

**COMPARISON OF MORPHOLOGY, PATHOGENICITY
AND DRUG RESPONSE AMONG THREE ISOLATES
OF *SCHISTOSOMA JAPONICUM*
IN THE MAINLAND OF CHINA**

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SUMMARY. Geographic differences among isolates of *Schistosoma japonicum* from Anhui Province (A) in the east, Guangxi Autonomous Region (G) in the south and Yunnan Province (Y) in the southwest of mainland of China were studied. C57BL mice were used to compare these isolates. Morphologically, both male and female worms of isolate Y were shorter than those of isolates A and G, and the ovary of female worm of isolate G was the smallest. In isolate A, the percentage of male worms bearing 7 testes was 87 %, while 64 % of isolate Y and 75 % of isolate G were with 7 testes. As to the volume of hepatic egg granuloma which was used as a parameter of pathogenicity in our experiment, that of isolate G was the smallest. The sensitivity to praziquantel of isolates was estimated by parasite reduction rate at ED₅₀ (230 mg/kg) level. It was found that isolate Y's response was significantly higher than that of isolates A and G, and the latter two were similar in drug response. The results indicate significant differences among the three isolates studied.

Key-words: *Schistosoma japonicum*. The Chinese isolates. Morphology. Pathogenicity. Egg granuloma. Praziquantel.

Comparaison en morphologie, en pathogénicité et en réponse thérapeutique des trois isolats de *Schistosoma japonicum* en Chine continentale.

RÉSUMÉ. Les différences géographiques parmi les isolats de *Schistosoma japonicum* en province d'Anhui (A) à l'est, en région autonome de Guangxi (G) au sud et en province d'Yunnan (Y) au sud-ouest en Chine continentale ont été étudiées. Des souris C57BL ont été employées pour comparer ces trois isolats. Morphologiquement, les adultes mâles et femelles de l'isolat Y furent plus courts que ceux des isolats A et G, et l'ovaire du vers femelle de l'isolat G fut le plus petit parmi les trois. Le pourcentage des vers mâles portant sept testicules fut 87 % pour l'isolat A, 64 % pour l'isolat Y et 75 % pour l'isolat G. Pour évaluer la pathogénicité des différents isolats, nous avons mesuré la dimension des granulomes ovulaires dans le foie et nous avons trouvé que celle de l'isolat G fut la plus petite. La sensibilité au praziquantel des trois isolats a été estimée par le taux de réduction des vers au niveau de ED₅₀, soit 230 mg par kilogramme

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du poids. La réponse thérapeutique de l'isolat Y fut significativement plus haute que celle des isolats A et G, tandis que la différence entre les deux derniers ne fut pas significative. Les résultats obtenus indiquent qu'il existe des différences assez importantes parmi les trois isolats de *S. japonicum* qui sont géographiquement séparés en Chine continentale.

Mots-clés : *Schistosoma japonicum*. Isolats en Chine continentale. Morphologie. Pathogénicité. Granulome ovulaire. Praziquantel.

Introduction

Schistosome species of medical importance possess some different intraspecific features by which specific worm strains are recognized. Hunter *et al.* (1952) were the first to put forward the strain problem in *Schistosoma japonicum*. Since then, studies by various authors have shown that *Schistosoma japonicum* includes at least four geographic strains, i. e. the Japanese (J), the Philippines (P), the mainland of China (C) and Taiwan Province (F). They differ in many aspects. In the first place, strain F is zoophilic while the others are pathogenic to humans (Hsu *et al.*, 1955). Morphologically, the adult worms of F are larger than those of J (Hsu *et al.*, 1959). In male worms, the number and arrangement of testes are somewhat different among the four strains (Hsu *et al.*, 1957). The size of eggs of the four strains, recovered from experimentally infected mice, when compared by their respective index, was in the order of $J > F > P > C$ (Hsu *et al.*, 1958). Physiologically, the periodicity of cercarial shedding of strain P was found to be nocturnal by Bauman *et al.* (1948) and that of C to be diurnal by Mao *et al.* (1949). According to Hsu *et al.* (1958), the prepatent period in experimental infection with J and P was shorter than that with C and F. As to pathogenicity, studies made by Hsu *et al.* (1960) demonstrated that the survival time of infected mice with strains J and P was shorter than that with C and F. Warren *et al.* (1972) reported that so far as the development of hepatosplenic disease in experimental animals was concerned, strains P and F were the most virulent, J was intermediate in virulence and C the least virulent. With respect to biochemistry and genetics, Ruff *et al.* (1973) and Maeda *et al.* (1984) found that the electrophoretic protein patterns also reflected interstrain divergence in *Schistosoma japonicum*, while Merenlender *et al.* (1987) reported large genetic distance of about 0.575 between strains C and P. Immunologically, when the four geographic strains were observed with COPT, Hsu *et al.* (1961) found that the precipitating reaction was stronger between homologous eggs and antisera. The strain-specific nature of protective immunity by attenuated cercariae was observed by Moloney *et al.* (1985). As to the drug response, under similar experimental conditions, strain J was less sensitive to stibophen than strains F and P (Chu, 1961). Besides, many other authors have conducted artificial cross infection between miracidia and snails from various localities and demonstrated the diversity of host-parasite compatibility (Hunter *et al.*, 1952; De Witt *et al.*, 1954; Hsu *et al.*, 1960; Yuan *et al.*, 1984; Cross *et al.*, 1984).

In the mainland of China, the endemic areas of schistosomiasis japonica is discontinuous, showing conspicuous geographic and topographic isolations (*fig. 1*). In the past, apart from studies on the snail-parasite compatibility showing the refractoriness of *Oncomelania* snails from the southwest to miracidia from east China (Shao *et al.*, 1956) and vice versa (Yuan, 1958), little was known in the characteristics of schistosomes isolated from various localities in the mainland. We try, therefore, to tackle the problem by comparing first the morphology, pathogenicity and drug response of schistosomes isolated from Anhui Province in the east, Guangxi Autonomous Region in the south and Yunnan Province in the south-west which were selected on the basis of previous investigations showing different degree of snail-parasite incompatibility.

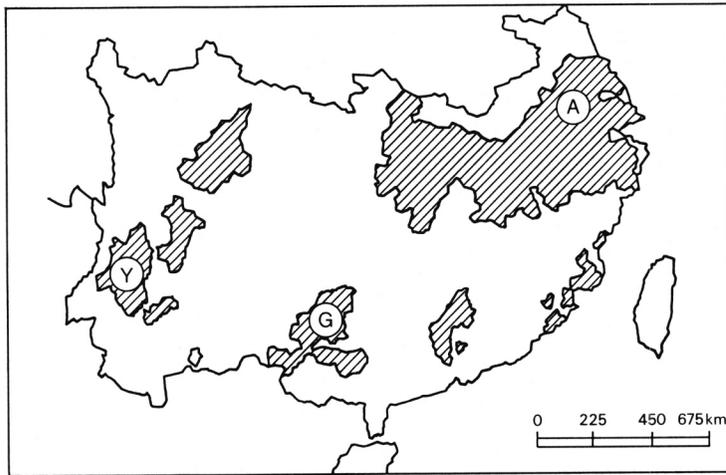


FIG. 1. — Southern part of the mainland of China showing historical distribution of schistosomiasis japonica (barred area) and source of schistosome isolates used in the present study.

Materials and methods

1 — SCHISTOSOME CERCARIAE

Naturally infected *Oncomelania* snails were collected from Anhui (A), Yunnan (Y) and Guangxi (G) (*fig. 1*) before experiments. Cercaria shedding was performed with the conventional method in our Institute and these cercariae were coded isolate* A, Y and G, respectively. In addition, cercariae of Anhui line* (LA) which has been passed through rabbit-snail cycle in laboratory for long time were occasionally used. Generally, cercariae from at least 50 infected snails were pooled and used for inoculation.

* Terminology used as defined by Rollinson, 1984.

2 — EXPERIMENTAL ANIMAL

C57BL inbred mice weighing 16-18 g and of both sexes from the Centre of Experimental Animals of Chinese Academy in Shanghai were used.

3 — DRUG

Praziquantel in powder form synthesized in 1979 by the Department of Pharmaceutical Chemistry, Institute of Parasitic Diseases, CAPM was used.

4 — INFECTION, TREATMENT AND AUTOPSY OF MICE

Each mouse was inoculated with 40 cercariae on its shaven abdominal skin. Infected mice were divided into four groups according to the Random number Table. One served as infected control and the others for experimental treatment. Mice in the control group were sacrificed 42 days post-infection and their livers and worms were examined for pathological changes and parasite morphology.

Three dosage-levels of praziquantel, i. e., 150, 230 and 310 mg/kg body weight of mouse in single dose were used in our therapeutic experiments. They were arbitrarily chosen as representing ED40, ED50 and ED60 of praziquantel in mice infected with *Schistosoma japonicum* by considering both the theoretical values of ED40 (165.5 mg/kg), ED50 (229.3 mg/kg) and ED60 (317.7 mg/kg) from Yan (1978) and the experimental data from the Department of Pharmacology, IPD, CAPM (Yue, personal communication). Drug powder was added to 1 % gum tragacanth and stirred thoroughly to form suspension of desired concentration. The amount of drug given was calculated by mouse weight prior to treatment. In the first experiment, mice were treated intragastrically once 30 days post-infection while in the second, 35 days post-infection. They were sacrificed 28 days after treatment and schistosomes were collected by perfusion. Livers and mesenteries were torn and searched for remaining worms. Worms recovered by both techniques from each mouse were mixed and counted.

5 — OBSERVATION ON MORPHOLOGY

Among the 42-day-old worms from untreated mice, intact paired worms were picked out, put in tapwater and, when relaxed and manually separated, fixed in 5 % formalin. The body length was measured with a paper ruler. Subsequently, they were stained with acid carmin and mounted *in toto* in neutral agar. Under light microscope, male worms were observed for the number and arrangement of testes and female worms, for the area of ovary. As the ovary was oval shaped, its area was calculated by the formula $\Pi AB/4$, A and B standing for the two perpendicular axes.

6 — OBSERVATION ON PATHOLOGY

Livers from six mice infected with line A or isolates Y or G were fixed in 10 % formalin. A small piece of $1.0 \times 0.5 \times 0.2$ cm was cut from the largest lobe, dehydrated, embedded in paraffin, serially sectioned and stained with hematoxylin-eosin. The developmental stages of granulomas were divided as previously described by Hsu *et al.* (1972). At least 30 exudative granulomas containing a single mature egg were measured for each group. The volume of egg granuloma was calculated by the formula $\Pi AB^2/6$, in which A and B stand for the two perpendicular axes as suggested by He *et al.* (personal communication). Logarithmically transformed means of granuloma volumes between two groups were compared with Student's t-test.

7 — EVALUATION OF DRUG RESPONSE

The worm reduction rate was calculated by the equation :

$$\left(1 - \frac{\text{worm survival rate in treated group}}{\text{worm development rate in control group}}\right) \times 100 \%$$

Chi square test was used to compare worm reduction rates between every two groups.

Results

1 — WORM MORPHOLOGY

For isolates A, Y and G, the average body length of 42-day-old male worms was 13.4, 9.9 and 10.5 mm respectively, the difference being statistically significant ($p < 0.01$), and that of female worms was 15.2, 14.2 and 15.5 mm, respectively, with isolate Y distinctly smaller than isolates A and G, between which no significant difference was found (*table I*).

TABLE I. — Body length of adult worms and ovary area in female worms of isolates A, Y and G in the mainland of China.

Iso- late	No. of pairs	Body length (mm) ($\bar{X} \pm S. D.$)		No. of females	Area of ovary ($10^4 \mu\text{m}^2$)
		Male	Female		
A	140	13.4 ± 1.2	15.2 ± 1.4	107	6.59 ± 1.84
Y	97	9.9 ± 0.8	14.2 ± 1.1	85	5.87 ± 0.94
G	104	10.5 ± 0.9	15.5 ± 1.5	88	5.35 ± 1.38

Difference in the average areas of ovary among the three isolates was highly significant ($p < 0.01$) and this is unrelated to the difference in length of female worms, for the body length of isolate G was the biggest, whereas its ovary was the smallest (*table I*).

The percentages of male worms with testes arranged in column and in cluster were 53 and 47 % in isolate A and 68 and 32 % in both isolates Y and G. No significant difference was found. The number of specimens bearing seven testes in isolate A was significantly higher than that in isolates Y and G (*table II*).

TABLE II. — Numerical feature of testes in male schistosomes of isolates A, Y and G.

Isolate	No. of males	Worms with 7 testes		Worms with non-7 testes	
		No.	%	No.	%
A	78	68	87	10	13
Y	95	61	64	34	36
G	109	82	75	27	25
A vs. Y		χ^2 11.92		p < 0.005	
A vs. G		4.09		< 0.05	
G vs. Y		2.94		> 0.05	

2 — LIVER PATHOLOGY

Granulomas in exudative stage were characterized by eosinophil aggregates arranged concentrically around mature egg(s). The average volume of such granulomas containing a single mature egg of line A, isolates Y and G was 8.1, 6.5 and 4.5×10^{-3} mm³, respectively. As measurements showed big deviation among granulomas in the same group, logarithmically transformed data were used for comparison (*table III*) which showed that granulomas produced by isolate G are significantly smaller than those by either Y or LA.

TABLE III. — Volume of exudative granulomas induced by single egg of LA, isolates Y and G of *S. japonicum* from the mainland of China.

Worm source	No. of granulomas measured	Log transformed volume ($\bar{X} \pm$ S. D.)	Comparison	t	p
LA	36	2.4803 ± 0.4470	LA vs. Y	1.0900	> 0.05
Y	30	2.3688 ± 0.3700	Y vs. G	2.4907	< 0.01
G	33	2.1546 ± 0.3128	G vs. LA	3.4760	< 0.01

3 — DRUG RESPONSE (*fig. 2*)

The development rate of isolates A, Y and G in untreated C57BL mice 42 days post-infection was 61.6, 60.0 and 63.5 % respectively. In animals treated 30 days post-infection with praziquantel at the middle dosage-level of 230 mg/kg and sacrificed 28 days post-treatment, the worm reduction rate was 40.1, 60.8 and 38.1 %, respectively, showing that the sensitivity of isolate Y to praziquantel was significantly higher than that of isolates A and G ($p < 0.05$). The same was true at the low dosage-level of 150 mg/kg.

In another experiment, only 35-day-old schistosomes from isolates Y and G were compared for drug sensitivity. Their development rate in untreated animals was 51.7 and 50.0 % respectively. At the dosage-level of 230 mg/kg, the worm reduction rate of isolate Y was still higher than that of isolate G, the difference being statistically significant. Furthermore, the worm reduction rate of 35-day-old isolate G at the three dosage-levels tested was increased by 10 % as compared with the drug response of 30-day-old worms to the same dosage-levels. No such difference was observed between worms of different ages for isolate Y.

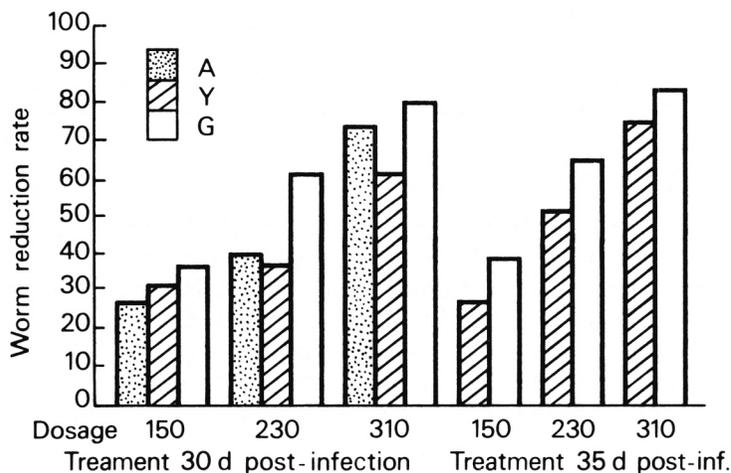


FIG. 2. — Worm reduction rate of different isolates treated with praziquantel.

Discussion

Two million years ago, i. e., in Quaternary Age, dramatic changes took place in Nature, including variations of climate characterized by repeated turnings of ice age and interglacial age, global eustatic movement, and quaternary neotectonics. These changes resulted in geographic separation of Japan, the Philip-

pinas and Taiwan island from the continent. Studies by Fletcher (1978) suggested that the isolation of four schistosome populations in the above-mentioned areas was consistent in time with geographic isolation.

In China, the mainland also underwent such fluctuation simultaneously, in that the southwest part was elevated and the east part lowered. Topographic change may result in discontinuous distribution of *Oncomelania* snail as well *Schistosoma japonicum*. According to molecular drive (Dover *et al.*, 1982), individuals in a population can slowly and gradually undergo evolutionary divergence through inner mechanism even without natural selection or genetic drift. Change in phenotype and its rate in various schistosome populations in the mainland and existence of geographic strains are to be expected.

In the present study, schistosome isolates from three geographically distant localities were selected for comparison. Of various parameters used by previous authors, morphology, pathogenicity and drug sensitivity were selected as differential characteristics because morphological study which was rather simple had been proved to be of value and pathogenicity and drug response were of practical importance.

Among the three isolates, we demonstrated the difference in body length of adult worms, in the area of ovary and in the percentage of male worms bearing seven testes. Males and female adults of isolate Y measured respectively 9.9 ± 0.8 and 14.2 ± 1.1 mm in length which was not in agreement with 14.26 and 16.51 mm reported by Chen *et al.* in 1985 (personal communication). It is to be noted that the material used by Chen, though originated from Yunnan, was not of definite worm age (36 ~ 62-day-old) nor from the same host (rabbit and mouse). Ito, in 1955, pointed out that the Japanese strain developed larger in rabbit than in mouse, guinea-pig and rat. Therefore, the two sets of data was barely comparable. Under our experimental conditions, the difference in body length among three isolates observed is of significance.

The percentage of male worms bearing seven testes in isolate A was 87 %, corresponding well with 89 % in line A reported by He *et al.* (1983). In isolate Y, we observed 64 % which was higher than 44.3 % in Chen's study. As the numerical feature of testes was not host-specific (Hsu *et al.*, 1955), explanation has to be sought elsewhere. Nevertheless, it is evident that the percentage of male worms bearing seven testes for schistosomes from Yunnan is smaller than that from Anhui.

For schistosome of a given origin, the area of ovary is proportional to the length of female worm. By studying the measurement published by He *et al.* in 1983, we found a positive correlation between female worm length and ovary size ($r = 0.8999$, $p < 0.01$). In the present study, the difference in ovary areas among the three isolates is not correlated with that in body length, suggesting the strain-specific character of the ovary area.

To determine the pathogenicity of a schistosome species or strain, many criteria should be used, such as killing effect, egg granuloma formation, development of hepatosplenic disease, etc. In our experiment, the size of granuloma

produced by isolates Y and G and line A was compared, without implying that granuloma formation was the sole pathologic manifestation of schistosomiasis. We compared egg granulomas 42 day post-infection, or about one week after the average patency of *S. japonicum*. Since we know little about the possible variation in the prepatent period of the isolates studied, the choice of egg granulomas at exudative stage for comparison was justified and it was shown that granuloma produced by isolate G was significantly smaller than that by either Y or line A.

Finally, our experimental results showed that the sensitivity of isolates A, Y and G to praziquantel was different. Although 230 mg/kg was not an accurate ED50 for any isolate, it has been shown to be able to sensitively demarcate the parasite response to praziquantel, especially, when compared with its upper and lower dosage-levels. The sensitivity of isolate Y to praziquantel was distinctly higher than that of isolates A and G, indicating probably that drug response might be a significant parameter for inter- and intra-specific differentiation, though it might be unwise to recommend the reduction of therapeutic dosage in clinics.

It is well known that the response of schistosomes to praziquantel is age-dependent, in that when 28-day-old worms were exposed to this drug, the tegumental lesions were mild and occurred slowly, whereas 35 ~ 42-day-old worms were affected more seriously and quickly (You *et al.*, 1986). In the present study, the reduction rates of 35-day-old worms in isolate G following praziquantel treatment were 10 % higher than those of 30-day-old worms, suggesting a possible age-dependence. Yet, in case of isolate Y, 30- and 35-day-old worms were equally sensitive, indicating that the higher sensitivity expressed by isolate Y was not due to its possible prematuration.

Under our experimental conditions, schistosome isolates from Anhui, Yunnan and Guangxi certainly differ in morphology, pathogenicity and drug sensitivity. Whether they can be considered as separate geographic strains awaits further studies which are being undertaken in our Institute.

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