in other infectious diseases.[31,32] Our designed primer set was suitable for detection of the 28S ribosomal region from *S. japonicum*, and it performed better than had been previously reported, indicating that the sensitivity was 15 pg.[19] Non-LTR retrotransposon, SjR2, was also detected by PCR at a sensitivity of 1 pg, nearly coinciding with the previous result (0.8 pg).[16] Our results indicated that the sensitivity of 28S rDNA is higher than that of SjR2, because R2 sequences were specifically inserted into the 28S ribosomal region and the copy numbers of SjR2 were restricted by that of the target 28S ribosomal DNA.[33]

The LAMP assay is a rapid, specific, and convenient assay employing four primers and isothermal DNA polymerase, and this tool can be applied as a new molecular diagnosis in the field in endemic areas. Our results showed that the sensitivity of LAMP was the same as that of conventional PCR. In general, the LAMP method is more sensitive than the PCR method,[20,22,34,35] although similar sensitivities between the two have also been reported.[24] This may reflect the fact that the sensitivity is dependent on the designed primers. However, the present study found that the sensitivity of PCR assay was sufficient to amplify the 28S ribosomal DNA from a single miracidium, and the results between the LAMP and PCR assays were completely consistent. Therefore, the LAMP assay seems capable of detecting a single miracidium rapidly and inexpensively in the field. Recently, Xu and others[25] investigated LAMP targeting SjR2 and found that its sensitivity was 0.08 fg. We repeated their experiment using the reported primers,[25] but we found that the DNA from *S. japonicum* was not adequately amplified, because each sequence of the reported primers was not identical to the SjR2 regions.

By contrast, PCR, using 28S rDNA primer sets amplifying the *S. japonicum* DNA from the snails infected with a single miracidium at 1 day after infection, was able to detect a single individual of *S. japonicum* throughout the snail stages, whereas conventional microscopy can detect only mature cercaria of *S. japonicum*. Furthermore, PCR is useful for beginners without skill and knowledge, because this method can be specific to *S. japonicum* and can distinguish it from the other species. To evaluate whether our PCR method could be applied in endemic areas, we collected wild snails from endemic areas in Anhui province of China. After crushing the snails and checking for infection by microscopy, total DNA extracted from each snail underwent PCR using 28S rDNA primer sets. PCR amplified the product band from not only snails including the matured cercariae of *S. japonicum* but also snails where cercaria could not be seen by microscopy. This is further support for our hypothesis that PCR could detect the potential infection in the snails with early sporocysts. However, PCR never detected schistosomal DNA from cercaria-negative snails collected from Tongling in the autumn (September). It may be that the differences in the findings between the two areas reflect differences in the timing of new infections in snails as a function of the season and local factors. These areas were part of the marshlands of the Yangtze River where water levels fluctuate markedly because of rainfall and flooding. In autumn, domestic animals exit the marshlands because of the rising water level, which usually reaches the highest level of the year. Thus, transmission of miracidia to snails may be the most difficult in autumn, although domestic animals known to contain hosts of *S. japonicum* were found to be repeatedly infected throughout the year.[36,37] These data suggested that the PCR method has the potential to monitor the timing of the infection of snails in endemic areas.

Several previous reports have suggested that LAMP is useful for the detection of the infections in pathogen-carrying vectors.[38,39] To evaluate the efficiency of the LAMP method for detecting infected snails, a large number of snails contaminated with a single infected snail were crushed together, and the total DNA was extracted in one tube. We then investigated whether LAMP could amplify the schistosomal DNA alone and found that LAMP could detect infection from a snail infected with *S. japonicum* in a group of 100 non-infected snails, indicating its use for detecting infection at a 1% infection rate. If snails (1,000–10,000 individuals) collected from several locations (e.g., 10–100 locations) were assayed, we expect that this method could precisely identify the infection rate in that area. The LAMP assay using 28S rDNA primers may be an effective tool, having the benefits of being rapid, easy, and inexpensive. Although the microscopy method is inexpensive, it is difficult to crush and observe a large number of snails. In particular, the novel LAMP method will make it possible to easily monitor very low infection rates of snails in endemic areas, where the new integrated strategy will be implemented.[7,8]

In the present study, we evaluated PCR and LAMP assay targeting to 28S rDNA from *S. japonicum*. We found that PCR amplifying 28S rDNA could detect 100 fg of DNA from *S. japonicum* but none from *S. mansoni*. Furthermore, the PCR (and LAMP) method could detect the infection of *S. japonicum* in every stage inside the snail. In fact, PCR could detect potential infection from snails deemed negative for infection by microscopy that were sampled from wild snails collected from endemic areas. LAMP, which is rapid, easy, and safe to use in the field, was able to amplify the schistosomal DNA from a single infected snail in a total of 100 snails without marked inhibitions. PCR and LAMP targeting to 28S rDNA may be useful for monitoring the infection rate of snails in endemic areas and for confirming complete eradications against infected snails in the areas where the new integrated strategy is implemented.

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EFFICACY OF SODIUM METAPERIODATE (SMP)-ELISA FOR THE SERODIAGNOSIS OF SCHISTOSOMIASIS MEKONGI

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Abstract. Schistosomiasis mekongi is an important public health issue in endemic countries. In this study, we evaluated an indirect immunodiagnostic ELISA method using *Schistosoma mekongi* soluble egg antigen. Sodium metaperiodate (SMP)-ELISA was utilized in order to remove the glycosylated epitopes responsible for false positive reactions and the results using this method were compared with those using conventional ELISA (conv-ELISA). Forty-two serum samples from schistosomiasis mekongi egg-positive patients and 100 serum samples from schistosomiasis-negative Cambodian subjects were tested using both ELISA methods. The ranges of ELISA values for positive and negative sera were distinct on SMP-ELISA, but the ranges of the two groups of sera overlapped on conv-ELISA. Therefore, diagnostic criteria may be established based on the highest ELISA value on negative sera and the lowest ELISA value on positive sera. In the present study, both the sensitivity and specificity of SMP-ELISA reached 100% using the criteria in which an ELISA value ≥ 0.2 was positive.

Keywords: SMP-ELISA, *Schistosoma mekongi*, diagnosis

INTRODUCTION

Schistosomiasis mekongi is a significant public health problem in Lao PDR and Cambodia. A mass drug administration campaign by the National Center for Parasitology, Entomology and Malaria Control (CNM), Ministry of Health, Cambodia, with international cooperation was initiated in 1994 using praziquantel in Kratie Province. At the beginning of the control program, the disease prevalence in primary schools in the Kratie Province, as determined by stool examination using the Kato-Katz method, was 72.9% (Stich *et al*, 1999). As a result of the control program, the prevalence of schistosomiasis decreased dramatically. In 2004, when stool

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surveys were conducted at five sentinel villages, no schistosome eggs were detected and only three cases of schistosomiasis were reported (Sinuon *et al*, 2007).

Despite the success of the control program, the limited sensitivity of the Kato-Katz method is a matter of concern. The detection of eggs from patient stool has become difficult because the intensity and prevalence of the disease have decreased. Therefore, a more sensitive diagnostic technique is required to determine progress of the schistosomiasis control program (Urbani *et al*, 2002; Fenwick *et al*, 2006). Bergquist *et al* (2009) stated that diagnostic methods need to be continually adapted based on the stage of control. Serological diagnosis, such as using an enzyme-linked immunosorbent assay (ELISA), is a potential alternative diagnostic method for the schistosomiasis mekongi control program (Ohmae *et al*, 2004).

We previously conducted a seroepidemiological survey of schistosomiasis japonica using circum oval precipitin (COP) tests (Tanaka *et al*, 1987) and ELISA (Matsumoto *et al*, 1999; Chigusa *et al*, 2006) and have studied *Schistosoma mekongi* control in Cambodia (Ohmae *et al*, 2004).

Immunodiagnosis of schistosomaisis mekongi using antigens of *Schistosoma japonicum* (Zhu *et al*, 2005) and keyhole limpet hemocyanin (Ittiprasert *et al*, 2000) have been carried out, but immunological diagnosis using *S. mekongi* antigen has not previously been reported.

In this study, we performed an ELISA using the soluble egg antigen (SEA) of *S. mekongi* and utilized sodium metaperiodate (SMP)-ELISA. The method of Alarcón de Noya *et al* (2000) was used in order to remove the glycosylated epitopes responsible for false positive reactions occurring with immunodiagnosis of

S. mansoni infection. In this study, a comparison between SMP-ELISA and conventional ELISA was performed, and we discus suitable criteria for the methods.

MATERIALS AND METHODS

Serum samples

A mass drug administration campaign was conducted in Cambodia: individuals in the target population were diagnosed by stool examination and serological testing as part of the National Schistosomiasis Control Program for Cambodia. The 42 positive control sera (Group A) used in this study were collected from schistosome egg-positive residents in 2003 (*n* = 34) and 2009 (*n* = 8) in Kratie Province, Cambodia. The 34 sera collected from *S. mekongi* infected patients in 2003 were pooled and used as the positive reference serum, which was distributed into sample tubes and stored at -40°C until use.

Negative control sera were collected from volunteers without risk for schistosomiasis mekongi infection: 100 Cambodians (Group B) in 2002 and 25 Japanese (Group C) in 2003. Group B was comprised of schoolchildren in Phnom Penh, located more than 120 km away from the endemic area. Furthermore, these subjects had no history of visiting the endemic area. Serum samples were collected by CNM as a part of the parasitic diseases control program.

Individuals in Group C were healthy Japanese volunteers without a history of traveling abroad. The purpose of the study was explained and written informed consent was obtained from all volunteers.

Antigen

Schistosoma mekongi (Laotian strain) was maintained in *Neotricula aperta* snails and mice (ICR strain) at the Laboratory of Tropical Medicine and Parasitology,

Dokkyo Medical University, Japan. SEA was prepared using the method described by Matsuda *et al* (1984). Eggs of *S. mekongi* were isolated from infected mice intestines by digestion method using actinase E (No. 122, Kaken Pharmaceutical, Tokyo, Japan) and collagenase (C6885, Sigma-Aldrich, St. Louis, MO), and the collected eggs were subsequently lyophilized. SEA was extracted from lyophilized eggs with carbonate buffer (0.05 M, pH 9.6). After protein content measurement using Bradford reagent (#500-0006, Bio-Rad Laboratories, Hercules, CA), 1 ml aliquots of SEA solution were placed in small tubes and stored at -80°C until use.

ELISA

The ELISA was performed as described by Matsuda *et al* (1984) and Hirose *et al* (2005) with some modifications. In this study, the standard ELISA technique used in our laboratory was designated as conventional-ELISA (conv-ELISA). *S. mekongi* SEA was dissolved in carbonate buffer at a concentration of 2 μg protein/ml just before sensitization of the ELISA plate (No.762070, Greiner Bio-One, Frickenhausen, Germany). Each well of the ELISA plate was sensitized overnight with 0.1 ml of *S. mekongi* SEA diluent. After washing the wells with T-PBS (0.15 M phosphate buffered-saline containing 0.05% Tween 20), the inner surfaces of the wells were blocked with 0.12 ml of BSA/T-PBS solution (T-PBS containing 1% bovine serum albumin) for 10 minutes.

We used horseradish peroxidase (HRP)-conjugated anti-human IgG goat serum (55252, MP Biochemicals, LLC-Cappel Products, Soton, OH) for the enzyme-conjugated antibody and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) di-ammonium salt (ABTS)(A-1888, Sigma-Aldrich, St. Louis, MO) as a substrate for

HRP. The optimal concentrations of antigen, test sera, and enzyme conjugate were determined using checkerboard titration. The test serum was diluted to 1:200 with BSA/T-PBS, and 0.1 ml of the dilution was put in each well. After incubation of the plate at 37°C for 45 minutes, the wells were washed three times with T-PBS. Zero point one milliliter of HRP conjugate diluted to 1:1,200 was then added, and the plate was incubated at 37°C for 60 minutes. After washing, 0.2 ml of ABTS solution (0.03% ABTS, 0.25 M citric acid, 0.25 M sodium dihydrogen phosphate, 0.003% H_2O_2) was put in each well and the plate was kept at room temperature for 1 hour to allow enzyme reactions. The optical density (OD) of each well was read by a microplate reader (MTP-120, CORONA ELECTRIC, Ibaraki, Japan) at 415 nm.

For SMP-ELISA, SMP treatment of antigen was performed as described by Alarcón de Noya *et al* (2000) except for the concentration of SMP solution. The *S. mekongi* SEA-sensitized plate was prepared using the same method as conv-ELISA and was washed with 50 mM sodium acetate buffer (pH 4.5). Each well was treated with 0.1 ml of 0.5 mM SMP solution (0.5 mM SMP in sodium acetate buffer) at room temperature for 1 hour in the dark. After briefly washing the treated plate with PBS, 0.12 ml of 50 mM sodium borohydride in PBS was added to the wells and the plate was incubated for 30 minutes at room temperature. After washing three times with T-PBS, the plate was subjected to ELISA in a similar manner as conv-ELISA, except the dilution of HRP conjugate was 1:6,000. Optimal concentrations of SMP solution and enzyme conjugate were determined using checkerboard titration to make the positive reference serum produce an OD value comparable with that of the conv-ELISA.

Evaluation of cross-reaction with other parasites

A parasitological survey was conducted in Kratie Province, Cambodia in May 2005 as part of the National Schistosomiasis Control Program. In this survey, eggs from other helminths were found at positivity rates of 14.6-25.8% by stool examination in a village, Talous, located 5 km south of the city of Kratie. According to a previous survey, Talous was known as an area with low transmission of *S. mekongi*. Therefore, we compared the results of stool examination with those of the two ELISA methods in samples obtained from Talous to evaluate cross-reaction with other parasite infections by ELISA. Blood and stool were collected from 151 residents in Talous. Blood samples were collected on blood sampling filter papers (Advantec Toyo Kaisha, Tokyo, Japan) after finger pricking. The blood samples on filter paper were dried and transported to the laboratory in Japan. Discs (3 mm in diameter) were cut out from the blood sampling filter paper and placed individually into wells of deep-well microplates. Each disc with blood was immersed in 400 μl of BSA/T-PBS containing 0.5% skimmed milk, shaken vigorously, and incubated overnight at 4°C after vigorous shaking. The resulting extract was estimated to have a 1:200 dilution of the plasma specimen and was tested by the ELISA methods as described above. For reasons described in the Discussion section, we used the criterion that ELISA values ≥ 0.2 were positive. Stool examination was conducted using a formalin-detergent technique (Waikagul *et al*, 1997). In brief, 0.5 ml of each stool sample was suspended in a formalin-detergent solution (10% liquid dish-washing detergent, 2% formalin in water) and incubated for 30 minutes at room temperature. The suspension was filtered through gauze into another tube and then shaken vigorously. The suspension was allowed to settle for 3 hours, after which the supernatant was discarded. The remaining pellet was dissolved in 10% formalin to give a volume of 1 ml. The resulting specimen was mixed well, then 0.04 ml of the specimen was examined under the microscope. Each sample was examined twice.

Statistical analysis

The software program Microsoft Excel (Microsoft Office Excel 2003, Microsoft, Washington, WA) was used to calculate the correlation coefficient (r).

The two-sided probability (P) for the Fisher's exact probability test was calculated by standard statistical software (Dr. SPSS 2 for Windows, Version 11.0.1J, SPSS, Chicago, IL). *P*-value < 0.05 was considered significant.

RESULTS

The results of the two ELISA methods are shown in Fig 1 and Table 1. The range of ELISA values in Group A was wider with the SMP-ELISA on both the higher and lower sides. The highest ELISA value of Group B with the conv-ELISA (0.578) was markedly lower with the SMP-ELISA (0.198). The range of ELISA values from Group C was very narrow in comparison with the other groups, and there was little difference between the two ELISA methods. With conv-ELISA, the lowest value in Group A (0.330) was lower than the highest value in Group B. Therefore, 10 individuals (23.8%) from Group A and 6 individuals (6%) from Group B had ELISA values ranging from 0.330 to 0.578. In contrast, the distribution of the ELISA values from Group A was different than those from Groups B and C with SMP-ELISA.

The correlation between conv-ELISA and SMP-ELISA, from Groups A and B is

Table 1

A comparison of the ELISA methods for Schistosomiasis mekongi.

	conv-ELISA			SMP-ELISA		
	Group A	Group B	Group C	Group A	Group B	Group C
No. examined	42	100	25	42	100	25
Minimum	0.330	0.006	0.004	0.233	0.015	0.010
Maximum	1.232	0.578	0.054	1.526	0.198	0.058
Mean	0.755	0.089	0.018	0.694	0.058	0.029
SD	0.211	0.107	0.014	0.314	0.041	0.011
Mean + 3SD		0.410	0.059		0.180	0.061

Table 2

Relationship between the ELISA results and other parasitic infections detected by stool examination (n = 151).

			A. lumbricoides		Hookworms		Echinostoma sp	
			Positive	Negative	Positive	Negative	Positive	Negative
			22	129	35	116	39	112
conv-ELISA[b]	Positive	25	3	22	7	18	9	16
	Negative	126	19	107	28	98	30	96
	Fisher's exact probability test (P)		1.000		0.605		0.217	
SMP-ELISA[b]	Positive	8	2	6	3	5	3	5
	Negative	143	20	123	32	111	36	107
	Fisher's exact probability test (P)		0.329		0.388		0.426	

[a]Stool examination was conducted using the formalin-detergent method.
[b]ELISA values ≥ 0.2 were considered positive.

shown in Fig 2. There were significant correlations between the two ELISA methods in both groups. The correlation coefficient of Group A was higher (r = 0.951, <0.001) than the correlation coefficient of Group B (r = 0.744, <0.001).

Stool examination and ELISA were conducted to determine cross-reactivity with other parasites (Table 2). Of 151 subjects, 25 (16.6%) and 8 (5.3%) had positive

ELISA results with the conv-ELISA and the SMP-ELISA, respectively. No *S. mekongi* eggs were detected. Eggs from *Ascaris lumbricoides*, hookworms, and *Echinostoma* sp were detected in samples from 22, 35, and 39 individuals, respectively. Most individuals who had eggs of other parasites had negative ELISA results on both ELISAs, but some individuals had positive ELISA results. The ELISA positivity rates

Table 3
False positive and false negative in each criteria.

	conv-ELISA			SMP-ELISA		
	Group A	Group B	Group C	Group A	Group B	Group C
≥0.2	42 (100%)	9 (9%)	0 (0%)	42 (100%)	0 (0%)	0 (0%)
<0.2	0 (0%)	91 (91%)	25 (100%)	0 (0%)	100 (100%)	25 (100%)
≥X (B)[a]	41 (98%)	2 (2%)	0 (0%)	42 (100%)	2 (2%)	0 (0%)
<X (B)[a]	1 (2%)	98 (98%)	25 (100%)	0	98 (98%)	25 (100%)
≥X (C)[b]	42 (100%)	45 (45%)	0 (0%)	42 (100%)	33 (33%)	0 (0%)
<X (C)[b]	0	55 (55%)	25 (100%)	0 (0%)	67 (67%)	25 (100%)

[a]X (B) Mean + 3SD of Group B in each the method. The values are shown in Table 1.
[b]X (C) Mean + 3SD of Group C in each the method. The values are shown in Tabele 1.

among egg-positive patients were 13.6-23.1% with conv-ELISA and 7.7-9.1% with SMP-ELISA. The Fisher's exact probability test did not show bias with the ELISA for intestinal parasites.

DISCUSSION

SMP treatment of SEA was performed in order to destroy the glycosylated epitopes responsible for false-positive results (Alarcón de Noya *et al*, 2000). Because glycosylated epitopes can be recognized by antibodies in *Schistosoma mekongi* infected patients, the OD values of the patients decreased with the SMP-ELISA when we used enzyme conjugate at the same dilution rate as that used for conv-ELISA (data not shown). In this experiment, we used a higher concentration of enzyme conjugate with the SMP-ELISA. SMP treatment can increase or decrease ELISA values based on serum type and result in expansion of the range of ELISA values for Group A with SMP-ELISA. Differences in the effect of SMP treatment on ELISA values appear to depend on the composition of target epitopes in each serum sample. Most antibodies in sera with high ELISA values in Group B, which consisted of subjects from a non-endemic area in Cambodia, recognized the glycosylated epitopes in SEA.

Diagnostic criteria are established based on the protocol and/or purpose of the study. Many researchers have used mean + 3SD for the OD values of negative control sera as a cut-off limit (Alarcón de Noya *et al*, 2000). Some authors used criteria that depended on the distribution of ELISA values for positive and negative controls (Hirose *et al*, 2005). In this study, Cambodian (Group B) and Japanese (Group C) individuals were examined as negative controls. Using the mean + 3SD for the negative control for Group B as a cut-off value (Table 3), the sensitivity and specificity of conv-ELISA were both 98%, and the sensitivity and specificity with SMP-ELISA were 100% and 98%, respectively. Using the same criteria for Group C (Table 3) results in false positives with conv-ELISA of 45% and with SMP-ELISA of 33%.

The range of ELISA values for positive sera was distinct from that for negative sera with SMP-ELISA. Therefore,
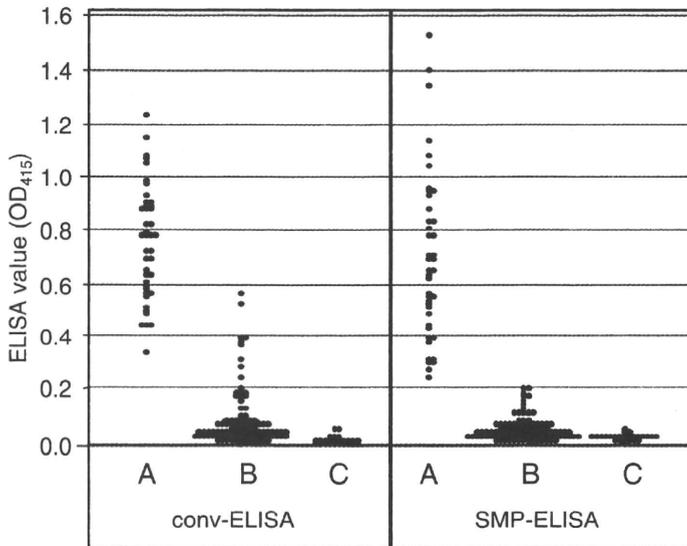
Fig 1–Distribution of ELISA values for sera from *S. mekongi* infected patients and from subjects in non-endemic areas by the two ELISA methods. A: *S. mekongi* infected patients ($n = 42$); B: subjects in a non-endemic area in Cambodia ($n = 100$); C: subjects in Japan ($n = 25$).
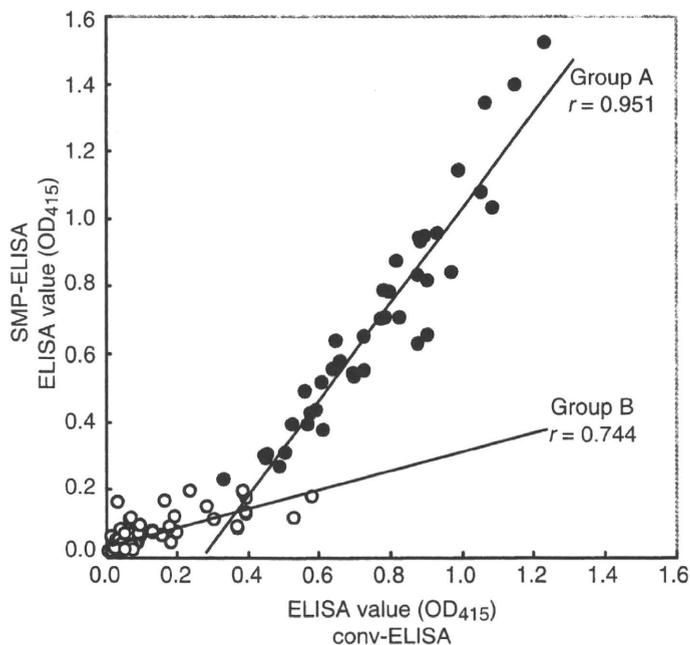

Fig 2–Plot of OD values comparing conv-ELISA to SMP-ELISA to evaluate the correlation between the two ELISA methods. The OD values of sera from *S. mekongi* infected patients (solid circle) and Cambodians living in a non-endemic area (open circle) are shown. Group A: *Schistosoma mekongi* infected patients ($n = 42$); Group B: persons in a non-endemic area in Cambodia ($n = 100$).

diagnostic criteria can be established based on the highest ELISA value for negative sera and the lowest ELISA value for positive sera. Using the criterion in which an ELISA value ≥ 0.2 was positive, both the sensitivity and specificity of SMP-ELISA reached 100% (Table 3).

Concerning negative control sera, the range of ELISA values for Group B (Cambodian) was higher than for Group C (Japanese). This is possibly due to a difference in antigens to which each group were exposed, both qualitatively and quantitatively. Our results suggest SMP-ELISA can reduce the influence of cross reactive antigens.

A positive ELISA reaction without schistosome eggs indicates one of three scenarios: 1) active infection with *S. mekongi* but no eggs were detected, 2) *S. mekongi* has already been treated but residual antibodies still existed, and (3) non-specific cross-reaction was detected. The lower sensitivity of the stool examination compared to the ELISA may explain why scenario 1) might occur. In low transmission areas, such as in Talous, it is difficult to detect eggs in stool samples because of lower disease intensity (Urbani *et al*, 2002; Fenwick *et al*, 2006). Scenario 2) is a characteristic feature of diagnostic methods detecting specific antibodies. This reaction indicates the subject had a risk for infection. In

general, residual antibodies tend to diminish progressively, and withdrawal periods vary between individuals (Hayashi *et al*, 2000). In scenario 3), a non-specific cross-reaction, might be one of the causalities, at least with the conv-ELISA (Fig 1). Most patients with intestinal parasite eggs had negative ELISA values, although some had positive values. The present data suggest infection with one of these three intestinal parasites should not result in a significant cross-reaction. Given the results of Table 3, it can be seen that the positive reactions on SMP-ELISA are most likely caused by scenarios 1) or 2). Assuming that all positive ELISA tests were due to cross-reaction with other parasites, the maximum rate of false positives with SMP-ELISA would be 9.1% (2/22). In another survey in a *Schistosoma mekongi* endemic area in Champasack Province, Lao PDR in 2006, we detected *Opisthorchis viverrini* eggs without *S. mekongi* eggs in 13 of 41 individuals who took the survey (Nakamura *et al*, 2006; unpublished data). Three of 13 opisthorchiasis patients had positive ELISA values, and 14 out of 41 had positive ELISA values on SMP-ELISA. The ELISA positivity rate for *O. viverrini* egg-positive persons (23%) was lower than the positivity rate of the targeted 41 persons (34%). These data suggest cross-reaction should not be significant with these helminths, although further studies are required for validation.

We conclude the use of SMP-ELISA improves diagnostic specificity and sensitivity for schistosomiasis mekongi. This method should become a powerful tool for diagnosing infection.

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抗蠕虫薬
プラジカンテル，メベンダゾール，アルベンダゾール，トリクラベンダゾール

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プラジカンテル *praziquantel*

（±）-2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino-〔2,1-a〕isoquinolin-4-one，商品名：ビルトリシド(バイエル薬品)

解　説●

プラジカンテルはピラジノイソキノリン誘導体で，ヒト使用例でも動物実験でも，多くの異なった種の吸虫や条虫がこの薬物に良く反応するが，線虫に対してはほとんど効果がない[1~3]．有効で安全な住血吸虫の駆虫薬を目指したバイエル薬品により，1970年代中頃に開発され，世界保健機構(WHO)は，住血吸虫症治療の essential medicine としているが，輸入例を除き同症が問題となることがなくなった日本では，保険適応は，肝吸虫，肺吸虫，横川吸虫など他の吸虫に限られる[2,4]．

抗蠕虫作用は，もっぱら住血吸虫で研究されてきており，成虫では，カルシウムイオンの関与で被膜から吸収され，主に2つの効果を示す．比較的低い有効濃度では，筋の活性を増強し，収縮と痙性麻痺を起こすので，作用を受けた成虫は血管壁から剥離し，腸間膜静脈系から肝臓へと血流に乗って移行する．やや高濃度では，プラジカンテルは成虫の外被に損傷を与える[1,3,5]．また，プラジカンテルは住血吸虫の虫卵にも作用し，組織内で肉芽腫を形成する虫卵を孵化し排出させる働きがある[6]．

プラジカンテルは経口投与後，速やかに吸収され，血漿濃度は1~2時間で最高となる．血漿中の半減期も短く，正常な腎・肝機能をもつ成人では，0.8~2時間だが，重篤な肝障害のある例では，3~8時間と伸びる[1,3]．プラジカンテルと代謝産物は，70~80％が，24時間以内に尿中に検出される．プラジカンテルは肝臓のチトクロームP450 3A4(CYP3A4)で代謝されるので，同酵素の反応を誘導するフェニトインやカルバマゼピン，リファンピシンで，クリアランスが上昇し，反応を抑制するシメチジンでクリアランスが低下する．リファンピシンと併用すると，有効な血中濃度が得られないので併用は禁忌とされる．

使用法●

プラジカンテルは，ヒトに感染するすべての住血吸虫の種に対し有効で，1回量40 mg/kgの1回内服，あるいは20 mg/kgでの6時間ごとに2，3回内服で，90％以上の治癒率が得られる．1回量25 mg/kgを同一日に3回投与すると，肝吸虫や横川吸虫，あるいは肥大吸虫，異形吸虫，横川吸虫などの消化管寄生吸虫にも高い治癒率を示す．同じ用量での1日3回投与を2日間続ける方法は，ウェステルマン肺吸虫症の治療にも有効である．ただし，肝蛭の場合は，用量を多くしても治療効果は期待できない[1~3]．

ヒトでの条虫成虫の腸内感染には，吸虫よりも少量のプラジカンテルで効果がみられる．広節裂頭条虫(日本海裂頭条虫)，有鉤および無鉤条虫に対しては用量10~20 mg/kgでの1回の内服でよい[1,2]．一方，幼虫寄生の場合，治療にはアルベンダゾールのほうがよいが，有鉤嚢虫(有鉤条虫幼虫)の組織感染では，プラジカンテルによる長期大量療法も，利用される[2,7]．しかし，多包条虫の幼虫による包虫症(エキノコックス症)での嚢胞病変には，プラジカンテルの効果はみられない．

副作用●

通常の使用例では，プラジカンテルの副作用が問題となることは少なく，嘔気，下痢，頭痛，腹

プラジカンテルは住血吸虫症治療薬として開発され，
WHO のエッセンシャル・ドラッグリストにも載るが，
日本での保険適応は，肝吸虫，肺吸虫，横川吸虫に限られる．
消化管に寄生した条虫の成虫に対しては，吸虫よりも
低用量のプラジカンテルで効果がみられる．

痛，発疹といった副作用は，みられたとしても軽度で一過性である．ただ，プラジカンテルの効果により体内で死滅した寄生虫の内容物や排泄物に対して，宿主の免疫反応が生じるので，多数寄生の場合は，重篤な症状を示すことがある．これらの副作用は，副腎皮質ホルモンの投与により緩和されるが，中枢神経系に占拠性病変を作っている有鉤嚢虫症の場合は，治療に細心の注意を払わねばならない．神経症状や画像所見からみて，治療後に，死滅虫体により不可逆性の失語症や片麻痺，眼障害などを起こす危険性がある場合，プラジカンテルの投与は禁忌である．

プラジカンテルは，小児にも安全に使用できるが，母乳中に出現するので，授乳中の婦人に投与する場合，服用して72時間は授乳を控えるのが望ましい．また，高用量のプラジカンテル投与はラットの流産率を高めるので，妊婦に用いるのは避けたほうがよいが，スーダンでの後ろ向き調査では，妊娠中のプラジカンテル服用の有無にかかわらず，流産や異常出産の比率に差はなく，目立った先天奇形もみられなかった[8]．

メベンダゾール mebendazole

methyl (5-benzoyl-1H-benzimidazol-2-yl) carbamate
　商品名：メベンダゾール(ヤンセンファーマ)

アルベンダゾール albendazole

methyl [6-(propylthio)-1H-benzoimidazol-2-yl] carbamate
　商品名：エスカゾール(グラクソ-スミスクライン)

トリクラベンダゾール triculabendazole

5-chloro-6-(2,3-dichlorophenoxy)-2-(methylthio)-1H-benzimidazole
　商品名：Fasinex (ノバルティス)懸濁液が獣医用薬として国内で市販
　Egaten (ノバルティス)ヒト用錠剤だが，国内では市販されていないので，"熱帯病治療薬研究班"から入手するなど入手法は限られる．

解　説

ベンズイミダゾールは，ベンゼン環とイミダゾール環が一辺を共有して結合した複素環式化合物で，上記の3種類の薬剤は，ベンズイミダゾール系化合物 benzimidazoles (BZAs) に属する．従来，メベンダゾール，アルベンダゾール，チアベンダゾールの3つの化合物が，ヒトの寄生蠕虫感染の治療に用いられてきたが，チアベンダゾールは他の BZAs に比して毒性が強く，近年，世界的に使用例が減少する傾向がある．日本国内でも，チアベンダゾールは糞線虫症に対して保険適応があったが，2002年にイベルメクチンが同症に対する治療薬として認可された後，生産・販売の中止に至った．メベンダゾールとアルベンダゾールは，消化管寄生蠕虫症の治療薬として，WHO Model List of Essential Medicines に記載されており，トリクラベンダゾールは，肝蛭の治療薬として吸虫症治療薬にあげられている[4]．一方，日本では，パモ酸ピランテル(コンバントリン)が，回虫，鉤虫，蟯虫など主要な線虫に対する保険適応薬とされており，メベンダゾールの適応は鞭虫症，アルベンダゾールの適応はエキノコックス症と限られている[2]．

BZAs が寄生蠕虫の体内で起こす生化学的変化は，ミトコンドリア内のフマル酸還元酵素や酸化

メベンダゾールやアルベンダゾールは，ベンズイミダゾール系化合物に属し，
国際的には消化管寄生線虫に対する標準的治療薬だが，
日本国内での保険適応は，メベンダゾールが鞭虫症，
アルベンダゾールがエキノコックス症（多包条虫の幼虫寄生）と限られている.

表1　プラジカンテルおよびベンズイミダゾール系薬剤の適応と使用法

	保険適応のある寄生虫症と使用量		保険適応のない寄生虫症と使用量	
プラジカンテル（ビルトリシド）	肝吸虫	20〜40 mg/kg/日　分2　3日または　75 mg/kg　分3　1日	住血吸虫　40 mg/kg/日　分2　2日	
	横川吸虫	50 mg/kg　早朝空腹時に1回内服し，2時間後に塩類下剤 50 mg/kg/日　分3　1〜2日　この場合下剤は不用	消化管寄生条虫（成虫寄生の場合）　20 mg/kg を1回服用後，下剤を併用有鉤嚢虫　50 mg/kg/日　分3　30日マンソン孤虫　75 mg/kg/日　分3　3日	
	肺吸虫	40〜75 mg/kg/日　分2〜3　2〜3日		
メベンダゾール（メベンダゾール）	鞭虫	200 mg/日/分2　3日間	蟯虫　100 mg を単回服用し，2週後に再度服用回虫　200 mg/日/分2　3日間鉤虫　200 mg/日/分2　3日間糞線虫　200 mg/kg/日/分2　4日上記を1クールとし陰性化まで旋毛虫　5 mg/kg　分3　5〜7日間	
アルベンダゾール（エスカゾール）	エキノコックス症（包虫症）	600 mg/日　分3　28日間服薬した後，14日間休薬のサイクルを繰り返す	イヌ・ネコ回虫（トキソカラ症）　10〜15 mg/kg/日　分2〜3で，4〜8週間顎口虫　10〜15 mg/kg/日　分2　3〜7日最大3週間まで有鉤嚢虫　15 mg/kg/日（最大800 mg/日）　分2　8〜30日間旋毛虫　400 mg　分2　5日	
トリクラベンダゾール（Egaten）			肝蛭　10 mg/kg　食直後　20 mg/kg　分2　食直後（重症例）	

（文献2）より引用）

的リン酸化の阻害，糖輸送の抑制など多様だが，重要な作用点として，β-チューブリンと結合することによる微小管重合の阻害をあげることができる[9]．これらの薬物では，哺乳類での蛋白質との結合に比して，寄生蠕虫のβ-チューブリンとの結合が，はるかに低い濃度で起きるので，多くの寄生蠕虫に対して駆虫効果を示す.

BZAs には，水に溶けにくく脂肪に移行しやすいものが多いので，投与後血漿中の濃度が急速に高くなることはなく，高脂肪食で吸収が促進され

ることが多い．また，速やかに肝臓での代謝経路に移行するが，代謝産物にも抗蠕虫作用がみられる．血漿中では蛋白と結合しやすいものが多く，メベンダゾールの95% は血漿蛋白と結合し代謝される．アルベンダゾールの代謝産物も，約70% が血漿蛋白と結合する．以上のような体内動態の特徴から，BZAs の血漿半減期は4〜15時間と長くばらつきも大きい傾向がある[1]．シメチジンと一緒に投与するとメベンダゾールの血漿濃度が増加するが，それは，肝臓のチトクローム

線虫や条虫の幼虫寄生の場合，治療にはアルベンダゾールが使用されることが多いが，有鉤嚢虫症（有鉤条虫の幼虫寄生）では，プラジカンテルも利用される．また，いずれの薬剤でも，幼虫寄生の場合は，成虫の駆虫よりも高用量で長期間の内服を必要とするのが一般的である．

系における代謝が妨げられるためと考えられる[10]．また，肝内の同じチトクローム系で代謝されるプラジカンテルと併用した場合も，メベンダゾールの血漿濃度は増加すると思われる．

使用法●

メベンダゾールやアルベンダゾールは，感染者の多い回虫，鉤虫，鞭虫などでは，成虫のみならず幼虫にも活性を示し，混合感染にも有効である．これらの消化管に寄生する線虫成虫では，運動停止は緩徐に起こるので，消化管から蠕動運動により寄生虫が完全に除去されるのに治療後数日を要する．蟯虫症は，国内でもまだ経験することが多い線虫症だが，メベンダゾールを使用する場合，100 mg錠剤を1回投与し，2週間後に2回目の投与を行う．回虫症・鞭虫症・鉤虫症には，メベンダゾール500 mgを1回投与する方法もあるが，1回量100 mgのメベンダゾールを朝夕2回，連続3日間投与することで確実な効果が得られる[1,2]．治療後3週間たっても患者が治癒しない場合は，同一の投薬スケジュールを繰り返す．特に，線虫の混合感染には，メベンダゾール3日間投与のほうが，メベンダゾール（500 mg）やアルベンダゾール（400 mg）の1回投与よりも効果がある．同じ1回経口投与法では，アルベンダゾールの治療効果のほうが高いという報告が多く，土壌伝播線虫感染の簡便な治療法として，アルベンダゾール400 mgの1回内服は，国際的には広く認知されている．また，アルベンダゾールは，フィリピン毛頭虫など，メベンダゾールが効かない線虫に対しても効果を示すが，糞線虫に対する効果は安定しない．さらに，アルベンダゾールは，イヌやネコの回虫・鉤虫による幼虫移行症に対しても効果的で，腸管にいる時期であれば旋毛虫にも活性がある[2]．

アルベンダゾールは，種によっては条虫の幼虫にも効果を示し，エキノコックス症や有鉤嚢虫症の治療にも使用されるが，その場合は，他の治療法や薬剤と併用されることが多い[11]．エキノコックス症では，単独で使用されることもあるが，嚢胞に対する除去手術や硬化剤注入術の前後に併用すると効果が増す．成人であれば，1回量300～400 mgを1日2回で，休薬しながら数ヵ月続けることになる[1,2]．また，有鉤嚢虫症に対しては，やはり1回量400 mgの1日2分服を，嚢虫の数・型・部位により8～30日間継続するという方法がある[1,2]．有鉤嚢虫の寄生部位によっては，アルベンダゾール内服前に副腎皮質ホルモンを投与し，死滅する嚢虫や放出される内容物死に対する生体の炎症反応の軽減をはかる配慮が必要である．また，アルベンダゾールは，免疫不全者に腸管感染を起こすある種の微生胞子虫 microsporidia に効果を示し，腟トリコモナスやランブル鞭毛虫のような嫌気性原虫に対しても，ある程度の有効性を示すが，日本国内で実際に使用される機会はほとんどない．トリクラベンダゾールは，もともとは獣医用肝蛭症治療薬（懸濁液）として開発されたBZAsだが，ヒト用治療薬（錠剤）も開発された．BZAsであるにもかかわらず，腸管内寄生線虫に対する駆虫薬としての効果はほとんどないが，プラジカンテルやアルベンダゾールといった薬剤が効かない肝蛭症には，感染早期の幼虫から慢性期の成虫まで有効である．

副作用●

BZAsは総じて安全な薬剤で，世界中で消化管寄生線虫症の治療に汎用されているが，副作用の頻度は，軽い消化器症状を中心に1%程度でしかない[1]．大量に感染している腸管内寄生虫が排出される際，一過性の腹痛や下痢が起こることがあ

ベンズイミダゾール系化合物に属するトリクラベンダゾールは，
寄生線虫に対する効果はほとんどないが，他の薬剤が効かない肝蛭症に
効果を示す．
寄生蠕虫症は，熱帯の途上国では最も日常的な感染症で，
WHO の指定する Neglected Tropical Diseases（NTDs）の過半を占めているが，
近年，対策の一環として，駆虫薬が集団投与される機会が増えている．

る．エキノコックス症や有鉤嚢虫症の治療に，アルベンダゾールを長期間用いる場合も，まず問題なく服用を続けられる．最も多くみられる副作用は，血清アミノトランスフェラーゼ活性の増加で，治療を終了すると正常域に戻るが，肝硬変の患者では禁忌と考えたほうがよい[12]．また，白血球や血小板の減少もみられるので，アルベンダゾールの長期連用中は，肝機能や骨髄抑制をモニターすべきである．

実験動物では，メベンダゾールやアルベンダゾールに，強力な胎仔毒性・催奇形性が報告されている．ヒトに催奇形性があるという証拠はないが，妊婦や2歳以下の小児には原則的に禁忌である[1]．しかしながら，妊娠初期に不注意にメベンダゾールを内服してしまった妊婦の調査で，通常の妊娠時に比して，有意に高い流産や先天奇形の合併はみられなかったとの報告もある．また，2歳以下の小児に対する使用について，WHO による非公式な会議で，土壌伝播蠕虫の治療によって得られる効果が大きいと判断されれば，1歳以上の例について投与量を半分に減量したうえで投与してもよいとコメントされた[13]．

集団治療による国際的寄生虫症対策

最近，WHO は，途上国を中心に世界的には感染者，罹病者が多いにもかかわらず，死亡にまで至る例が少ないうえ，先進国では感染者自体が限られているので，その健康被害の把握や対策の実施が遅れてきた一群の感染症を，Neglected Tropical Diseases（NTDs）として，国際的な取り組みを強化している．NTDs としては，ハンセン病やブルリ潰瘍などもあげられているが，各種の線虫，吸虫，条虫などによる寄生蠕虫症は，その過半を占めている．世界的には何十億という感染者数が推定される．回虫，鉤虫，鞭虫などによる

土壌伝播寄生線虫症 soil transmitted helminthes（STH）は，代表的な NTDs で，熱帯の途上国においては，最も一般的な感染症である．これらの寄生虫による健康被害は，就学期の小児で他の世代よりも高いので，WHO などの国際機関は，学校組織を通じた定期的で頻回の集団治療を進めている．罹病率を下げるために汎用される駆虫剤は BZAs で，特にアルベンダゾールやメベンダゾールの1回投与法が利用される．BZAs の1回投与によって駆虫されると，結果として宿主の鉄代謝やヘモグロビンの生成が安定，身体的発育や性的成熟も改善し，教育効果があがって欠席も少なくなるといった効果が期待される．線虫症では，リンパ性フィラリア症とオンコセルカ症の世界的制圧計画も進んでいるが，そこで中心となるジエチルカルバマジンやイベルメクチンといった抗フィラリア薬である．アルベンダゾールは，これらの薬剤との併用にも適しているとされ，合併する STH の治療にも有用であるのみならず，抗フィラリア作用が増す可能性も示唆されている．住血吸虫症も，NTDs にあげられるが，プラジカンテルは流行地の住民を対象にした集団的化学療法に広く使用されてきた．淡水性陸生貝から游出する幼虫によって感染する住血吸虫では，健康被害は小児以外に，農作業や野外作業に従事する青年層にも及ぶので，集団治療のターゲットとなる年齢層の範囲は広くなる．

このように，BZAs やプラジカンテルの使用頻度が世界的に増すにつれ，最近はこれらの駆虫薬に対する薬剤耐性の問題が関心を集めるようになった．現在，ヒトの線虫では，BZAs に対する薬剤耐性のはっきりした報告はないが，家畜の線虫では耐性の拡大が問題になっている．ベンズイミダゾール耐性株では，実験室由来株，野外分離株のいずれにおいても，薬物の β-チューブリン

プラジカンテルやベンズイミダゾール系薬剤の使用頻度が，世界的に増加する状況下で，今後は，薬剤耐性の出現にも注意を払わねばならない．

との結合の高い親和性が減退し，薬剤耐性と関連するβ-チューブリン・アイソタイプの遺伝子発現が変化している[1,14]．マンソン住血吸虫や日本住血吸虫では，プラジカンテル耐性株が実験室で分離されているが，その遺伝学的背景は明らかではない．しかし，プラジカンテルに対する臨床効果が減少したマンソン住血吸虫感染の例が，集団治療が行われた複数の高度浸淫地で報告されている．BZAsやプラジカンテルの定期的な頻回投与の機会が急速に増していることから，今後，これらの薬剤に対する耐性は，地球規模できちんとモニタリングしていく必要があると思われる．

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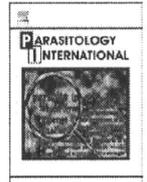
Transcripts analysis of infective larvae of an intestinal nematode, *Strongyloides venezuelensis*

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ABSTRACT

Free-living infective larvae of *Strongyloides* nematodes fulfill a number of requirements for the successful infection. They need to endure a long wait in harsh environmental conditions, like temperature, salinity, and pH, which might change drastically from time to time. Infective larvae also have to deal with pathogens and potentially hazardous free-living microbes in the environment. In addition, infective larvae must recognize the adequate host properly, and start skin penetration as quickly as possible. All these tasks are essentially important for the survival of *Strongyloides* nematodes, however, our knowledge is extremely limited in any one of these aspects. In order to understand how *Strongyloides* infective larvae meet these requirements, we examined transcripts of infective larvae by randomly sequencing cDNA clones constructed from *S. venezuelensis* infective larvae. After assembling successfully sequenced clones, we obtained 162 unique singletons and contigs, of which 84 had been significantly annotated. Annotated genes included those for respiratory enzymes, heat-shock proteins, neuromuscular proteins, proteases, and immunodominant antigens. Genes for lipase, small heat-shock protein, globin-like protein and cytochrome *c* oxidase were most abundantly transcribed, though genes of unknown functions were also abundantly transcribed. There were no hits found against NCBI or NEMABASE4 for 37 (22.3%) EST out of the total 162 EST. Although most of the transcripts were not infective larva-specific, the expression of respiration related proteins was most actively transcribed in the infective larva stage. The expression of astacin-like metalloprotease, small heat-shock protein, *S. stercoralis* L3Nie antigen homologue, and one unannotated and 2 novel genes was highly specific for the infective larva stage.

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1. Introduction

Strongyloidiasis is endemic in tropical and subtropical regions, such as Southeast Asia, Latin-America, and sub-Saharan Africa, and endemic foci are present in temperate countries as well, e.g. Mediterranean coast of Spain, southern United States, and Satsunann-Ryukyu Islands in Japan [1–3]. Hundreds of millions of people have been possibly affected globally, though no precise estimate is available.

The key for the control of strongyloidiasis is in the infective larva because the infection starts with this stage of the worm. Free-living infective larvae develop from eggs deposited by free-living females in the soil or parasitic females in the infected host. Infective larvae then wait a host to be infected for some time in the external environment. Considering the life of infective larvae, they obviously face a number of hard tasks before they finally find the host.

First, they are required to endure physical and chemical conditions among them. Temperature, salinity, and pH might change drastically during the wait and chemical compounds could contaminate their surroundings. Second, they have to get rid of or get along with pathogens and potentially hazardous free-living microbes. Among them are various kinds of viruses, bacteria, parasites, and fungi. Infective larvae should be equipped with a kind of defense mechanisms as free-living nematodes are [4,5]. Otherwise they would be heavily infected before they infect the host. In addition to the protection against a number of environmental factors, they must recognize appropriate host animals and start skin penetration as quickly as possible. Failure in host-finding and infection processes would result in the extinction of the species.

Strongyloides infective larvae definitely have solutions to all of the problems and situations described above, however, little is known about their survival and infection strategies [6]. It would be of great scientific and practical significance to understand the biological processes taking place in *Strongyloides* infective larvae. For example, because infection control cannot be done solely with a mass treatment of humans and animals due to the adverse side effects of drugs on biological diversity, new strategies to control the infection have to be explored based on the biology of the nematodes [7,8].

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In the present study, we examined the transcripts of infective larvae by randomly sequencing cDNA clones to clarify the biological processes activated in *S. venezuelensis* infective larvae. We found that the transcripts for respiratory enzymes, heat-shock proteins, neuromuscular proteins, proteases for infection, and an autophagy-related protein were observed. In addition, infective larvae of *S. venezuelensis* abundantly expressed genes which cannot be found in nucleotide databases with significant match. Further analysis of these molecules of unknown functions would greatly facilitate our understanding of the survival strategy of *Strongyloides* nematodes.

2. Materials and methods

2.1. Parasites and animals

Strongyloides venezuelensis has been maintained in male Wistar rats in the Division of Parasitology, Department of Infectious Diseases, University of Miyazaki [9]. ICR mice and Wistar rats were purchased from Kyudo (Kumamoto, Japan). All animals were kept and handled under the approval of the Animal Experiment Committee, University of Miyazaki. The third-stage infective larvae (L3i) were obtained from faecal culture by the filter paper method [10]. L3i were used right after they emerged from the feces (2–3 days after starting faecal culture). Parasitic adult female worms were collected from infected rats 8–10 days post infection (p.i.) [11].

Preparation of larvae in different developmental stages was carried out as previously described [12]. Lung-stage larvae (LL3) were collected as follows; male ICR mice were subcutaneously inoculated with 30,000 L3i, and lungs were removed 72–75 h p.i., homogenized with a Polytron PT-MR3000 (Kinematica AG, Littau, Switzerland) at 20,000 rpm for a few seconds. Lung homogenates were wrapped with Kimwipe papers and incubated in phosphate-buffered saline (PBS) at 37 °C for 1.5 h and emerging worms were collected. Since *S. venezuelensis* has been reported to molt twice in the intestinal mucosa, we designated lung larvae as LL3 [13]. For the preparation of tissue-migrating larvae (L3tm), L3i were injected into the peritoneal cavity of ICR mice, and recovered 20 h p.i. from the peritoneal cavity.

Collected worms were washed with sterile distilled water or PBS extensively, pelleted at the bottom of 2 ml centrifuge tubes then stored at −80 °C until used for RNA preparation (see below).

2.2. cDNA library construction

Infective larvae were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with glass beads for 1.5–2.5 min by a Mini-BeadBeater (Bio Spec Products, Bartlesville, OK, USA), followed by total RNA purification according to the manufacturer's instruction. After treatment with DNaseI (Promega, Madison, WI), poly (A)$^+$ RNA was purified with GenElute mRNA Miniprep Kit (Sigma, Saint Louis, MO).

A cDNA library was constructed using a SMART cDNA library construction kit (Clontech, Mountain View, CA). Reverse transcription (RT) of purified poly (A)$^+$ RNA was performed using MMLV reverse transcriptase with the SMART IV oligonucleotide primer and the CDS III/ 3′ PCR primer provided in the kit. Double-stranded cDNA (ds-cDNA) was synthesized by long distance PCR with the 5′ PCR primer and the CDS III/3′ PCR primer using the Advantage 2 PCR kit (Clontech). The ds-cDNA was treated with proteinase K and then digested with SfiI. After size fractionation, cDNA was ligated to SfiI-digested pDNR-LIB. The ligation product was then transformed into *Escherichia coli* ElectroMAX DH10B electrocompetent cells (Invitrogen).

2.3. DNA sequencing

Clones were transferred to LB agar plates containing 50 μg/ml chloramphenicol and grown for 20 h prior to colony direct PCR. Insert DNA was amplified from 500 randomly selected clones with a M13 primer set, and PCR products were purified with Post-Reaction Purification Columns (Sigma). Single-pass sequencing was performed from the 5′-end only using M13 forward primer (5′-GTAAAAC-GACGGCCAGT-3′) in ABI PRISM 3130×1 Genetic Analyzer (Applied Biosystems, Carlsbad, CA), using ABI PRISM Big-Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

2.4. EST processing, contig assembly and analysis

All ESTs were edited out flanking vector and adaptor sequences. After removing rRNA sequences, high quality ESTs longer than 250 bp (408 sequences) were assembled into clusters of contiguous sequences by using Sequencher software (Gene Codes, Ann Arbor, MI, USA). Reads with more than 99% identity were assembled into the same contig. The consensus sequences of contigs and singletons

Table 1
Primer sequences for real-time PCR.

NCBI accession	Associated annotation	Forward primer (5′→3′)	Reverse primer (5′→3′)	Expected product size
HO652180	FMRFamide-related peptide	CATTCCACCATCCGAGGTTACTT	GCAAGGGCTTGTTGTAGGG	115
HO652210	Cytochrome P450	GGGAAAAAGAATATGTGCAGGA	TGATAACACCAGAATCGGATGA	132
HO652258	NADH dehydrogenase subunit 6	TGTTGGTTGTTTTGCCAGTT	CCATAACCACAAAAATTCCACTT	99
HO652261	Poly(A)-binding protein	GGTGTCAATGCAGGACAAGTTA	TTGGATAATTCTGTGGGTTTCC	94
HO652553	Hsp90	CCAAGAGGATGCTGGTGATT	TCGAGACAATGCCGACTGTA	84
HO652556	L3Nie Ag (SvL3Nie-2)	GAACCAGAAAAATAAATTGGGAGA	ACTTCATCGTACCAACCTTTTACTCC	87
HO652576	Astacin-like metalloproteinase	GGTGTTGTTACACCCCAAGC	AACAACAATATATTAGTGCAATCGTT	145
HP429054	Globin-like protein	TCCCGGAAATGGCAGATTATT	AGGCCGGAAGAAACACTGAAA	111
HP429055	Novel[a] (SVC L3ist-1)	TAACCTAAATACTATCTTTTACAATTCC	CAATTCAGTTAATTTCCCTCCA	89
HP429056	Novel[a] (SVC L3ist-2)	TGGAGAGAAATTAACTGAATTGTTGG	TTTTCAATGTTTTTATGCAGGTTC	114
HP429057	*S. stercoralis* Hsp20 (SvHsp-Ss2)	TGGGATTGGCCTTTAAGTCA	GCAGTGAATGTCAATTTTGTCC	147
HP429058	None[b] (PTC 00570_1)	CGTAGAGGATCCACTGGACA	CATTGATACCAGTATCCATTACAGAGG	85
HP429059	*A. suum* Hsp20 (SvHsp20-As1)	TTTCCTTCCGCCATTCCTAT	TTGAAACGCCCTCATCTCTAA	108
HP429060	Lipase, class 2	TGCAAGTGTGGTAAAGATTGCACATGT	CCTCACAACATTTTGATTCAGCACAGC	88
HP429062	Cytochrome *c* oxidase subunit I	TGCTGGTGGTAATCCTTTGA	ATCCCAAGGGTTCCAAAAAC	151
HP429068	L3Nie Ag (SvL3Nie-1)	CTTGCAGCAAAAGCACAAAA	CAATGGTGAACCAAATGCAA	117
HP429073	None[c] (FC810578.1)	TGGCCATGGTATGTAGCAAT	ACCCCTAAATGATAGAATGCAGTTGA	131
HP429091	*S. stercoralis* Hsp20 (SvHsp-Ss1)	TTGTGATTGGCCACTTAATGTA	CCTGAATATCCTGTGGGTCAA	113
AB453330.1	18S rebosomal RNA	CCAGCTTTCCAAGTGCATAA	CATCCAAGATGCTCATTACACA	86

[a] Novel; No hits were found in major databases.
[b] Hits were found in NEMABASE4 with no associated annotation.
[c] No hit in NEMABASE4. Match was found in NCBI EST database.