reduce to half. The simulations predicted that the suppression of schistosomiasis would be possible in Cambodia by maintaining control strategies for humans such as biyearly universal treatment or yearly targeted mass treatment with a 85% coverage rate.

2. Materials and methods

2.1. Study area

Kratie province is located on northern Cambodia where the Mekong River runs from north to south. The population at risk of schistosomiasis mekongi was estimated to be about 50,000 in the province [20].

In Cambodia, universal treatment with praziquantel has been conducted annually since 1995 (except for 1998 because of a luck of funds and 2003 when targeted mass treatment for ages of 6–22 years-olds was applied) [4,20]. Annual parasitological surveys were conducted in Achen, Chatnaol, Srekoeun, and Sambok, which served as sentinel villages, reported that the prevalence of *S. mekongi* in these villages decreased from 50–70% in 1994 to less than 5% in 2002 [4].

In this study, we chose Chatnaol as the study area where the population was about 500 in 1999. The average prevalence and intensity of infection were estimated as approximately 52% and 115 eggs per gram of stool, respectively, in 1994–1995 before the launching of control programs in Cambodia [21]. The age-dependent prevalence and intensity of infection showed a peak in the age group of 10–14 years-old [21].

2.2. Water level of the Mekong River

The rainy season begins in March in Cambodia, and heavy rainfall lasts from June to October (Fig. 1). The rainfall dramatically drops in November, and thereafter the dry season lasts from December to February.

The heavy rainfall in June results in rising water levels in the Mekong River, so the high water period begins in June. The water level reaches a peak during September–October. After the arrival of the dry season, the water level drops gradually, and the low water period begins in February (Fig. 1).

It is recognized that the available transmission period for *S. mekongi* begins in February when water contact of humans is practicable [1]. We determined that the low water period lasts from February to mid-May on the basis of water level data in Kratie province from 1989–2002 measured by the Mekong River Commission (Fig. 1), when water contact and water contamination of the definitive hosts can occur.

2.3. Life cycle of S. mekongi

2.3.1. Definitive hosts

Schistosomes can infect various mammalian hosts including humans. Due to the involvement of animal reservoirs with schistosomiasis transmission, human chemotherapy alone is insufficient to reduce the prevalence of infection [22]. Dogs and pigs have been known to act as animal reservoirs for *S. mekongi* [23,24]. In Laos, the prevalence in dogs was estimated at 11% [23] and 29.2% [25]. SMHF has conducted several surveys to detect animal reservoirs in Cambodia by stool examinations, which revealed that dogs were the definitive host of *S. mekongi* [5,19]. Despite the low prevalence in dogs, one infected dog showed high egg density in its feces [19]. We consider dogs to be definitive hosts besides humans in the model.

Cercarial penetration of an individual through the skin can occur when in contact with the water of the Mekong River. A pair of adult worms commences egg production 4–6 weeks after invasion [26]. The life span of a worm is estimated at 3–5 years [10]. In this study, we supposed that the duration of infection in definitive hosts is 5 years.

2.3.2. Intermediate hosts

Neotricula aperta, which is composed of three strains (α, β, and γ) is recognized as the intermediate host of *S. mekongi* [2,27]. *N. aperta*, which is penetrated by a miracidia releases cercariae after a latent period of 45–53 days [28]. Thus, we adopted 6 weeks as the latent period in the model. Experimental studies with *N. aperta* showed that the mortality per week was approximately 1.8% [29] to 2.1% [30]. It

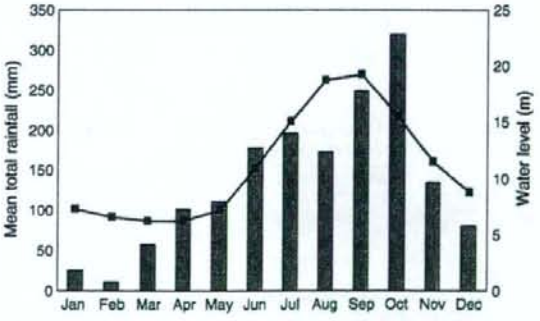

Fig. 1. Monthly average rainfall levels (bars) for 5 years during 1997–2001 in Phnom Penh [World Weather Information Service] and monthly average water levels of the Mekong River (line) for 14 years during 1989–2002 in Kratie province, Cambodia [Mekong River Commission].

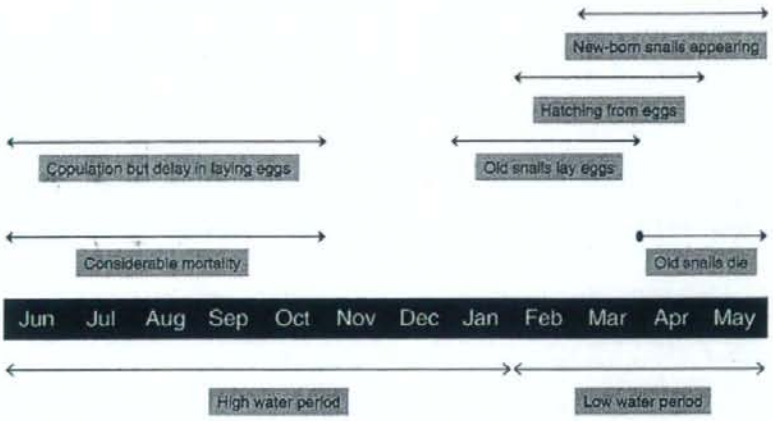

Fig. 2. Population dynamics of *N. aperta* on the basis of the Post-Spate Survival hypothesis.

was confirmed that for the other schistosome species there is a significant difference in mortality among negative and infected snails. [31]. However, such a difference was not observed for *S. mekongi* [32]. In this study, we assume that the mortality of infected snails is equivalent to that of negative snails, and that the value of the mortality rate (d) was estimated at about 2% per week. Due to the fact that infection rate of *N. aperta* in the field is very low, 0.22% [29] to 0.14% [30], we held the infection rate below 1% at all times in the model.

The biology of *N. aperta* is still largely unknown because of the impracticality of field observations during the high water period of the Mekong River, although the population dynamics of the snails will affect schistosomiasis transmission. To represent the population dynamics of *N. aperta*, we adopted a "Post-Spate Survival (PSS)" hypothesis that *N. aperta* survive and copulate during high water period of the Mekong River, but that laying eggs would be delayed until next January, and that thereafter the eggs would hatch from February [18]. Fig. 2 shows briefly the life cycle of *N. aperta* based on this PSS hypothesis. The snail population is divided into two age-groups, old and new-born snails. New-born snails survive during the high water period, June to October, by sticking to rocks [33]. It is accepted that the severe water conditions cause considerable mortality in snails during this period [34]. The proportion of females to males (ξ) is estimated to be about 0.67. New-born snails that pass the year-end join the old snails group. The number of eggs produced per female per month (b_v) is approximately 10 [35]. Old snails may die out in late March, because of exhaustion following a period of prolonged egg-laying. Eggs begin to hatch in February after a 4–5 week incubation period [36]. There are no data available about the time necessary to grow to participate in *S. mekongi* transmission. Because an abundance of snails is observed in April–May in the field, we assume a maturity time (τ_m) of 1 month.

Table 1
The population size, initial prevalence, and intensity of infection (the number of eggs per gram of stool) in each age category of humans

Age category	Population*	Initial prevalence (%)§	Intensity (egg/g)§
1–4	75	16	105
5–9	75	58	130
10–14	65	72	195
15–19	60	71	170
20–29	85	62	100
30–39	65	52	95
40–49	40	41	75
49<	25	28	45
Average		50	115

* According to population census of Kratie province in Cambodia in 1998 [The national Institute of Statistics of Cambodia].
§Estimated based on epidemiological data of Chatnaoi in 1994–1995 [21].

2.4. Mathematical model

We built a transmission model for *S. mekongi* based on Van Druten's and Barbour's works [37,38]. Our model contains three host populations: humans (H) and dogs (D) as the definitive hosts, and snails (V) as the intermediate hosts. The two definitive hosts are separated into two epidemiological classes: negative (H_1, D_1) and infected (H_2, D_2). The snail population consists of two subpopulations: old snails (V_O) and new-born snails (V_N). Each subpopulation was divided into three epidemiological classes: negative, latent, and infected (which are represented by V_1, V_2, V_3 for old snails and by V_4, V_5, V_6 for new-born snails, respectively). Because both the prevalence and the intensity of infection vary by age, the human population was subdivided into 8 age categories (which are indexed by k). The human population was assumed to be 500 with 50% initial overall prevalence of *S. mekongi*. Each age category is assigned to the initial prevalence and the intensity of infection as shown in Table 1. Although several surveys of animal reservoirs revealed the prevalence in dogs was from 0.3% [19] to 29.2% [25], the dog population was assumed to be 200 with 10% initial prevalence in the model.

In this study, it was assumed that each transfer rate of the definitive hosts (humans and dogs) from negative to infected (α_H, α_D) was in proportion to the total number of infected snails. The proportional coefficients for humans and dogs are expressed by β_H and β_D, respectively. The estimated values of proportional coefficient for age-categories of humans and also dogs are shown in Table 2.

Table 2
Estimation of the proportional coefficient values among hosts

Hosts	Age category (years)	Estimated value of proportional coefficient
Human (β_H)	1–4	1.13×10^{-4}
	5–9	7.52×10^{-4}
	10–14	1.18×10^{-3}
	15–19	1.03×10^{-3}
	20–29	6.39×10^{-4}
	30–39	3.97×10^{-4}
	40–49	2.47×10^{-4}
	49<	1.39×10^{-4}
Dog (β_D)		6.32×10^{-5}
Snail (β_V)		5.15×10^{-6}

Table 3
Estimated values of model parameters

Symbol	Interpretation	Estimated value
Human		
β_H	Birth rate (/week)	0.16
δ_H	Death rate (/week)	3.26×10^{-4}
β_H	Proportional coefficient	See Table 2
γ_H	Recovery rate (/week)	0.0038
f_H	Amount of fecal output (gram/day)	160
e_H	Number of eggs per gram of stool	See Table 1
Dog		
β_D	Birth rate (/week)	0.38
δ_D	Death rate (/week)	0.002
β_D	Proportional coefficient	see Table 2
γ_D	Recovery rate (/week)	0.0038
f_D	Amount of fecal output (gram/day)	100
e_D	Number of eggs per gram of stool	100
Snail		
p_h	Probability of egg hatching	0.8
ξ	Ratio of female to male	0.67
b_v	Average number of eggs produced (/female/month)	10
τ_h	Incubation period (week)	4
τ_m	Maturity period to participate in transmission (week)	4
τ_c	Latent period (week)	6
d	Mortality (/week)	0.02
θ	Additional mortality for old snails	$6–12\times d$
Φ	Additional mortality for new-born snails	$1–4\times d$
β_V	Proportional coefficient	see Table 2
Transmission		
c_t	Probability of water contact	0 (high water), 1 (low water)
c_n	Probability of water contamination	0 (high water), 1 (low water)

Hence, we obtained the following formulae for the transfer rate of the definitive hosts:

$$\alpha_H^{(k)}(t) = \beta_H^{(k)} c_t(t)(V_3(t) + V_6(t)),$$

$$\alpha_D(t) = \beta_D c_t(t)(V_3(t) + V_6(t)).$$

Herein, $c_t(t)$ stands for the probability of water contact of definitive hosts at time t.

The transfer rate of snails from negative to latent (α_V) was assumed to be in proportion to the number of eggs per snail where the proportional coefficient for snails is represented by β_V. The total number of eggs that are excreted by infected humans and dogs is expressed by the product of the amount of fecal output (f_H, f_D), the number of schistosome eggs per gram of stool (e_H, e_D), and the number of infected definitive hosts (H_2, D_2). The transfer rate of snails is expressed as:

$$\alpha_V(t) = \beta_V c_n(t) \frac{\left(\sum_k f_H e_H^{(k)} H_2^{(k)}(t) + f_D e_D D_2(t)\right)}{V_O(t) + V_N(t)}.$$

Herein, the probability of water contamination of definitive host stands for $c_n(t)$ at time t.

After the latent period (τ_C), snails are transferred from the latent class to the infected class. The other relevant parameter values in the model are estimated by experimental and field data (**Table 3**). The flowchart of the model is shown in Fig. 3.

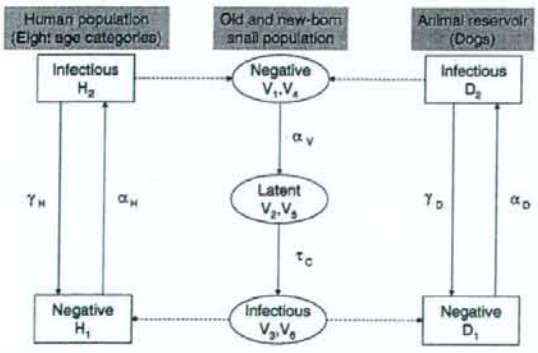

Fig. 3. The basic scheme of the transmission model for *S. mekongi*. Deaths of hosts are omitted in this scheme. The solid line shows the transfer among epidemiological classes of hosts. The dotted line shows miracidial and cercarial infections.

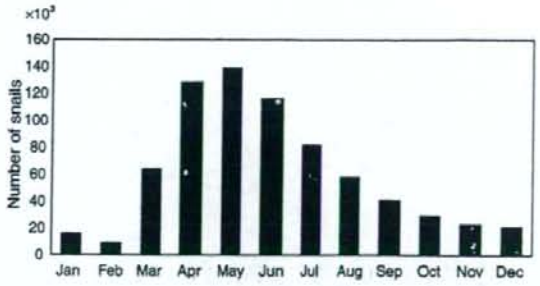

Fig. 4. The monthly variation of the total snail population.

3. Results

3.1. Seasonal variation of *N. aperta* in the transmission model

It is infeasible to observe *N. aperta* throughout the year due to the seasonal spate of the Mekong River. We postulate that there are 20,000 old snails in January every year and that the population dynamics of *N. aperta* follow the PSS hypothesis. Then, we estimated the seasonal variation of the snail population (**Fig. 4**). The snail population had a peak between April–May, and afterwards it reduced dramatically during the high water period due to severe mortality. Female snails that survive start to lay eggs next January. The transmission of *S. mekongi* occurs actively during the low water period, especially late March to early May.

3.2. Prevalence in definitive hosts

The initial prevalence in humans and dogs were set to be 50% and 10%, respectively. Fig. 5 shows the variation in the prevalence of schistosomiasis mekongi in both humans and dogs without control

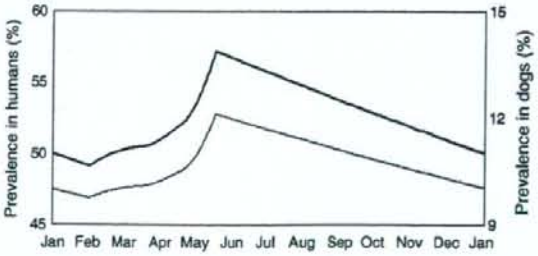

Fig. 5. Variation of prevalence in both humans (black line) and dogs (gray line) without control measures.

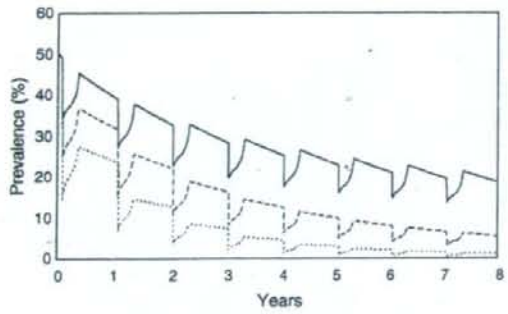

Fig. 6. Variation of the prevalence of *S. mekongi* in humans with yearly universal treatment (UT) for three coverage rates: 30% (solid line), 50% (dashed line), and 70% (dotted line).

measures. The prevalence gradually declines in January. For the low water period, prevalence rises swiftly in February–March, and rises steeply in April–May together with an increase in the snail population. Thereafter, the rate decreases in the high water period due to the absence of water contact.

3.3. Simulation of control measures for S. mekongi

Mass drug treatment combined with health education has been applied in Cambodia. We carried out simulations on the situation resulting from the execution of several control measures for humans: universal treatment (UT), targeted mass treatment (TT), a combination of UT and TT, and health intervention.

Firstly, we conducted a series of simulations of yearly UT with three coverage rates: 30%, 50%, and 70% (Fig. 6). Yearly UT with 50% and 70% coverage rates decreased the prevalence in humans from 50% to less than 5% after 8 years, while yearly UT with a 30% coverage rate only decreased the prevalence to almost 20%.

Secondly, we compared the effects of the suppression of *S. mekongi* between yearly UT and biyearly UT (Fig. 7). Yearly and biyearly UT for 8 years with a 70% coverage rate reduced the prevalence in humans to 1% and 10%, respectively. Biyearly UT for 8 years with a 85% coverage rate reduced the prevalence to 5%, which was similar to yearly UT with a 50% coverage rate (Fig. 6).

Thirdly, we observed the effect of TT after three courses of yearly UT on the prevalence in both humans and dogs (Fig. 8). We assumed that children of 5–19 years-old, who show higher prevalence and intensity of infection, were treated by TT. Three courses of yearly UT

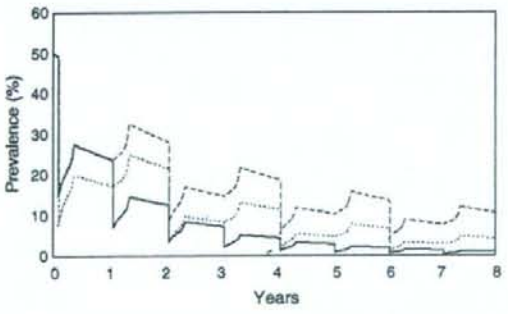

Fig. 7. Variation of the prevalence of *S. mekongi* in humans with universal treatment (UT) by changing the interval between treatments with two coverage rates: yearly UT with a 70% coverage rate (solid line), biyearly UT with a 70% coverage rate (dashed line), and biyearly UT with a 85% coverage rate (dotted line).

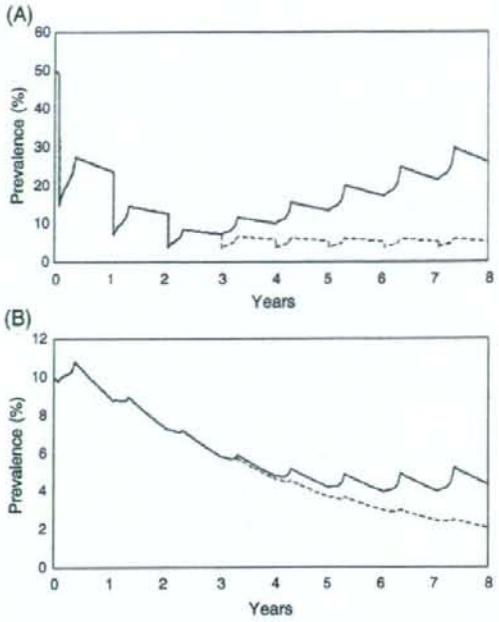

Fig. 8. Variations of the prevalence in both humans (A) and dogs (B) with 2 control measures. 1: yearly universal treatment (UT) with a 70% coverage rate for the initial 3 years (solid line). 2: after 3 years of annual UT yearly targeted mass treatment (TT) with a 85% coverage rate (dashed line).

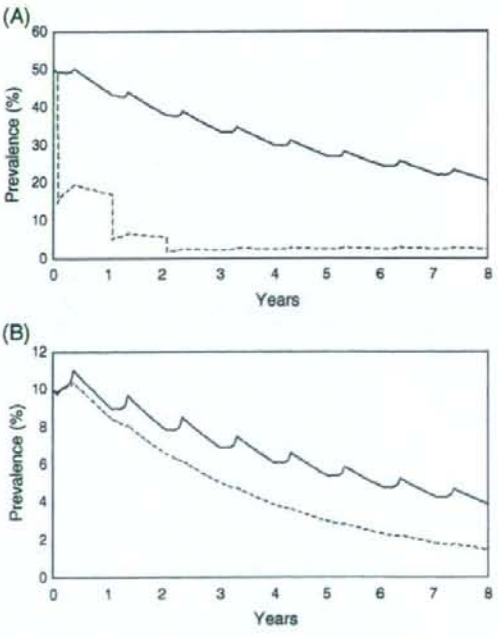

Fig. 9. Variations of the prevalence in both humans (A) and dogs (B) with 2 control measures. 1: only health intervention for 8 years (solid line). 2: health intervention for 8 years with yearly universal treatment (UT) with a 70% coverage rate for the initial 3 years (dashed line).

with a 70% coverage rate reduced the prevalence in humans to 10% and in dogs to 6%. Yearly TT with a 85% coverage rate after three courses of yearly UT kept the prevalence in humans low and also reduces the prevalence in dogs throughout the 8-year simulation. For the situation of an interruption of mass treatment after three courses of yearly UT, the prevalence in humans increased swiftly and the prevalence in dogs was restored gradually after the interruption.

Finally, we checked the effect of health intervention on the prevalence in both humans and dogs (Fig. 9). We assumed that health intervention reduced to half both the probability of water contact and amount of fecal output by humans. Health intervention for 8 years without UT or TT slightly reduced the prevalence in both humans and dogs, while health intervention for 8 years with yearly UT for initial 3 years drastically reduced the prevalence in humans.

4. Discussion

General mathematical models are helpful to understand the dynamics of schistosomiasis transmission [10–12], although, these models should be expanded to fit the local condition of endemic areas with a view to aiding to design schistosomiasis control programs. In this paper, a mathematical model incorporating with some key transmission factors was developed to evaluate the effect of control measures against schistosomiasis mekongi in Cambodia, quantitatively.

In most endemic countries, the highest prevalence and intensity of *Schistosoma* infection are found in young children [39]. A similar trend was confirmed at Chatnaol, which was chosen as our study area and was one of the sentinel villages selected in Cambodia in 1994–1995 [21]. This trend probably resulted from frequent water contact by children and the acquired immunity of adults caused by past repeated infections, which reduces susceptibility [40]. We assigned proportional coefficients to each age category in humans instead of the effect of acquired immunity (Table 2).

One of features of our model is the allowance for the dynamics of the *N. aperta* population. We adopted the PSS hypothesis [18] for *N. aperta* dynamics to predict the seasonal variation of the snail population. Although the life cycle of *N. aperta* is still largely unknown [5], the seasonal variation of the snail population is of great influence in transmitting *S. mekongi* to the definitive hosts. The simulation showed that the snail population reached a peak in April–May due to a delay of egg-laying during the high water period (Fig. 4). Since there is some difficulty in estimating the acute mortality in snails during the high water period, we chose its value to maintain a constant snail population size every year. In the field, the living sites of *N. aperta* and their population vary from year to year because of changes in water flow, water level, and the form of the riverbed, etc [5]. It is desirable to conduct further surveys of *N. aperta* to make the transmission model more realistic.

We carried out simulations of conditions where the initial overall prevalence in humans was 50% based on the epidemiological data of Chatnaol in 1994 [21]. The transmission of *S. mekongi* to humans is considered to occur mainly in April when humans comes into contact with the water in the Mekong River frequently and an abundance of snails is observable [18]. The model simulation showed the high prevalence of schistosomiasis mekongi in humans in May when the *N. aperta* population reaches a peak (Fig. 5). We assumed simply that the transmission from snails to the definitive hosts, humans and dogs, can occur during the low water period, ($c_t=1$) and that it cannot occur during the high water period ($c_t=0$). Future observations of the frequency of water contact and exposure time of humans in the low water period will be reflected in improvement in the simulations of the transmission model.

Following on from stool examinations for animal reservoirs in Cambodia, we involved dogs as a definitive host in the model. Dogs were observed swimming in the Mekong River, and one infected dog was revealed to have a high density of schistosome eggs per gram of stool [19]. Therefore, dogs are considered to play an important role in schistosomiasis transmission in Cambodia. The simulation under the assumption that the number of dogs was 200 with 10% initial prevalence shows that only UT for humans had a good effect on the reduction in prevalence in dogs (Fig. 8 (B)).

Some of model simulations indicated that snail control such as applying chemical molluscicide had an impact on the reduction of disease infection [16,41]. In the Mekong River, an application of chemical molluscicide was ineffective due to long reaches of the river and a large of volume of water flow [6]. Therefore, this study aimed at evaluating effects of control measures for humans only.

We estimated the effect of control measures for humans including UT, TT, and health intervention on the prevalence of schistosomiasis mekongi in the definitive hosts. In Cambodia, mass drug administration with coverage rates between 62% and 86% has been conducted annually since 1995, which reduced the prevalence in 4 sentinel villages to below 5% on average in 2002 [4]. The simulation results showed that yearly UT for 8 years with a 70% coverage rate reduced the prevalence in humans from 50% to 2% (Fig. 6), which suggested an effective coverage rate for MDA in Cambodia to suppress endemic of the disease. It was suggested to prolong the interval between UT with a view to cost saving [4]. The simulation indicated that biyearly UT with a 85% coverage rate also sufficiently reduced the prevalence in humans (Fig. 7). TT aimed at schoolchildren is another cost-effective alternative method [39]. Yearly TT with a 85% coverage rate aimed at 5–19 years-old following three courses of yearly UT with a 70% coverage rate achieved low prevalence below 5% in humans and below 2% in dogs (Fig. 8). Health intervention such as health education and provision of latrines has an important role in the control of helminth infection [42]. In this study, we assumed that the probability of water contact (c_t) and an amount of fecal output of humans (e_H) were reduced to half as the result of health intervention. The performance of health intervention for 8 years without mass drug administration reduced the prevalence in both humans and dogs to half the initiate level in the simulation (Fig. 9). The combination of yearly UT with health intervention had a strong effect on reduction of the prevalence in both definitive hosts in the simulation (Fig. 9).

With regard to the re-emergence of schistosomiasis in Laos, it is necessary to continue performing control programs and surveillance using ELISA in Cambodia [2]. In addition, there is a need to convert control measures with good cost-effectiveness because few positive cases were detected in recent years [4]. The simulation results show that biyearly UT or yearly TT is efficacious in restricting *S. mekongi* infections if the coverage rate is kept at more than 85%. The reduction in the probability of water contact or the amount of fecal output by infected humans also impacts on the suppression of transmission of *S. mekongi*. The simulation results suggested that the suppression of *S. mekongi* in Cambodia would be possible by sustaining the control program and surveillance.

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MOLECULAR DISCRIMINATION BETWEEN *PARAGONIMUS HETEROTREMUS* AND TWO FORMS OF *P. WESTERMANI* OCCURRING IN THAILAND

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Abstract. In areas of central Thailand where paragonimiasis is endemic, metacercariae of *Paragonimus westermani* (large metacercarial form) and *P. heterotremus* have been detected in a single crab species. Of these two species, only the latter has been confirmed to infect humans. In southern Thailand, we have previously identified another form of *P. westermani* (small metacercarial form) in another crab species, which also acts as host for *P. westermani* (large metacercarial form). In this study, we established a new multiplex PCR method and evaluated its applicability for discriminating between *P. heterotremus* and two forms of *P. westermani* at the metacercarial stage. We found that multiplex PCR in combination with restriction enzyme digestion (PCR-RFLP with *Bsa*HI) was effective for the discrimination.

INTRODUCTION

During an intensive field survey for lung flukes in southern Thailand, we found two forms of *Paragonimus westermani* metacercariae in a single crab species, *Phricotelphusa aedes* (Binchai *et al*, 2007; Sugiyama *et al*, 2007). Metacercariae of these two forms had the same shape, but were of different sizes: the diameter of metacercarial cysts of the large form is about twice that of the small one. As the nuclear ribosomal DNA (rDNA) second internal transcribed spacer (ITS2) sequences obtained from the large metacercarial form were identical to those of *P. westermani*, whose sequence was deposited in the GenBank/EMBL/DDBJ nucleotide databases under the accession number of AF159604 (referred to as *P. westermani* strain Thailand), we referred to the small metacercarial form as *P. westermani*-like for descriptive purposes.

In Thailand, human infections with *P. westermani* have not been confirmed, although *P. heterotremus* is known to affect humans (Srisont *et al*, 1997; Blair *et al*, 1998). The metacercariae of these two species have been detected in the same crab host in paragonimiasis-endemic areas (Miyazaki, 1991). Therefore, we had developed methods that could be used as reliable tools for discriminating these two lung fluke species. We demonstrated that multiplex PCR method was the most efficient because species identification involved a single round of PCR in a single tube (Sugiyama *et al*, 2005). In this study, we modified the previously established multiplex PCR method and evaluated its applicability for discriminating between *P. heterotremus* and two forms of *P. westermani* at the metacercarial stage.

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MATERIALS AND METHODS

Parasite material and DNA isolation

Metacercariae of *P. heterotremus* were harvested from the freshwater crab, *Larnaudia larnaudii*, captured in a mountain stream in Saraburi Province, Thailand (Kawashima *et al*, 1989). Metacercariae of *P. westermani* and *P. westermani*-like were isolated from the freshwater crab, *Phricotelphusa aedes*,

captured in a mountain stream in Surat Thani Province, Thailand (Sugiyama *et al*, 2007). This study also includes *P. siamensis*, the species known to be closely related to *P. westermani* (Blair *et al*, 1998). Metacercariae of *P. siamensis* were harvested from the freshwater crab, *Sayamia germaini*, captured in paddy fields in Prachin Buri Province, Thailand (Srisont *et al*, 1997). DNA samples were prepared from the metacercariae as previously described (Sugiyama *et al*, 2002).

DNA amplification and sequencing

For multiplex PCR amplification, we constructed new species-specific forward primers based on the respective rDNA ITS2 sequences in order to generate the products that would remain uncut by further restriction enzyme digestion. The sequences (and alignment positions) (Fig 1) of the primers used for the multiplex PCR are as follows:

1) Interspecies-conserved forward primer (3S): 5′ GGTACCGGTGGATCACT CGGCTCGTG 3′;

2) Interspecies-conserved reverse primer (A28): 5′GGGATCCTGGTTAGTTTCTTTTC CTCCGC 3′(Bowles *et al*, 1995);

3) *P. heterotremus*-specific forward primer (PhTF2): 5′ CAAATCCGGGCGTAT CCATGTTGTG 3′ (positions 238 to 262);

4) *P. westermani*-specific forward primer (PwTF4): 5′ TCTGCGTTCGAT GCTGACCTACG 3′ (positions 368 to 390, a sequence common between the two forms of *P. westermani*).

These four primers were included in a single-tube reaction. Multiplex PCR amplification was performed using 0.1 μM of PhTF2 and PwTF4 primers, 0.5 μM of 3S and A28 primers, 2.5 units of the *Taq* polymerase (Invitrogen, USA) and 10 ng of DNA template. The resulting PCR products were separated by electrophoresis in 3% (w/v) agarose gels.

The amplicons were extracted from agarose gels and sequenced using the corresponding primers and BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) in an automated sequencer (ABI310, Applied Biosystems). The sequence alignment and comparison were conducted using GENETYX-WIN (ver. 7.0, Software Development, Japan) program.

Restriction enzyme digestion of the multiplex PCR products (PCR-linked restriction fragment length polymorphism (PCR-RFLP))

Amplicons (4 to 10 μl) were also digested with five units of *Bsa*HI (New England Biolabs, USA) at 37 °C for 1 hour. The samples were then separated by electrophoresis in 3% (w/v) agarose gels.

RESULTS

Using multiplex PCR method with the new species-specific primers, two products were amplified from each of the metacercarial DNA samples of *P. heterotremus* (ca. 520 bp and 250 bp), *P. westermani* (ca. 520 bp and 125 bp), and *P. westermani*-like (ca. 520 bp and 125 bp) (Fig 2, lanes 1 to 3). However, a single 520-bp product was generated from the DNA samples of *P. siamensis* (Fig 2, lane 4). Sequence analysis of the amplification products (520 bp and others) revealed that the products corresponded to the rDNA ITS2 region of the respective species (Fig 1).

For species discrimination by RFLP using the multiplex PCR products, we selected restriction enzyme *Bsa*HI based on the putative restriction maps generated from ITS2 region sequences (Fig 1). Digestion of multiplex PCR products of *P. westermani*-like produced three fragments (ca. 270, 170 and 90 bp) from the 520-bp amplicon (Fig 2, lane 7). However, the 520-bp amplicons of the other 3 species (*P. heterotremus*, *P. westermani* and *P. siamensis*) produced two fragments (ca. 350 and 170 bp; Fig 2, lanes 5, 6 and 8). Multiplex PCR products of less than 520 bp in size (250-bp product for *P. heterotremus* and 125-bp products for *P.*

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                                                                    BsaHI
Ph 001: TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTGAACTGCATACTGCTTTGAACA 060
Pw 001: .....................................C..................... 060
PL 001: .....................................C..................... 060

Ph 061: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pw 061: ............................................................ 120
PL 061: ............................................................ 120

                          BsaHI
Ph 121: TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT 180
Pw 121: ............C.....[.....]..................................... 180
PL 121: .........G.....[.....]..................................... 180

                                                          BsaHI
Ph 181: GATTTCCCCAACGTGGCCTTGTGTCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACAA 240
Pw 181: ...C.......TC...T......C.....................A..............T 240
PL 181: ...C.......TC...T......C.....................[.....C].....T 240

          PhTF2-->
Ph 241: ATCCGGGCGTATCCATGTTGTGGCTGAAAGCCTTGATGGGGATGTGGCAACGGAGTCGTG 300
Pw 241: .CT..C...C.C...C....C..............C.....................A..... 300
PL 241: .CT......C.C...C....C..............C.....................A..... 300

Ph 301: GCTCAGTGAATGATTTATGTGCACGTTCCGCTGTCCCGTCATCATCTATGGTTGAAGTTG 360
Pw 301: .......A.............G....T........T...T......G.....C.T.... 360
PL 301: .........T..G.........G.............T...T......G.....T.T.... 360

Ph 361: CGCGTGGTGTG--TCCGATGCTGACCTATATATGTGCCATGTGGCTCATTTTCCTGACCT 420
Pw 361: .......C..CG.T.............CG..............TC....C.T....... 420
PL 361: .......C..CG.T.............CG..............T.....C......... 420
                  PwTF4-->

Ph 421: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA 463
Pw 421: .....T..................................... 463
PL 421: ........................................... 463
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Fig 1- Sequences alignment of the ITS2 region from *P. heterotremus* (Ph), *P. westermani* (Pw) and *P. westermani*-like (PL) metacercariae. The 5' and 3' ends of the sequences include 5.8S rDNA and 28S rDNA, respectively. A dot in the *P. westermani* and *P. westermani*-like sequences indicates identity with *P. heterotremus* sequence. The locations of the *P. heterotremus*-specific forward primer (PhTF2; 5' CAAATCCGGGCGTATCCATGTTGTG 3') and *P. westermani*-specific forward primer (PwTF4; 5' TCTGCGTTCGATGCTGACCTACG 3') are underlined. The recognition sites of *Bsa*HI (GR/CGYC) are located in boxes. The numbers refer to the alignment positions.

westermani and *P. westermani*-like) remained undigested by *Bsa*HI.

DISCUSSION

We previously reported that the multiplex PCR method we developed (Sugiyama *et al*, 2005) was effective for discriminating among the five *Paragonimus* species occurring in Thailand when used in combination with *Scr*FI digestion (Sugiyama *et al*, 2006). However, this method was not applicable for discriminating among *P. heterotremus* and two forms of *P. westermani* because the latter two forms showed identical PCR-RFLP patterns. Therefore, in this study, we treated the amplicons with *Bsa*HI chosen based on the sequence differences between these two forms of *P. westermani*. In addition, new species-specific primers were constructed to generate products that would remain uncut by *Bsa*HI digestion. The improved method was shown to be effective in discriminating among *P. heterotremus* and two forms of *P. westermani*.

Two forms of *P. westermani* were found

Fig 2- Multiplex PCR and multiplex PCR plus PRLP analysis of ITS2 amplification products from the metacercarial DNA samples of *P. heterotremus* (lanes 1 and 5), *P. westermani* (lanes 2 and 6), *P. westermani*-like (lanes 3 and 7) and *P. siamensis* (lanes 4 and 8). After digestion of the multiplex PCR products with *Bsa*HI, three bands were observed for *P. heterotremus* (ca. 350, 250 and 170 bp, lane 5), three bands for *P. westermani* (ca. 350, 170 and 125 bp, lane 6), four bands for *P. westermani*-like (ca. 250, 170, 125 and 90 bp, lane 7) and two bands for *P. siamensis* (ca. 350 and 250 bp, lane 8). Both the 25-bp and 100-bp DNA ladders were used to estimate the sizes of the bands (lanes M1 and M2, respectively).

to occur in Surat Thani, southern Thailand, and they both used a single crab species as the second intermediate host (Sugiyama *et al*, 2007). Possible discovery of *P. westermani*-like metacercariae were reported from crabs occurring not only in Surat Thani (Shibahara *et al*, 1995) but Nakhon Si Thammarat (Tsuzuki *et al*, 1995), the neighboring province of Surat Thani. However, little attention has been paid to *P. westermani*-like and its infection of humans has not been determined. To obtain accurate epidemiological information about the prevalence of the lung fluke species and forms in Thailand, various methods for identification are needed on parasitological materials obtained from host animals. The method developed in this study has the potential for this purpose.

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MOLECULAR ANALYSIS OF JAPANESE *ANISAKIS SIMPLEX* WORMS

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Abstract. In this study, we used sequence and RFLP analysis of the ribosomal DNA internal transcribed spacer region to identify the sibling species of *Anisakis simplex* worms isolated in Japan as third stage larvae (L3) from fish and patients and as adults from marine mammals. Worms from North Pacific Ocean were identified as *A. simplex* s. str., while those from the southern Sea of Japan were *A. pegreffii*. Worms from patients were mainly identified as *A. simplex* s. str, even though they were obtained from southern Japan. Worms of the hybrid genotype were only detected in fish and marine mammals. We also demonstrated that our newly established RFLP method for mitochondrial *cox1* enables us to unambiguously classify members of *A. simplex*, including hybrid genotype worms, into *A. simplex* s. str. or *A. pegreffii*.

INTRODUCTION

In Japan, over 2,000 cases of human anisakiasis have been reported annually due to the high consumption of raw fish as sushi and sashimi. The nematode *Anisakis simplex* is the parasite most frequently associated with the disease. *A. simplex* is widespread worldwide with no obvious variation in morphology. However, sequencing and/or restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA internal transcribed spacer region [(rDNA ITS region; namely, 5.8S rDNA and flanking ITS regions (ITS1 and ITS2)] have demonstrated that *A. simplex* morphospecies comprises three sibling species: *A. pegreffii*, *A. simplex* sensu stricto and *A. simplex* C (Mattiucci and Nascetti, 2006). Parasites with hybrid genotype between *A. simplex* s. str. and *A. pegreffii* were also detected from waters around the Iberian Peninsula (Abollo *et al*, 2003; Martin-Sanchez *et al*, 2005). In this paper, we have applied molecular methods for sibling species-level identification of Japanese *A. simplex* worms isolated from fish, marine mammals and patients with anisakiasis.

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MATERIALS AND METHODS

Parasite materials

A. simplex worms were collected from fish as third stage larvae (L3) and marine mammalian hosts as adults, as well as from patients with anisakiasis as L3. Worms from fish and marine mammals were stored at -20 °C and those from human patients were stored in 80% ethanol at room temperature until analysis. Host animals, the geographical location of collection and numbers of worms examined are listed in Table 1. Species was confirmed by DNA sequencing and/or RFLP analysis as described below.

DNA amplification and sequencing

We extracted DNA samples from individual worms using QIAamp DNA Mini Kit (Qiagen k. k., Japan). The entire ITS region (ITS1, 5.8S rDNA and ITS2) and mitochondrial cytochrome C oxidase 1 (*cox1*) gene was amplified by PCR using primer pairs A and B (D Amelio *et al*, 2000) and JB3 and JB4.5 (Hu *et al*, 2001), respectively. PCR conditions