lives in host tissues, mainly the liver, surrounded by thick connective tissues containing carbohydrate-rich laminated layers, which probably provide the parasite cells with an extremely-low-oxygen environment. Accordingly, it is not surprising that the parasite survives in the host by utilizing an anaerobic respiratory system.

Many anaerobic parasitic eukaryotes use the NADH-fumarate pathway, which is absent in mammals (2, 3, 10, 14, 22, 29). Therefore, this unique respiratory system is regarded as a promising chemotherapeutic target for the development of novel anthelminthics, as discussed in a recent review (9). In fact, Omura et al. previously found a natural compound, nafuredin, that is a potent inhibitor of the adult *A. suum* mitochondrial respiratory chain but much weaker against the mammalian mitochondrial respiratory chain (21). Yamashita et al. also found that quinazoline-type inhibitors were highly effective against adult *A. suum* complex I (35). Kinetic analyses using a series of quinazoline-type inhibitors revealed that *A. suum* complex I recognizes RQ_2 or UQ_2 in different ways, suggesting that mitochondrial complex I, which reacts preferably with RQs, could be a good target for chemotherapy. In the present study, we also tested several quinazoline-type compounds for their abilities to inhibit the anaerobic respiratory system of *E. multilocularis* protoscoleces. We found that all of the quinazoline-type compounds inhibited the NADH-fumarate reductase activity of *E. multilocularis* mitochondria to different extents. Furthermore, these compounds exhibited potent parasite-killing activities against *E. multilocularis* protoscoleces under in vitro culture conditions. Importantly, the nonsubstituted quinazoline, which has a higher inhibitory effect against NADH-fumarate oxidoreductase of the parasite mitochondria than the 8-OH derivative does, exhibited the parasite-killing activity even when used at 5 μM, whereas the 8-OH derivative did not do so at the same concentration. Such a correlation between the enzyme inhibition and the parasite-killing activities of these compounds suggests that the anaerobic NADH-fumarate reductase system of the parasite is a promising target for the development of antiechinococcal drugs.

Antiechinococcal drugs for chemotherapy of human AE should target not only protoscoleces but also the germinal layers of the *E. multilocularis* metacestode. The germinal layers in the larval parasite exhibit extremely unique characteristics. The parasite cells forming the germinal layers can differentiate into various tissues, including brood capsules and protoscoleces, and at the same time, they proliferate asexually as they remain in an undifferentiated state. This causes enlargement and, occasionally, metastasis of the lesions due to the formation of a large parasite mass. Therefore, for chemotherapy of AE, a complete cure cannot be achieved unless the germinal cells of the larval parasite are eliminated. Therefore, the mitochondrial respiratory system of germinal cells should be further characterized to aid in the development of a novel antiechinococcal compound(s) targeting the energy metabolism of larval *E. multilocularis*. However, it is presently quite difficult to obtain enough metacetode materials with homogeneous quality. Established methodologies for the in vitro cultivation of *E. multilocularis* metacestodes are now available (6, 23), and they will hopefully be applicable to large-scale preparations of metacestode materials in the near future.

During the life cycle of *E. multilocularis*, the parasite never undergoes active development and/or energy metabolism under aerobic conditions. The larval parasite lives mainly in the liver of intermediate host animals, whereas the adult worm dwells inside the small intestine of the final host, both of which are microaerobic conditions. Although the eggs of the parasite are exposed to air, they already contain a mature infective larva (oncosphere) waiting to be taken up by the next intermediate host. Therefore, the oncosphere does not develop or move under aerobic conditions. Taken together, these findings suggest that the respiratory system of *E. multilocularis* protoscoleces, as characterized in the present study, could represent the respiratory system used by the parasite throughout its developmental stages. Based on this speculation, the use of protoscolex materials in the first-step screening of candidate compounds by enzyme inhibition assays and subsequent in vitro parasite-killing assays appears to be reasonable, although it should be confirmed that the respiratory system of the *E. multilocularis* metacestode shares the same basic characteristics with that of the protoscolex stage of the parasite. We have already done preliminary experiments on the effects of the compounds used in this study, including the quinazoline derivative (8-OH), against in vitro-cultured metacestodes and found that the compounds exhibited high parasite-killing activities as evaluated by a modified MTT assay (data not shown). These results strongly suggest that our strategy is appropriate.

Highly effective chemotherapeutic compounds against human AE are not currently available despite the fact that the disease can be lethal unless the patient is appropriately treated during the early stage of the infection. Based on the findings presented here, it appears that the anaerobic respiratory system of *E. multilocularis*, which is distinct from that of host mammals, is a good target for the development of highly effective antiechinococcal drugs and, furthermore, that respiratory chain inhibitors (21, 35) are possible lead compounds for the development of antiechinococcal drugs.

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▣ MINI-REVIEW

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Modeling the Dynamics and Control of Transmission of *Schistosoma japonicum* and *S. mekongi* in Southeast Asia

Hirofumi Ishikawa[1,*] and Hiroshi Ohmae[2]

[1]Deptartment of Human Ecology, Graduate School of Environmental Science, Okayama University, Okayama 700-8530, Japan;
[2]Department of Parasitology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Abstract: A mathematical model for transmission of schistosomes is useful to predict effects of various control measures on suppression of these parasites. This review focuses on epidemiological and environmental factors in *Schistosoma japonicum* and *Schistosoma mekongi* infections and recent advances in mathematical models of *Schistosoma* transmission.

Key words: *Schistosoma japonicum*, *Schistosoma mekongi*, mathematical model, control strategies

INTRODUCTION

Schistosomiasis is an important disease problem in several Asian countries. Schistosomiasis japonica is prevalent in China and the Philippines, where millions of people are affected. Schistosomiasis mekongi is prevalent in the Mekong River basin (MRB) from the Khong district in southern Laos to Kratie province in northern Cambodia. The total population at risk for schistosomiasis mekongi is estimated to be 60,000 in Laos and 80,000 in Cambodia [1]. The first human case of *Schistosoma mekongi* infection was reported as *Schistosoma japonicum* infection in 1957 [2]. Afterwards, *S. mekongi* was identified as a new species of *Schistosoma* [3]. *S. japonicum* and *S. mekongi* have a complicated mode of transmission. As part of the life cycle occurs in the environment outside of the host, it is difficult to measure the transmission rate on the basis of field observations [4]. Therefore, a mathematical model for *Schistosoma* transmission could be useful for estimating its prevalence, and model simulations can be instrumental in managing various control strategies. There have been many studies involving the mathematical modeling of transmission for *S. japonicum* since 1965 [4-12], while there have been only 2 studies on mathematical modeling of the transmission of *S. mekongi* [13,14]. This review focuses on the epidemiological and environmental factors in *S. japonicum* and *S. mekongi* infections and the recent advances in mathematical models of *Schistosoma* transmission.

S. japonicum and *S. mekongi* carry out their transmission cycle in definitive and intermediate hosts. Humans are the major definitive hosts, and many domestic and wild mammals were found to be reservoirs [15-19], whereas the intermediate hosts are only snails. Cercariae, which are released from infected snails, penetrate into the definitive host via the skin and develop into mature adults in the host; female adults produce eggs throughout their life-span. A mathematical model that quantitatively describes *Schistosoma* transmission needs to include the following components [4,14].

1. Dynamics of the human population and behavior of humans related to water contact
2. Dynamics of the intermediate host population
3. Fluctuation of water level in the Mekong River (only for *S. mekongi*)
4. Contribution of animal reservoirs
5. Effect of control measures

DYNAMICS OF THE INTERMEDIATE HOSTS: SNAILS FOR *S. JAPONICUM*

Oncomelania spp. snails were recognized as intermediate hosts of *S. japonicum*; *O. quadrasi* and *O. hupensis* were identified in the Philippines [20,21] and in China [16], respectively. As snail density varies according to time and circumstances, it is difficult to make accurate estimates of snail density. In Bohol, the Philippines, a water area fluctuates seasonally according to rainfalls [4]. In regard to the longevity of snails, the mortality rate among infected snails is higher than that among uninfected snails [20,22].

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* Corresponding author (ishikawa@ems.okayama-u.ac.jp)

DYNAMICS OF THE INTERMEDIATE HOSTS: SNAILS FOR *S. MEKONGI* AND FLUCTUATION OF THE MEKONG RIVER FLOW

Neotricula aperta is recognized as the intermediate host of *S. mekongi*, which is composed of 3 races (α, β, and γ). Races α and γ range in the MRB from Khong to Kratie, whereas race β lives in the Mun River, a tributary of the Mekong River [23]. Race γ shows the highest susceptibility to miracidia among the 3 races [24].

In Cambodia, the rainy season begins in March, and heavy rainfall lasts from June to October; the rainfall drops dramatically in November, and thereafter the dry season lasts from December to February. The heavy rainfall and the arrival of the dry season results in rising and dropping water levels in the Mekong River (World Weather Information Service; Mekong River Commission) (Fig. 1). 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. The "Post-Spate Survival" hypothesis that *N. aperta* survive and copulate during the high water period of the Mekong River, but that laying eggs could be delayed until next January, and that thereafter the eggs hatch from February has been used to represent the population dynamics of *N. aperta* [14,25] (Fig. 2).

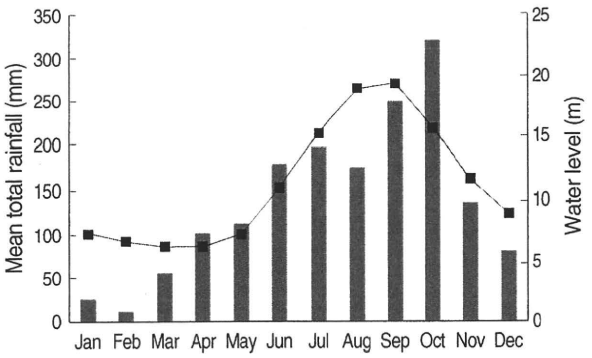

Fig. 1. Monthly average rainfall levels (bars) for 5 years during 1997-2001 in Phnom Penh (World Weather Information Service) and monthly average water levels of the Mekong River (line) for 14 years during 1989-2002 in Kratie province, Cambodia (Mekong River Commission) [14].

DEFINITIVE HOSTS: ANIMAL RESERVOIRS

When animal reservoirs play a part in the reproduction of *Schistosoma*, it is necessary to give careful consideration to them because of the difficulty in eliminating *Schistosoma* by means of chemotherapy for humans only [26]. In Lyte, the Philippines, many domestic animals such as dogs, pigs, and cows were found to be reservoirs for *S. japonicum* [15], while in Bohol, the Philippines, only rats were infected by *S. japonicum* with a low prevalence rate from the results of field surveys [21]. In China, more than 40 species of domestic and wild mammalians have been identified as reservoirs for *S. japonicum* [16]. In the MRB, dogs and pigs have been recognized as reservoirs for *S. mekongi* [18, 19]. In Laos, the prevalence in dogs was estimated as 11% [18] and 29.2% [17].

MATHEMATICAL MODELS OF *SCHISTOSOMA* TRANSMISSION

Macdonald [5] first proposed a mathematical model for *Schistosoma* transmission, and thereafter a number of mathematical models for *Schistosoma* transmission have been published, mainly from the theoretical point of view [5-8]. Anderson and May [22] studied the prevalence of snail infection based on empirical evidence. A stochastic model for schistosomiasis developed

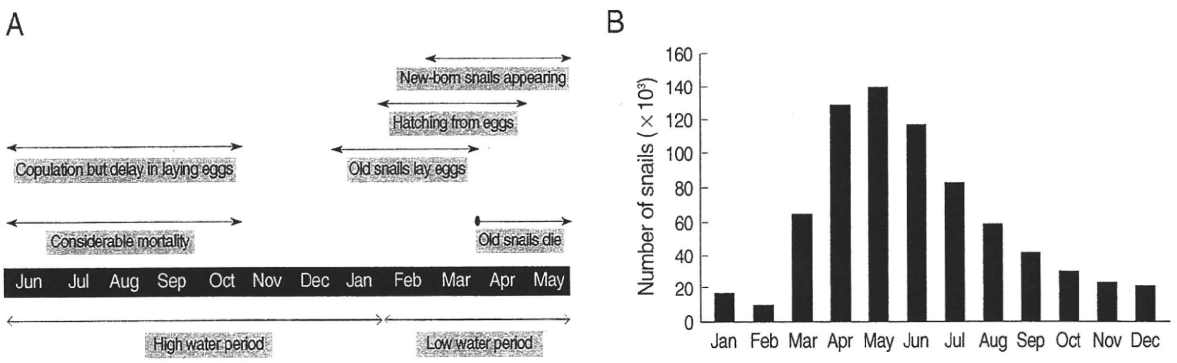

Fig. 2. Population dynamics of *Neotricula aperta* on the basis of the Post-Spate Survival hypothesis. (A) The life cycle of *N. aperta*, (B) The monthly variation of the total snail population [14].

from a model for onchocerciasis aimed to evaluate control strategies [9]. A series of studies on modeling of *S. japonicum* transmission and control in China have been performed [10-12]. Ishikawa et al. [4] developed a model of *S. japonicum* transmission that took account of seasonal variations in snail density, animal reservoirs, rats, and high and low cercarial shedding stages in snails to predict the effects of control measures against *S. japonicum* in Bohol, the Philippines (Fig. 3). A couple of studies proposed a mathematical model for the transmission of *S. mekongi* in Cambodia with age-structure, which was aimed at estimating the coverage rate and range of ages in targeted mass treatment (TT) to interrupt *Schistosoma* transmission [13,14]. The fluctuation of water level in the Mekong River, dynamics of the intermediate snail host population, and the contribution of an animal reservoir, dogs, were incorporated into this model [14].

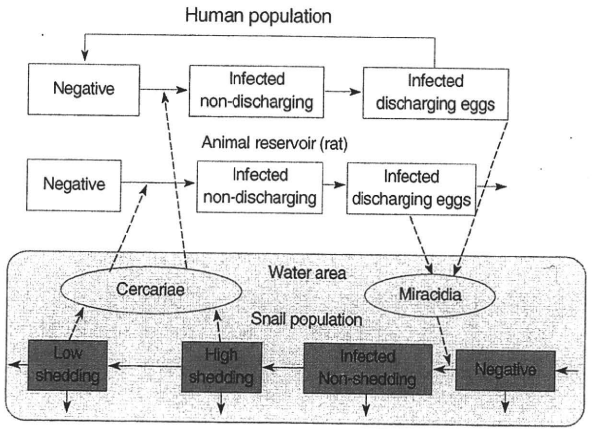

Fig. 3. The basic scheme of the transmission model for *S. japonicum* showing the transfers among epidemiological classes [4].

CONTROL MEASURES AND THEIR EVALUATION THROUGH SIMULATIONS

A collaborative project of the Schistosomiasis Control Service of the Philippine Department of Health and the Sasakawa Memorial Health Foundation of Japan has been continuing since 1981 in Bohol [21]. The major approach to the control of *S. japonicum* consists of 2 methods: the detection of infected individuals and chemotherapeutic treatment, snail control by environmental changes such as land reclamation and cement lining of ditches, and using molluscicides. The comparative simulations showed that the prevalence in inhabitants and the density of infected snails could be restored swiftly after the completion of 4 courses of yearly selected mass treatments without snail control, that the prevalence in inhabitants could be reduced gradually by snail control measures alone, and that the prevalence in inhabitants and the density of infected snails would be eliminated by human control together with snail control [4] (Fig. 4).

In the MRB, snail control measures such as using molluscicides are ineffective because of the Mekong River flow rate. In Cambodia, a universal treatment campaign (UT) was initiated by the Cambodian government, WHO, and Medecine Sans Frontieres in 1995. The Sasakawa Memorial Health Foundation has rendered support for control of schistosomiasis mekongi since 1997. In Laos, although the average prevalence of schistosomiasis mekongi decreased to less than 1% after mass treatment with praziquantel during a 10-year control program [27], a resurgence of schistosomiasis in the Khong district was confirmed by epidemiological surveys by WHO in 2003 [28]. Com-

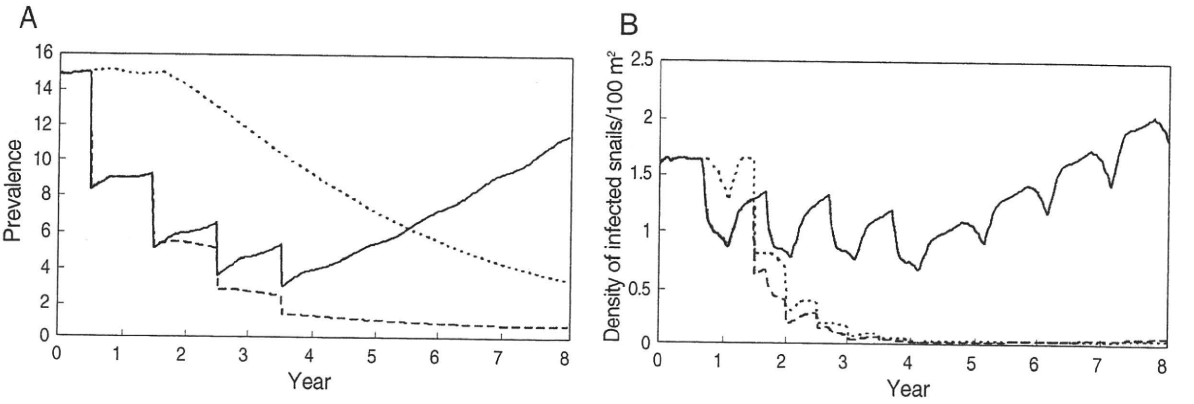

Fig. 4. Variations in the infection of *S. japonicum* in Bohol (Sto. Thomas) for the human-control case with selective mass treatment at 1-year interval with a coverage rate of 50% (solid line), the snail-control case with the use of molluscicides at half-year intervals under the assumption that its effective rate would be 50% (dotted line), and both the human and snail-control case (dashed line), respectively. (A) Variations in prevalence (%) in the human population, (B) Variations in infected snail densities per 1 *a* (100 m²) [4].

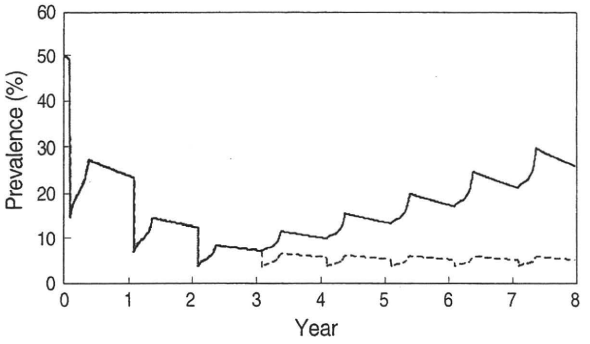

Fig. 5. Variations in the prevalence in humans with 2 control measures. 1: Yearly universal treatment (UT) with a 70% coverage rate for the initial 3 years (solid line), 2: After 3 years of annual UT targeted mass treatment (TT) with a 85% coverage rate (dashed line) [14].

parative simulations for the situation of an interruption in mass treatments after 3 courses of yearly UT showed that the prevalence in inhabitants increased swiftly, while the effect of yearly TT for children of 5-19 years old, who show higher prevalence and intensity of infection, after 3 courses of yearly UT would keep the prevalence in inhabitants low throughout an 8-year simulation [14] (Fig. 5).

PROSPECTS

Recent advances in mathematical modeling of the transmission of *S. japonicum* and *S. mekongi* are summarized here. There has been steady progress in the mathematical modeling of Schi*stosoma* transmission taking into consideration of the ecology of snails and the behavior of inhabitants. The model simulations suggested that, among various possible control measures, a selective mass treatment program coordinated with snail control would be effective for the elimination of *S. japonicum*. The model simulations also predicted that the suppression of schistosomiasis mekongi could be possible in Cambodia by maintaining control strategies for humans such as biyearly UT or yearly TT with high coverage.

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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

国立感染症研究所　寄生動物部
大前比呂思・朝日博子

獨協医科大学　熱帯病寄生虫病センター
千種雄一・桐木雅史

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

Hiroshi Ohmae*　Hiroko Asahi*　Yuichi Chigusa**　Masashi Kirinoki**　Orlando S Sy***

*Department of Parasitology, National Institute of Infectious Diseases
**Department of Tropical Medicine and Parasitology, Dokkyo Medical University
***Schistosomiasis Research Hospital

論文請求先：大前比呂思　〒162-8640　東京都新宿区戸山1-23-1　国立感染症研究所　寄生動物部

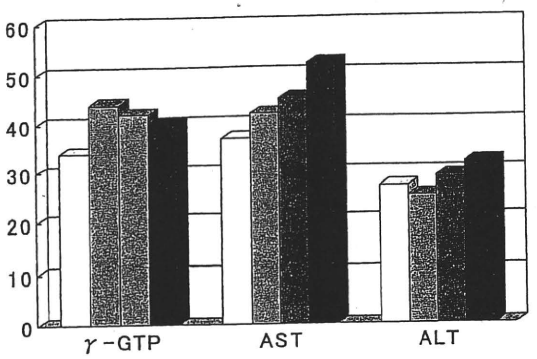

図1a　日本住血吸虫感染者における血清中のγ-GTP，AST，ALTと肝線維化
（1991年の調査結果）

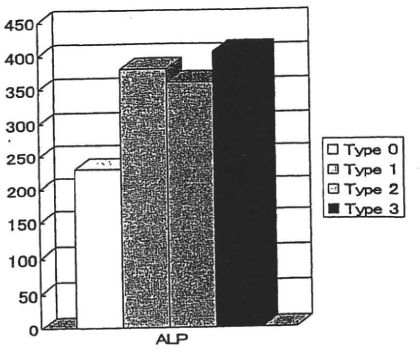

図1b　日本住血吸虫感染者における血清中のALPと肝線維化
（1991年の調査結果）

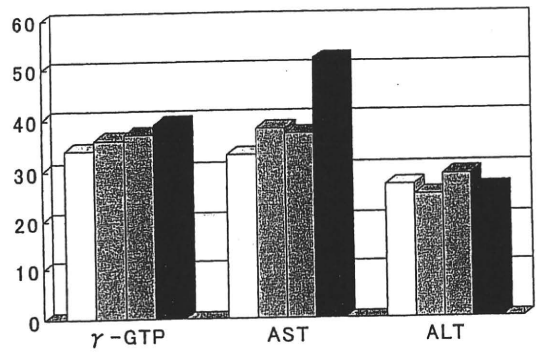

図2a　日本住血吸虫感染者における血清中のγ-GTP，AST，ALTと肝線維化
（2000年の調査結果）

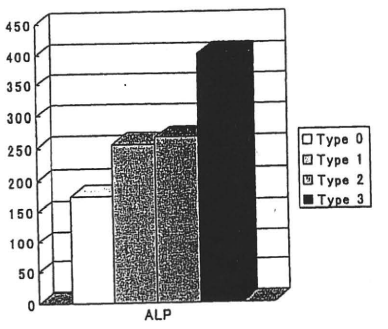

図2b　日本住血吸虫感染者における血清中のALPと肝線維化（2000年の調査結果）

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
	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	Trans R Soc Trop Med Hyg. 1997
	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および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)

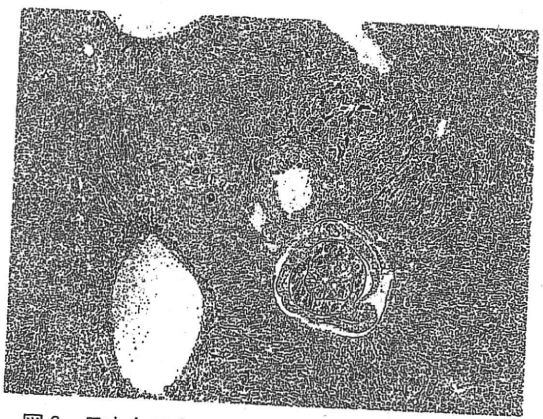

図3　日本血吸虫感染9週後のマウス肝臓病理組織標本（マッソン・トリクローム染色）

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

Naoto Hisakane [a], Masashi Kirinoki [b], Yuichi Chigusa [b], Muth Sinuon [c], Duong Socheat [c], Hajime Matsuda [d], Hirofumi Ishikawa [a,*]

[a] Department of Human Ecology, Graduate School of Environmental Science, Okayama University, 700-8530, Japan
[b] Center for Tropical Medicine and Parasitology, Dokkyo Medical University School of Medicine, 321-0293, Japan
[c] National Center for Parasitology, Entomology and Malaria Control, Ministry of Health, Cambodia
[d] Institute of International Education and Research, Dokkyo Medical University School of Medicine, 321-0293, Japan

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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*

* Corresponding author. Tel.: +81 86 251 8826; fax: +81 86 251 8837.
E-mail address: ishikawa@ems.okayama-u.ac.jp (H. Ishikawa).

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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

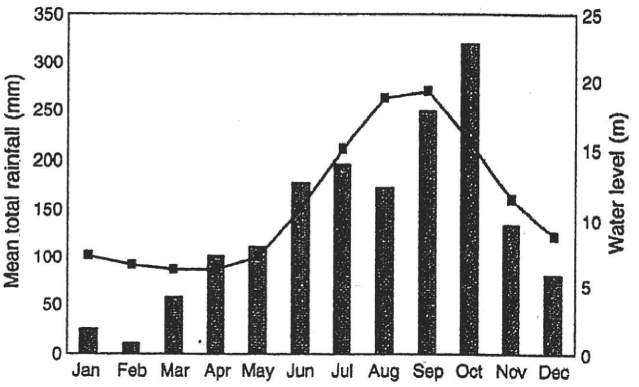

Fig. 1. Monthly average rainfall levels (bars) for 5 years during 1997–2001 in Phnom Penh [World Weather Information Service] and monthly average water levels of the Mekong River (line) for 14 years during 1989–2002 in Kratie province, Cambodia [Mekong River Commission].

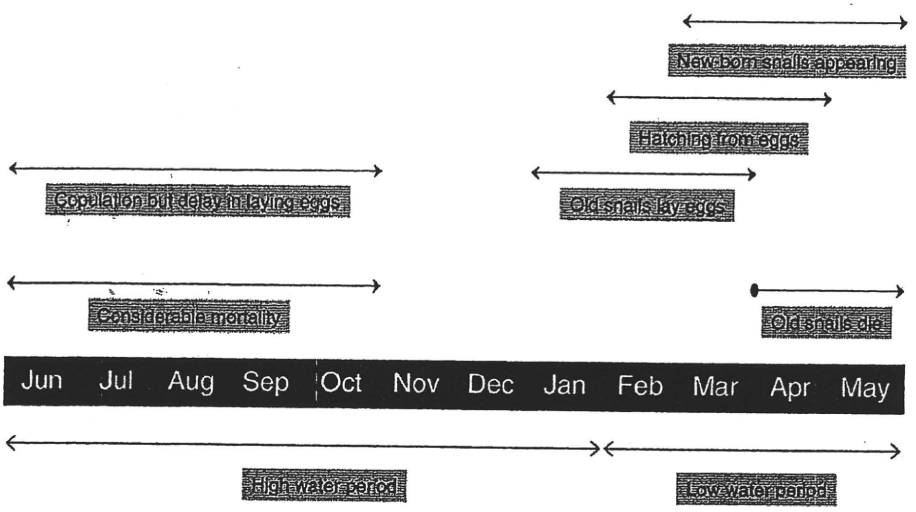

Fig. 2. Population dynamics of *N. aperta* on the basis of the Post-Spate Survival hypothesis.

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.

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 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 Chatnaol in 1994–1995 [21].

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 (δ_V)		5.15×10^{-6}

Table 3
Estimated values of model parameters

Symbol	Interpretation	Estimated value
Human		
B_H	Birth rate (/week)	0.16
μ_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		
B_D	Birth rate (/week)	0.38
μ_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
σ	Ratio of female to male	0.67
ν_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
μ_V	Mortality (/week)	0.02
ε_V	Additional mortality for old snails	$6\times10^{-12}\times\mu_V$
ε_N	Additional mortality for new-born snails	$1.2\times10^{-11}\times\mu_V$
ε_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:

$$\alpha_V(t) = \beta_V c_n(t) \frac{\left(\sum_k f_H e_H^{(k)} H_2^{(k)}(t) + f_D e_D D_2(t)\right)}{V_0(t) + V_N(t)}.$$

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.

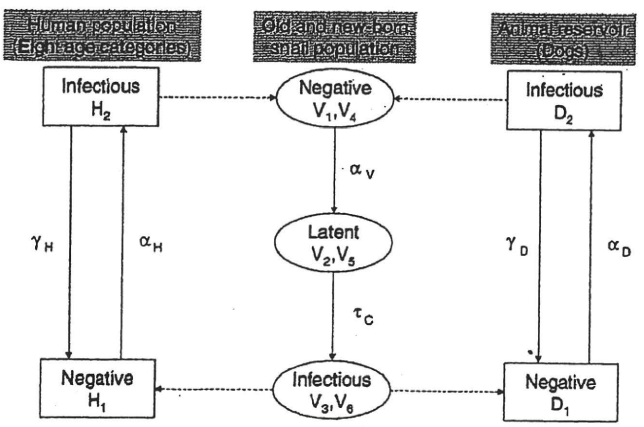

Fig. 3. The basic scheme of the transmission model for *S. mekongi*. Deaths of hosts are omitted in this scheme. The solid line shows the transfer among epidemiological classes of hosts. The dotted line shows miracidial and cercarial infections.

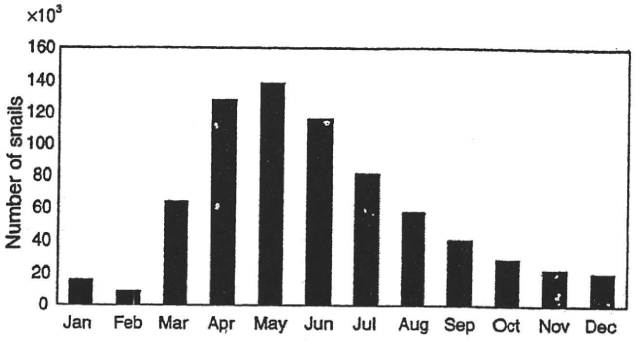

Fig. 4. The monthly variation of the total snail population.

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

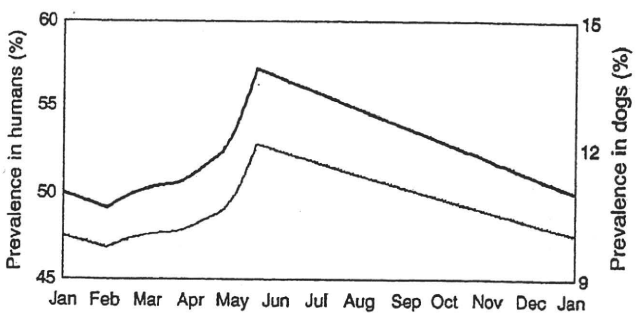

Fig. 5. Variation of prevalence in both humans (black line) and dogs (gray line) without control measures.

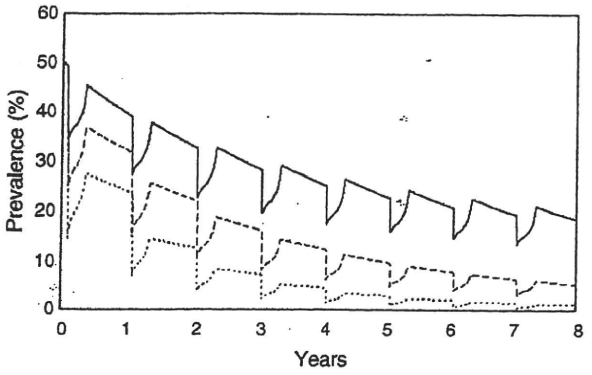

Fig. 6. Variation of the prevalence of *S. mekongi* in humans with yearly universal treatment (UT) for three coverage rates: 30% (solid line), 50% (dashed line), and 70% (dotted line).

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

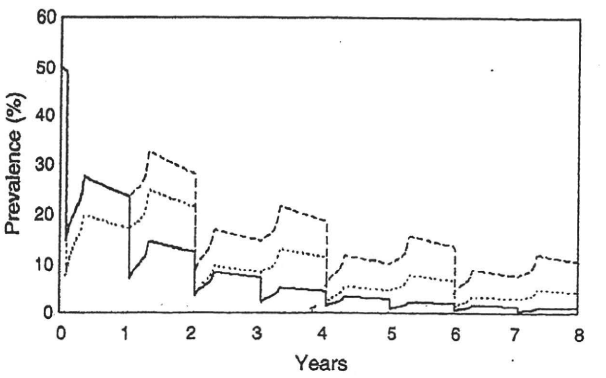

Fig. 7. Variation of the prevalence of *S. mekongi* in humans with universal treatment (UT) by changing the interval between treatments with two coverage rates: yearly UT with a 70% coverage rate (solid line), biyearly UT with a 70% coverage rate (dashed line), and biyearly UT with a 85% coverage rate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

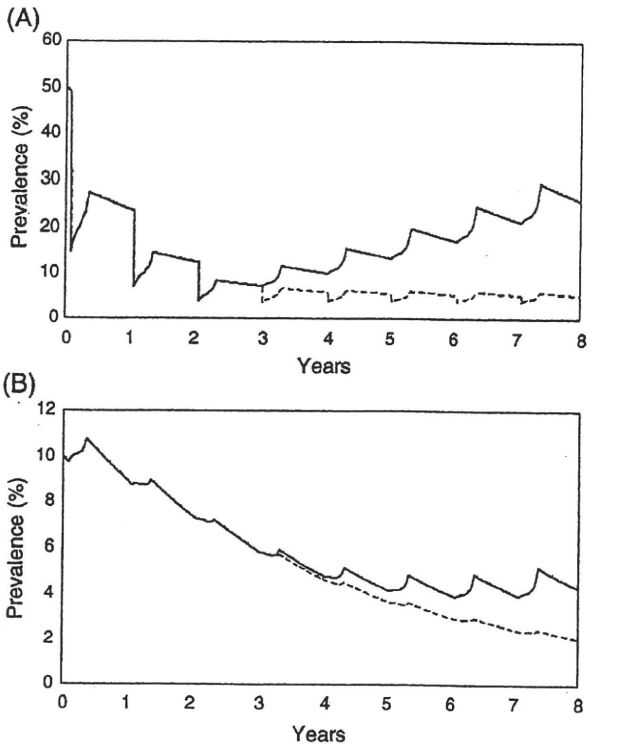

Fig. 8. Variations of the prevalence in both humans (A) and dogs (B) with 2 control measures. 1: yearly universal treatment (UT) with a 70% coverage rate for the initial 3 years (solid line), 2: after 3 years of annual UT yearly targeted mass treatment (TT) with a 85% coverage rate (dashed line).

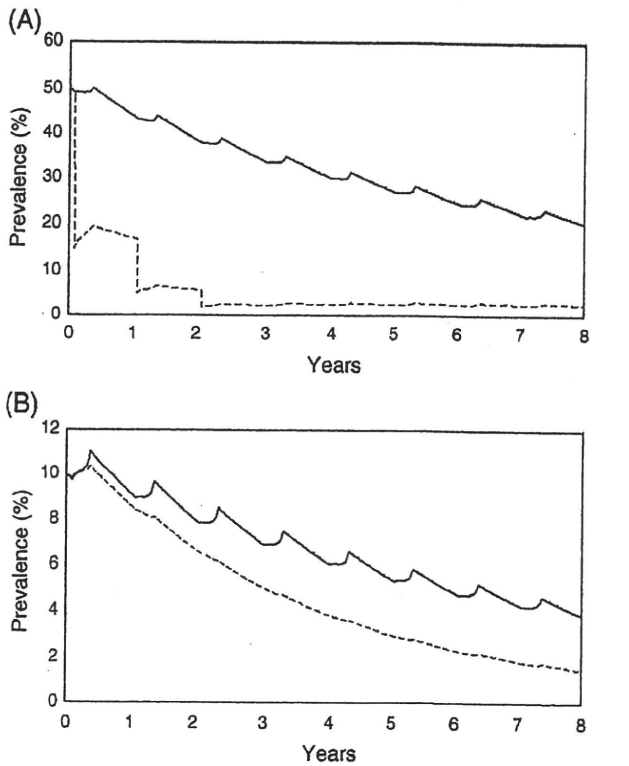

Fig. 9. Variations of the prevalence in both humans (A) and dogs (B) with 2 control measures. 1: only health intervention for 8 years (solid line), 2: health intervention for 8 years with yearly universal treatment (UT) with a 70% coverage rate for the initial 3 years (dashed line).

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

Hiromu Sugiyama[1], Yasuyuki Morishima[1], Sutheewan Binchai[2] and Achariya Rangsiruji[2]

[1] Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan;
[2] Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand

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.

Correspondence: Hiromu Sugiyama, Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel: +81-3-5285-1111; Fax: +81-3-5285-1173
E-mail: hsugi@nih.go.jp

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.*