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TABLE 1. *Relative expression levels of goblet cell- and mucin glycosylation-related genes in the gut mucosa of normal BN rats. Total RNA was extracted from epithelium separated from the jejunum, ileum or proximal colon, or was extracted from the glandular stomach mucosal scrape, and RT-PCR was performed. Levels of each gene expression were normalized to that of β-actin. In the Table levels in the jejunum were arbitrary expressed as 1.00.*

	Stomach	Jejunum	Ileum	Colon
MUC2 (mucin core peptide 2)	UD	1.0 ± 0.2	4.5 ± 1.8	153.2 ± 49.8*
MUC3 (mucin core peptide 3)	UD	1.0 ± 0.4	1.5 ± 0.3	7.5 ± 3.0*
MUC4 (mucin core peptide 4)	UD	1.0 ± 0.3	0.7 ± 0.1	41.0 ± 13.1*
Relmβ (resistin-like molecule β)	2.8 ± 1.7	1.0 ± 0.4	0.4 ± 0.1	47.3 ± 21.1*
TFF3 (intestinal trefoil factor)	0.2 ± 0.1	1.0 ± 0.4	2.7 ± 0.5*	3.9 ± 0.4*
Siat 4c (α-2,3-sialyltransferase IV)	3.6 ± 1.0*	1.0 ± 0.2	11.7 ± 4.1*	7.0 ± 3.0*
3ST1 (3-0 sulfotransferase-1)	12.7 ± 2.2*	1.0 ± 0.2	1.3 ± 0.2	15.4 ± 3.0*
3ST2 (3-0 sulfotransferase-2)	1.9 ± 0.7	1.0 ± 0.4	1.1 ± 0.3	3.3 ± 1.6
FUT1 (α-1,2-fucosyltransferase 1)	1.0 ± 0.2	1.0 ± 0.2	1.5 ± 0.1	13.8 ± 0.5*
FUT2 (α-1,2-fucosyltransferase 2)	3.6 ± 0.6*	1.0 ± 0.1	1.5 ± 0.3	14.3 ± 2.8*
FUT4 (α-1,3-fucosyltransferase 4)	4.7 ± 0.9*	1.0 ± 0.1	0.7 ± 0.1*	3.0 ± 0.2*
Lew 1 (Lewis type 1 antigen synthase: β1,3-N-acetylglucosaminyltransferase 5)	0.1 ± 0.0*	1.0 ± 0.3	0.3 ± 0.0	2.9 ± 0.9

Data shown are mean ± SE of 4 rats. *Significantly different from the levels in the jejunum ($p<0.05$). UD: undetectable.

TABLE 2. *Number of goblet cells in the jejunum and ileum after* N. brasiliensis *infection.*

Days after infection	Jejunum	Ileum
0	13.1 ± 0.7	12.3 ± 0.5
7	19.1 ± 1.2*	14.9 ± 1.2
14	21.5 ± 1.2*	20.0 ± 0.9*
21	11.3 ± 1.0	11.3 ± 1.3

Each measurement was performed on paraffin-embedded tissue sections. Figures in the table represent numbers of goblet cells/100 villus epithelial cells. All data are mean ± SE of 4 rats. *Significantly different from day 0 ($P<0.05$).

Figure legends

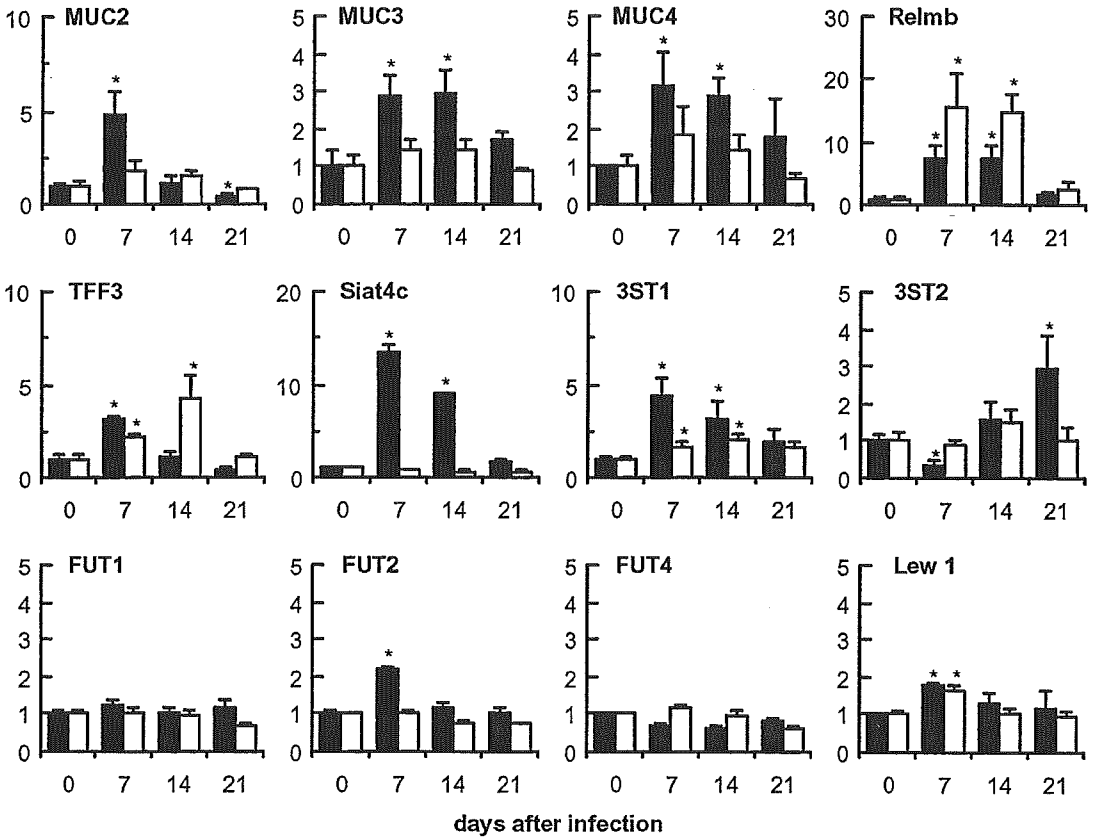
Fig 1. Expression of goblet cell- and mucin glycosylation-related genes in the jejunal (closed columns) and ileal (open columns) villus epithelium of BN rats after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction, reverse transcribed, and relative quantification was carried out by RT-PCR. The quantified value for each sample was normalized with respect to that for β-action. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 2. Expression of mucin glycosylation-related genes in the jejunal villus epithelium of mast cell-deficient *Ws/Ws* rats after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction and semi-quantitative RT-PCR was performed as described in Fig. 1. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 3. Expression of goblet cell- and mucin glycosylation-related genes in the jejunal villus epithelium of BN rats in the early period after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction and semi-quantitative RT-PCR was performed as described in Fig. 1. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 4. Successive upregulation of goblet cell- and mucin glycosylation-related genes in the intestinal epithelial cells during the course of *N. brasiliensis* infection.

Fig. 1


MUC2
MUC3
MUC4
Relmb
TFF3
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FUT1
FUT2
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Lew 1
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days after infection

Fig. 2.

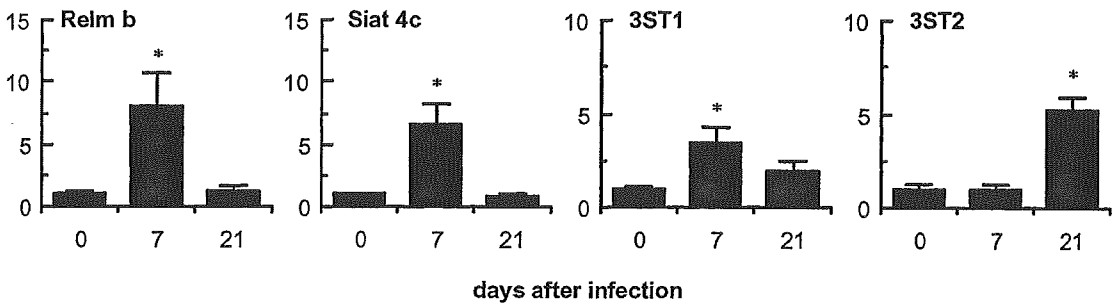


Fig. 3.

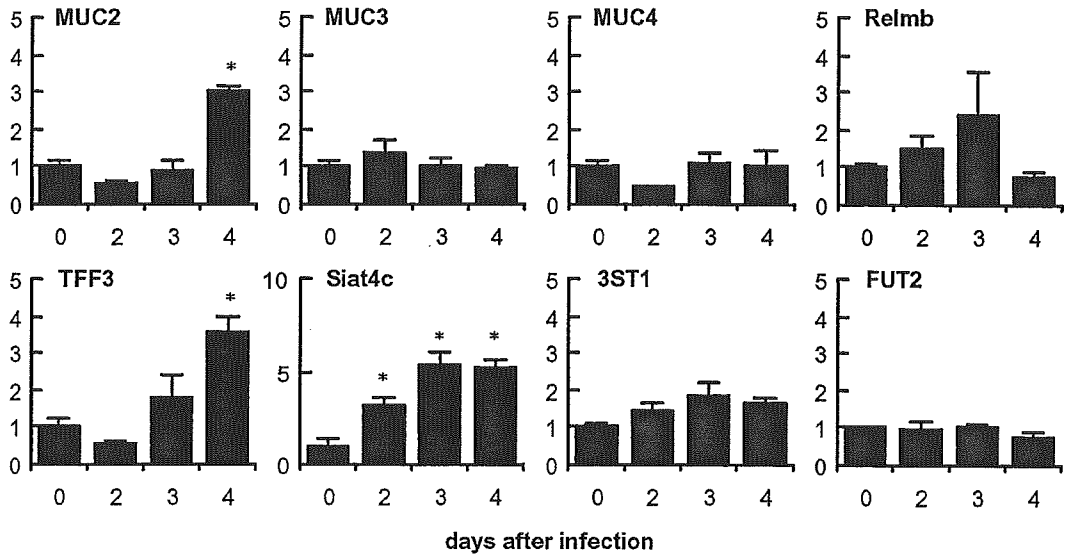
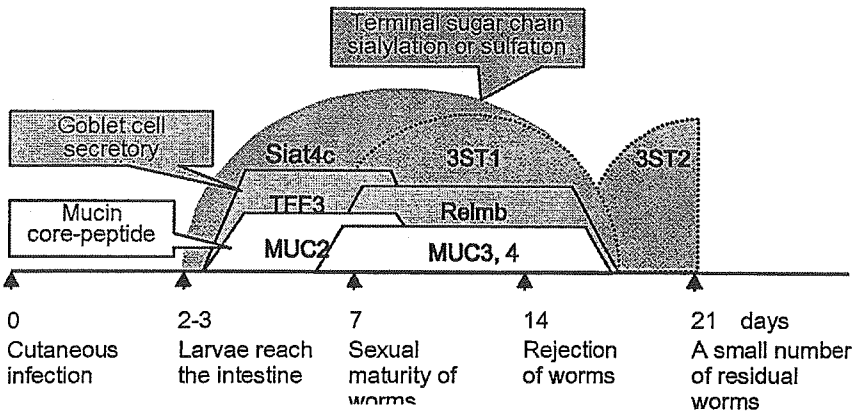

MUC2
MUC3
MUC4
Relmb
TFF3
Siat4c
3ST1
FUT2
days after infection

Fig. 4


Terminal sugar chain sialylation or sulfation
Goblet cell secretory
Mucin core-peptide
Siat4c
3ST1
3ST2
TFF3
Relmb
MUC2
MUC3, 4
0
Cutaneous infection
2-3
Larvae reach the intestine
7
Sexual maturity of worms
14
Rejection of worms
21 days
A small number of residual worms

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Epidemiological Report

Epidemiological Investigation on *Clonorchis sinensis* in Human Population in an Area of South China

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SUMMARY: To detect the prevalence of *Clonorchis sinensis*, one of the important helminthes in the human population of the Guangxi Region where *Schistosoma japonicum* was endemic but eliminated in the late 1980s, the Kato-Katz thick smear technique was used for examining fecal samples from selected townships in Hengxian County. Among 1,552 people examined, 491 (31.6%) were found infected with *C. sinensis*. By counting eggs per gram feces (EPG), it was found that the light, moderate, and heavy intensities of infection occupied 55.4, 33.0, and 11.6%, respectively, with an average EPG of 4,845 in the infected subjects. The survey revealed that the prevalence in the age groups of 0-9 and 10-19 years old was less than 10% but was 45-50% in the groups between 30-39 and 60-69 years old. A much higher prevalence was demonstrated in the male population (41.9%) than in the females (20.5%), and heavier intensity of infection was also found in the males than in the females. These results indicated that the prevalence of this liver fluke is increasing in the past decade in that region, and there is an urgent need to further assess the epidemiological factors in reference to the area's changing socio-economic conditions and human behavior, contamination of the environment and fish ponds, inadequate farming/fishery practices, and the infection of domestic animals.

INTRODUCTION

The Guangxi Zhuang Autonomous Region was one of the provinces in China where *Schistosoma japonicum* was prevalent. Since the mid-1950s, by extensive interventions of mass detection, chemotherapy for both human and cattle population, and intermediate host (*Oncomelania* snail) control, the elimination of schistosomiasis was announced in 1989 (1-3). Meanwhile, other helminthes are highly prevalent in the region due to its relatively poor socioeconomic development, particularly in areas with ethnic populations. In the first nationwide randomly sampled survey on human parasitic infections 10 years ago, the overall infection rate of intestinal parasites in Guangxi was 85%, and the average prevalence of *Ascaris lumbricoides*, hookworms, *Trichuris trichiura*, and *C. sinensis* in the region was 66, 38, 47, and 1.2%, respectively (4). Investigations of *C. sinensis* during 1978-1997 revealed a prevalence ranging from 0.08 to 75% in different areas in which the clonorchis infection had been previously reported (5). *Taenia* spp. was endemic in 30 counties, mostly those with ethnic minorities. An average prevalence was 0.36% with the highest rate of 39% in a township of Rongshui County (6). Natural nidi of *Paragonimus westermani* and other paragonimus species were detected in 15 counties. A positive rate of 42% by skin test and a rate of 7% by sputum or stool examination for eggs were reported in selected villages (7).

In order to understand the prevalennce of helminth infections other than schistosomiasis and to provide evidence for developing control strategies, an epidemiological study in selected areas was proposed. This paper reports the findings from a survey regarding *C. sinensis* infection.

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MATERIALS AND METHODS

Areas and subjects: Hengxian County, formerly an area in which schistosomiasis was present, was selected for the investigation of *C. sinensis* infection. Three spots (townships) in the county were identified and the whole population in each place, about 500 people, was included in the study.

Methods: The modified Kato thick smear method (Kato-Katz technique) was used for fecal examination (three slides for each fecal sample) to detect eggs of *C. sinensis* and intestinal nematodes, and an egg count was made to determine the intensity of clonorchis infection (number of eggs per gram feces [EPG]). Following the criteria set by the Chinese Ministry of Health (8), an EPG of <1,000, between 1,000 and 9,999, and >10,000 was identified as light, moderate, and heavy infection, respectively.

By using test kits purchased from Shenzhen Luhan Biotechnic Co. (Shenzhen, Guangdong, China), conventional enzyme-linked immunosorbent assay (ELISA), using crude antigen of clonorchis adult worms, was performed for sera from partial examinees with or without clonorchis eggs. The preparation of the antigen followed a routine procedure: ground powder of lyophilized adult worms was defatted by acetone, normal saline with thiomersalate was added for 72 h extraction, and the supernatant after centrifugation was then used as crude antigen. The stools of those with positive serological test but who were shown to be egg negative by Kato-Katz technique were reexamined using the formalin-ether concentration method.

Table 1. Fecal examination for the infection of *Clonorchis sinensis* in Hengxian

Township Surveyed	No. examined	Prevalence		Intensity of infection*			
		No. infected	%	Light %	Moderate %	Heavy %	Mean EPG
Fucheng	545	205	37.6	59.5	30.7	9.8	3034.8
Pinglang	502	157	31.3	64.3	30.6	5.1	2451.8
Pingma	505	129	25.2	38.0	39.5	22.5	9049.1
Total	1552	491	31.6	55.4	33.0	11.6	4845.2

* Light infection: eggs per gram feces (EPG) 1-999; moderate infection: EPG 1000-9999; heavy infection: EPG ≥10000.

Table 2. Sex distribution of *Clonorchis sinensis* infection in Hengxian

Township Surveyed	No. examined	Male			Female		
		No. examined	No. egg positive	%	No. examined	No. egg positive	%
Fucheng	545	283	147	51.9	262	58	22.1
Pinglang	502	266	100	37.4	236	57	24.2
Pingma	505	260	92	35.4	245	37	15.1
Total	1552	809	339	41.9	743	152	20.5

$\chi^2 = 25.2367$, $P < 0.001$.

The R x C chi square (χ^2) test was used for statistical analysis.

RESULTS

Prevalence of the infections of *C. sinensis* and intestinal nematodes: A population of 1,552 from the townships of Fucheng, Pinglang, and Pingma in Hengxian were sampled for the survey. The overall prevalence of helminth infections including intestinal nematodes and *C. sinensis* was 48.9% ranging from 44.2 to 53.8%. The average prevalence of *A. lumbricoides*, *T. trichiura*, and hookworm infections was 15.9, 12.8, and 7.5%, respectively. The prevalence of clonorchis infection was 37.6, 31.3, and 25.2% in the three townships, respectively, with an average of 31.6%.

The intensity of clonorchis infection: According to the Manual of Intestinal Parasite Control, the number of EPG is used to measure the intensity of clonorchis infection. Among the positive fecal specimens, light infection occupied 55.4% (ranging from 38 to 64.3% in the townships), moderate infection 33.0% (30.6-39.5%), and heavy infection 11.6% (5.1-22.5%). The average EPG was 5159.5 (Table 1). The highest EPG was 105,840 in one case in Pingma.

Age distribution of clonorchis infection: The prevalence was 4.3 and 8.7% in the 0-9 and 10-19-year-old age groups, respectively. It reached a plateau from the 30-39-year-old age group (50.2%) to the 60-69-year-old age group (45.4%) (Fig. 1). Statistical analysis showed an extremely significant difference in the prevalence among the age groups from 0-9 to 30-39 years old ($P < 0.0001$). In regard to the intensity of infection, all the cases in the 0-9-year-old group showed light infection; heavy infection occupied 6-7% in the 10-19 and 20-29-year-old groups, and from 11.3 to 16.7% in the other groups. Evidently, the adult population was more affected.

Sex distribution of clonorchis infection: As revealed in Table 2, the prevalence was much higher in males (41.9%) than in females (20.5%) ($P < 0.001$). The intensity of infection was also heavier in the male population than in the female population: light, moderate, and heavy infection

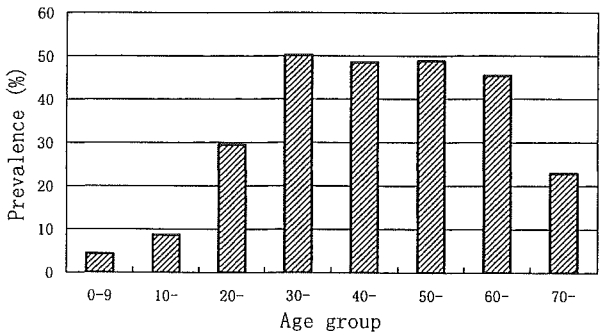

Fig. 1. Age distribution of *Clonorchis sinensis* infection. $\chi^2 = 184.7649$, $P < 0.0001$ (among age groups from 0-9 to 30-39).

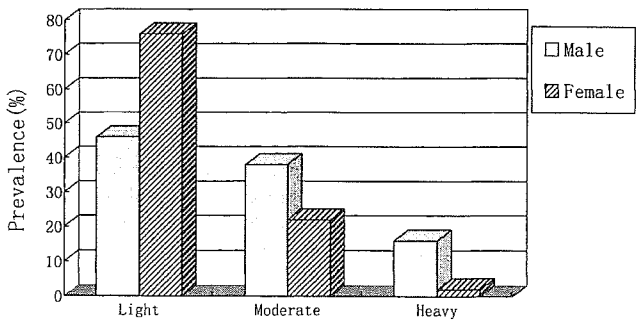

Fig. 2. Intensity of infection in male and female population groups.

occupied 46, 38, and 16%, respectively, in the males, while in the females, 76, 22, and 2%, respectively (Fig. 2) ($P < 0.0001$).

Result of serological test: ELISA was performed for serum samples taken from clonorchis egg-positives, with 191 out of 205 sera (93.2%) showing positive reaction. The serological test was also used in 207 subjects in which no eggs had been found by the Kato-Katz method; 12 (5.6%) were positive for the antibody. Fecal samples from these 12 cases were examined again using the formalin-ether concentration

method, in which approximately 2 g of fecal material was used, and clonorchis eggs were found in eight cases (66.6%).

DISCUSSION

In 1989, Hengxian County was identified as a pilot area for the national survey on human parasites (4), and the prevalence of clonorchiasis was 18% and those of ascaris, hookworm, and trichuris infection was 71, 34, and 55%, respectively. In more than a decade, the prevalence of ascaris, hookworms, and trichuris infection significantly decreased to 15.9, 7.5, and 12.8%, respectively. This was partly due to the local control activities in the 1990s, including deworming of students of primary and secondary schools. The improvement of living standards, environmental hygiene, and health literacy among local people also helped reduce the transmission of the soil-transmitted helminthes. The investigation in Hengxian County revealed again that clonorchis infection is apparently an important problem, with an increase of its prevalence from 18 to 31.5%. There may be epidemiological and socioeconomic factors that positively affected the prevalence of clonorchis infection. For instance, people in that area received higher incomes due to the socioeconomic reforms of the past decade and they preferred and could afford raw freshwater fish – a dish which was believed to be highly delicious, nutritious, and expensive. In addition, no specific control program was established for liver fluke, and the routine single-dose anthelmintics are not effective for the fluke.

People at any age can be infected by *C. sinensis*. Depending on the modes of fish-eating, the most affected population groups in China can be either children who became infected by eating small roast fish while playing in the field such as in some central provinces, or adults who contracted infection by consuming fish dishes which are raw or undercooked (9). This study showed a much higher prevalence in the adult population than in children and adolescents, a phenomenon often seen in the southern provinces.

As to the relationship between prevalence and sex, the investigation demonstrated that the infection rate was double in males in comparison to females. The intensity of infection also showed great degrees of difference with more heavy and moderate infections and less light infections in males than in females. It is not clear why the males contract more and heavier clonorchis infections. One possible reason might be the more frequent social activities and dining opportunities at restaurants for male adults than for females. It is interesting to note that the age and sex distribution of clonorchis infection in some areas of South Korea was quite coincident with what we found in Guangxi (10).

In some areas, serological tests have been used for epidemiological screening of clonorchis infection. In this study, ELISA was used in a portion of the subjects with or without a positive stool examination. It was interesting that by applying the formalin-ether concentration technique, eggs were found in fecal samples of two-thirds of the anti-clonorchis antibody positives. Apparently, the concentration method is more sensitive for detecting clonorchis eggs than is the Kato-Katz technique because the former technique requires a much larger quantity of fecal material and provides a clearer microscopical field for identifying the small eggs. This is virtually consistent with the previous studies which recommended that Kato-Katz method, with its practicality and feasibility, be used for mass survey in the field while the formalin-ether concentration technique be conducted for clinical diagnosis due to its higher sensitivity, though it is more time-consuming (9).

It seems necessary to indicate that it has been difficult to confirm an infection of *C. sinensis* only by eggs in cellophane-covered (Kato-Katz) thick smears in areas where intestinal trematodes in the family Heterophyidae may exist, which excrete similar small-type eggs in feces. In the southeastern provinces such as Guangdong and Fujian, by identification of adult worms expelled, trematodes other than *C. sinensis* were reported including *Hyterophyes hyterophyes*, *Metagonimus yokogawai*, *Haplochis pumilio*, and *Centrocestus formosanus*, and one case of the latter (*C. formosanus*) was recorded in Guangxi (4). Although these intestinal trematodes were not widely prevalent, mixed infection with *C. sinensis* is possible in some areas. Though intestinal trematodes other than *C. sinensis* were not found in the area surveyed previously and this time, it would be of interest to determine in further examination if these flukes are present in Hengxian County.

While the endemicity of soil-transmitted helminthes is decreasing, *C. sinensis* has become more prevalent than it was a decade ago in the region surveyed. It is not clear what important epidemiological factors may have contributed to the increase of the clonorchis prevalence. Further study is of great importance, especially in reference to the area's changing socioeconomic conditions and human behavior, contamination of the environment and fish ponds by the unhygienic latrines and human/animal excreta, inadequate farming/fishery practices, and the infection of domestic animals such as cats, dogs, and pigs. Epidemiological clarification of these factors will be essential for developing an effective control strategy for clonorchiasis.

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喀痰から虫卵が検出され形態と塩基配列から種同定したウェステルマン肺吸虫症の1例

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緒言

喀痰から肺吸虫卵が検出された症例について虫卵の形態を精査し，また虫卵を出発材料として塩基配列を解読したところ，2倍体型のウェステルマン肺吸虫（以下，ウ肺吸虫）と同定できた。排卵を認める肺吸虫症例は3倍体型のウ肺吸虫を原因とすることが多く[1]，本例は貴重な症例と考えられた。

症例（臨床的事項等は他の肺吸虫症自験例とともに既報[2]）

和歌山県に14年間在住する韓国人女性，44歳。半年間持続した血痰を主訴に受診した。胸部X線とCT検査で右肺上葉に硬化性病変（直径1-2cm，一部で空洞形成を伴う）が認められたため肺吸虫症を疑い，喀痰と糞便を調べて虫卵（図1）が検出されたので本症と確定した。マルチドットELISAで，患者血清はウ肺吸虫抗原と宮崎肺吸虫抗原とに反応したが，発色の程度は前者との方が強かった。プラジカンテルが体重1kgあたり75mgで3日間連続して経口投与された結果，排卵，画像所見および症状は消失・軽快し，治癒と判定された。患者に食歴を尋ねると，発症の約1年前より（モクズガニらしき）淡水産のカニを地元で採集したり，

A Case of Paragonimiasis westermani : Identification of the Causative Parasite as the Diploid Type of *P. westermani* from the Egg Morphology and DNA Sequence

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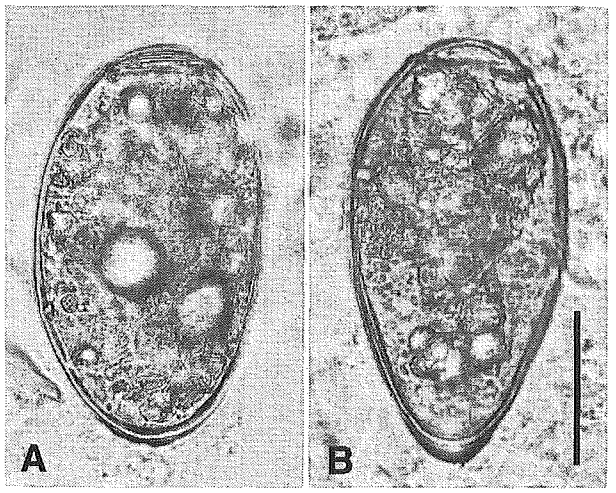

図1　症例の喀痰中に認められた虫卵，強拡大像。最大幅部が中央にあり，かつ無蓋端部の卵殻肥厚が明らかでない虫卵（A）が60%を占め，最大幅部が蓋端側で，かつ無蓋端部に卵殻肥厚を認めた虫卵は20%（B）に過ぎなかった。Bar=30 μm。

あるいは地元の市場で購入し，塩ゆでにして頻繁に摂食したと述べたことから，不完全加熱の淡水産のカニが感染源になったと考えられた。

虫卵の形態学的特徴

虫卵の形態学的特徴から原因虫種を推定するため，YM式喀痰固定液（50%エタノール他，武藤化学）に保存されていた喀痰中の虫卵のうち，形態が良く保持されたもの（20個）について計測・観察した（図1）。まず虫卵の大きさは，長径が77-88 μm（平均83 μm），短径は43-50 μm（平均46 μm）であった。次に最大幅部の位置（卵の幅が最も広い箇所）は，中央が14個(70%)，蓋端側（蓋のある側）は6個(30%)であった。また無蓋端部（蓋と反対側）における明らかな卵殻の肥厚（卵殻側部の2.5倍以上とした）は，「認めない」が16個(80%)，「認める」は4個（20%）にとどまった。

わが国に分布する人体寄生性肺吸虫は，ウ肺吸虫と宮崎肺吸虫の2種類である。このうちウ肺吸虫は，染色体構成により2倍体型と3倍体型の2タイプに分けることができる[1]。各々の虫卵には種（あるいはタイプ）に特有の形態学的特徴[3]がある。まず3倍体型ウ肺吸虫は，長径が時に90 μmを超え，最大幅部が蓋端側にあって，ほぼ総ての虫卵の無蓋端部に卵殻の肥厚を認め，その程度も顕著なものが多い。これに対して，2倍体型ウ肺吸虫と宮崎肺吸虫とは，虫卵の形態が相互に類似し，長径が3倍体型より10 μm以上も短く，最大幅部は中央にあるものが多い。しかしながら両者は無蓋端部の卵殻肥厚の程度が異なり，宮崎肺吸虫では無蓋端部の卵殻肥厚がないのに対し，2倍体型ではある程度の肥厚を半数以上の虫卵に認める。

本症例の虫卵は，最大幅部が概ね中央部にあり，長径も余り長くなかった。このような虫卵の特徴は，宮崎肺吸虫と2倍体型ウ肺吸虫とに共通するものである。そこで3倍体型ウ肺吸虫である可能性をまず除外した。一方で，無蓋端部における明らかな卵殻肥厚を20%の虫卵に認めた。しかしながらこの結果からは，本症例の原因虫が2倍体型ウ肺吸虫か，宮崎肺吸虫かを判定することが困難であった。そこで分子生物学的手法を用い，検討を加えることにした。

塩基配列の解読と比較検索

虫卵からのDNA抽出に先立ち，実体顕微鏡下に有柄針で虫卵を圧し，卵殻を破綻させた。処理した虫卵は1個ずつ，マイクロピペットを用いて，極少量の精製水（0.5 μl）とともに速やかにエッペンドルフチューブに移した。以降の手技，すなわちDNA抽出とPCR，塩基配列の解読・解析に関しては，概ね既報に準じた[4]。なお今回は，吸虫類のITS2領域に対してコンセンサスなプライマー（3SおよびA28）を用い，PCRと配列解読を行った。

卵殻を破綻させてDNA抽出を行うと，虫卵1個ずつからでも，電気泳動で明瞭な充分量の産物がPCR増幅された。ゲルからバンドを切り出して，塩基配列を解読・解析したところ，PCR産物は463 bpで，しかも2倍体型ウ肺吸虫の配列（U 96907）と完全に一致した。一方，宮崎肺吸虫（U 96912）とは92.6%の相同性に留まった。以上の結果から，本症例の原因虫を2倍体型ウ肺吸虫と判定した。

考察

わが国に分布する人体寄生性肺吸虫のうち，3倍

体型ウ肺吸虫は肺内に虫嚢を形成して産卵するのに対し，2倍体型ウ肺吸虫と宮崎肺吸虫とは肺に定着せず，胸腔内を移行して胸膜炎を引き起こすと説明されてきた[3]。しかしながら，宮崎肺吸虫が肺の虫嚢（画像では結節状陰影）に定着し，排卵を認める症例も報告されるようになってきた[5)6)]。相当数の虫体が寄生し，しかも長期間駆虫されない場合に，宮崎肺吸虫の感染でもこのような病態が発現すると考察されている[5]。ステロイド投与も関与すると推察されている[6]。本症例では，カニを繰り返し食べることで相当数のメタセルカリアが取り込まれたと考えられ，しかも血痰から半年後にようやく通院して投薬治療が開始されている。このような場合に，2倍体型ウ肺吸虫でも，宮崎肺吸虫と同様に，虫体が肺の虫嚢で同棲して成熟し，排卵を認めるようになると考えられた。わが国に分布する人体寄生性肺吸虫は，いずれも喀痰や糞便に虫卵を排出させることが示唆されたので，症例から得られた肺吸虫卵は，形態を精査し，また遺伝子配列を解析して，種（およびタイプ）を判定することが望まれる。

寄生虫卵を用いた塩基配列解読に関しては，線虫卵1個を出発材料としても可能であることが報告され[7]，また条虫卵でも可能であると示唆された[7]。一方，吸虫卵に関しては，肺吸虫卵を用いた成績がその後に報告された[8]。これらの方法を実験動物由来の宮崎肺吸虫卵を用いて追試したが，時にPCR増幅が上手くいかないことを経験した。そこで卵殻を破綻させてDNA抽出を行ったところ，配列解読にも充分な量の産物が，より確実にPCR増幅されることが分かった（杉山，他，未発表）。今回はこの方法をエタノール等で処理された人体症例由来の虫卵に適用した。その結果同様に，充分量の産物がPCR増幅され，さらに塩基配列も解読できた。肺吸虫卵を材料としてPCRを行う場合，増幅をより確実とするために試みるべき方法と考えられた。

本邦産人体寄生性肺吸虫に対する淡水産カニの感受性[1]については，サワガニはいずれにも感受性があり，モクズガニはウ肺吸虫（2倍体型・3倍体型）に感受性があることが記されている[1]。実際に和歌山県では，モクズガニでの肺吸虫寄生は見出されていないが，サワガニからは宮崎肺吸虫のメタセルカリアが検出されている（隣県の三重および奈良のサワガニからは2倍体型ウ肺吸虫も検出されている）[1]。和歌山県在住者の肺吸虫症例[2]の中には，県内のサワガニで宮崎肺吸虫に感染したものも含まれていた[9]が，今回報告した症例は，上に述べた疫学的な背景から，県外に由来する淡水産のカニで，恐らく市販されていたものが感染源になったのではないかと考えられた。

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A MULTIPLEX PCR FOR DISCRIMINATION BETWEEN *PARAGONIMUS WESTERMANI* AND *P. MIYAZAKII* AT THE METACERCARIAL STAGE

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Abstract. A multiplex polymerase chain reaction (PCR) system was developed for accurate species discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. The interspecies-conserved and species-specific primers designed from the sequences of the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA (rDNA) were all incorporated into single tubes and PCR amplification was carried out. The method allowed us to identify *P. westermani* and *P. miyazakii*, and discriminate them from *P. ohirai* at the metacercarial stage in a single tube reaction.

INTRODUCTION

The lung flukes, *Paragonimus westermani* and *P. miyazakii*, are of known medical importance as pathogens causing human paragonimiasis in Japan. The metacercariae of these species are found in the same freshwater crab species and are morphologically quite similar (Miyazaki, 1991). Therefore, the development of sensitive and objective diagnostic methods is required for accurate species discrimination and identification of the individual metacercariae of these species. These methods could be used for epidemiological investigations of the prevalence of the metacercariae in the crab host, and thus have important implications for controlling lung fluke disease.

We recently reported the establishment of molecular methods based on the use of polymerase chain reaction (PCR) for accurate discrimination of individual metacercariae of *P. westermani* and *P. miyazakii* (Sugiyama *et al*, 2002). The methods included direct cycle sequencing of the PCR products, PCR-restriction fragment length polymorphism (RFLP) and direct PCR-amplification using species-specific primers. All of these methods utilize nucleotide differences in the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA (rDNA) for discrimination. Of these methods, direct PCR-amplification provides a more rapid differential identification of species, because only a single-round PCR is required. However, this method limits identification to a single species and requires concurrently-run controls. In the present paper, we report the development of a multiplex PCR utilizing interspecies-conserved and species-specific primers designed from the sequences of the ITS2 region, which allows species discrimination of the individual metacercaria in a single tube reaction.

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MATERIALS AND METHODS

Parasite material and DNA isolation

Metacercariae of *P. westermani* (the diploid type) and *P. miyazakii* were harvested from the freshwater crab, *Geothelphusa dehaani*, and used for DNA isolation (Sugiyama *et al*, 2002). DNA was also extracted from the metacercariae of *P. ohirai*, which were isolated from the brackish water crab, *Chiromantes dehaani*, collected in Tokyo, Japan (Sugiyama *et al*, 2004).

Primers and amplification by PCR

The *P. westermani*-specific forward primer (PwF1; 5'-GTTTATGTTGCGCGTGGTCTGCTTTC-3', alignment positions 351 to 376 for *P. westermani* ITS2 region) and *P. miyazakii*-specific forward primer (PmF1; 5'- TTCCCCAACCTGGCCTCGTGG-3', alignment positions 184 to 204 *for P. miyazakii* ITS2 region) were newly designed in this study to target the 3'-terminal and the central portion of the ITS2 sequences of the corresponding species, respectively (Fig 1). In combination with the species-specific primers, consensus primers, 3S: 5'-GGTACCGG TGGATCACTCGGCTCGTG-3' (forward) and/or A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTC CGC-3' (reverse), which were designed based on the conserved sequences of the 5.8S and 28S genes (Bowles *et al*, 1995), were used.

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Pw    1 :TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACA
Pm    1 :. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . .

Pw   61 :TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG
Pm   61 :. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Pw  121 :TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT
Pm  121 :. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Pw  181 :GATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACAT
Pm  181 :. . . T . . . . . . C . . . . C . . C . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
             PmF1—>

Pw  241 :ACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTG
Pm  241 :. T C . . . . . T . . . . T . . . T . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . .

                                                                  PwF1-->
Pw  301 :GCTCAGTGAATGATTTATGTGCGCGTTCCGCTGTCCTGTCTTCATCTGTGGTTTATGTTG
Pm  301 :. . . . . . . . . . T . . . . . - - . . . . . . . . . . . . . A . . . . A . . A . . G . . . A . . . . . G . . . . . .

Pw  361 :CGCGTGGTCTGCTTTCGATGCTGACCTACGTATGTGCCATGTGGTTCATTCTCCTGACCT
Pm  359 :. . . . . . . . . . G . . T G C C . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . .

Pw  421 :CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA  :463
Pm  419 :. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . :461
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Fig 1- Aligned nucleotide sequences of an ITS2 region from *P. westermani* (Pw) and *P. miyazakii* (Pm) metacercariae. A hyphen indicates an alignment gap. A dot in the *P. miyazakii* sequence indicates a nucleotide identical to that in *P. westermani*. The 5' and 3' ends of the sequences include 5.8S rDNA and 28S rDNA, respectively. The locations of the *P. westermani*-specific forward primer (PwF1; 5'-GTTTATGTTGCGCGTGGTCTGCTTTC-3') and *P. miyazakii*-specific forward primer (PmF1; 5'-TTCCCCAACCTGGCCTCGTGG-3') are underlined. The numbers refer to alignment positions.

PCR amplification was performed as described previously (Sugiyama *et al*, 2002) using 0.25 μm of each primer and 2.5 units of Taq polymerase (Invitrogen, USA). In the present study, 1 ng of the DNA was added to each PCR reaction (final reaction volume, 100 μl); 1 ng of the DNA was equivalent to about 1/250, 1/400 and 1/100 of the DNA isolated from a single *P. westermani, P. miyazakii* and *P. ohirai* metacercaria, respectively. The resultant PCR products were separated by electrophoresis through 2% (w/v) agarose gels. the amplified PCR products were also excised from agarose gels and sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing Kit (Applied biosystems, USA) on an automated sequencer (ABI310, Applied Biosystems).

RESULTS

In the first step, the species-specificity of the newly designed forward primers, PwF1 and PmF1, was evaluated as to whether they could amplify different sized species-specific fragments from metacercarial DNA by PCR in combination with the consensus reverse primer A28. As was expected, the primer set PwF1-A28 amplified a PCR product of about 140 bp from *P. westermani* DNA, but not from *P. miyazakii* DNA (Fig 2). In contrast, PmF1-A28 amplified a product of about 300 bp from *P. miyazakii* DNA, but not from *P. westermani* DNA (Fig 2).

Having demonstrated the species-specificity of the primers PwF1 and PmF1, these two primers were mixed and incorporated into single tubes with the consensus primer A28 for the PCR reaction. As a result, a PCR product of about 140 bp was amplified from *P. westermani* DNA and that of about 300 bp was from *P. miyazakii* DNA (Fig 3). The same PCR amplification was carried out using DNA extracted from *P. ohirai* metacercariae but no product was amplified (Fig 3).

We previously reported that PCR products of the same size (520 bp) were amplified from *P. westermani* and *P. miyazakii* metacercarial DNAs using the