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Artificial preparation, indoor passage, and nature breed of *Oncomelania hupensis* infected with *Schistosoma Japonicum*

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Abstract: Objective To prepare the infected *Oncomelania hupensis* by artificial method for the research on the activity, vaccine, and genetic variation of *Schistosoma Japonicum* (*S. Japonicum*). **Methods** The mature eggs of *S. Japonicum* were collected by Nylon silk method and the miracidia were incubated under appropriate conditions. Negative snails were infected with miracidia in different proportion by means of individual or collective infection to seek the best method and proportion of infection between miracidia and snails. Infected snails were divided into 12 groups in total. Ⅰ - Ⅵ groups were for individual infection and Ⅶ - Ⅻ groups were for collective infection. There were 200 snails in each group. The infection ratios between snails and miracidia in Group Ⅰ - Ⅵ or Ⅶ - Ⅻ were 1∶0, 1∶5, 1∶10, 1∶15, 1∶20, 1∶25, respectively. The infected snails were screened, numbered, and reared singly. The amount of cercariae was calculated once every 10 days until the infected snails died. Then cercariae shedding quantity, infection quantity, and mortality of infected snails in every group were compared to find the best infection method and the best infection proportion between miracidia and snails. The cercariae were collected from the first generation of infected snails and were used to infect experimental animals. The mature eggs of *S. Japonicum* were saved from the infected experimental animals and incubated to get miracidia. The snails were artificially infected by miracidium to get the second generation of infected snails. The developmental rates of adult worms, the egg density in fecal and liver were compared between artificially and naturally infected snails. **Results** In individual infection Group Ⅰ - Ⅵ, the average infection value of snails were 0 ± 0, 22.7 ± 4.2, 31.7 ± 4.5, 53.0 ± 5.3, 39.3 ± 5.9, 32.7 ± 4.7, the average fatality of snails were 21.7 ± 3.1, 25.0 ± 3.6, 31.3 ± 4.9, 44.7 ± 6.5, 78.3 ± 9.5, 89.7 ± 13.6, and the average value of cercariae shedding from infected snails were 0.0 ± 0.0, 308.0 ± 96.6, 428.1 ± 146.2, 527.0 ± 171.1, 571.4 ± 148.9, 602.9 ± 356.3, respectively. In collective infection Group Ⅶ - Ⅻ, the average infection value of snails were 0 ± 0, 12.3 ± 2.5, 18.7 ± 4.7, 28.3 ± 4.2, 33.3 ± 4.7, 29.3 ± 5.5, and the average fatality of snails were 22.7 ± 3.8, 23.7 ± 4.5, 28.3 ± 5.5, 47.0 ± 9.5, 75.7 ± 8.5, 86.3 ± 12.2, and the average value of cercariae shedding from infected snails were 0 ± 0, 244.5 ± 57.3, 292.3 ± 74.8, 347.1 ± 100.8, 477.2 ± 142.1, 447.3 ± 161.4, respectively. The second

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Biography XIA Yingding, master, mainly engaged in the reseach of multi-culture of *Oncomelania hupensis*.

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generation of artificially infected snails was obtained successfully. The average infection rate and fatality rate for the second generation of artificially infected snails were 24.65% and 24.50%, both of which were not obviously different from that of the first generation of artificially infected snails ($P>0.05$). In the animal experiment, the worm growth rate for the naturally infected snails, the first or second generation of artificially infected snails were 68.50%, 73.50% or 71.00%. There was no obvious difference among them ($P>0.05$). The fecal (or liver) eggs per gram for the naturally infected snails, the first or the second generation of artificially infected snails were 1 503 ± 269, 1 683 ± 233, or 1 541 ± 117 (or 6 641 ± 1 819, 6 272 ± 1 419, or 7 263 ± 1 643). There was no significant difference among the 3 groups ($P>0.05$). **Conclusion** Infected snails can be obtained through the artificial method by using *S. Japonicum* miracidia to infect snails. Individual infection has the advantage over collective infection. The optimal proportion of infection between snails and miracidia is 1∶15. There was no significant difference between the first and the second generation of artificially infected snails in the average of cercariae shedding, infection, and fatality average of snails. There was no significant difference between artificially and naturally infected snails in the developmental rate of adult worms, fecal and liver eggs per gram.

Key words: *Schistosoma Japonicum*; *Oncomelania hupensis*; miracidia; artificially infected; passage

湖北钉螺人工感染、室内传代与模拟野外饲养的研究

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[**摘要**] **目的**:通过人工方法将湖北钉螺制备成血吸虫感染性钉螺,确定钉螺感染的最佳条件,建立钉螺人工感染传代的室内株,为研究其感染活性、遗传变异和疫苗等提供实验室依据。**方法**:用尼龙绢筛集卵法收集日本血吸虫成熟虫卵,常规法孵化毛蚴。将钉螺与毛蚴按不同比例进行感染,感染方式分为个体感染和集体感染。个体感染随机分6组(Ⅰ~Ⅵ组),每组200只钉螺,每只钉螺置单孔内分别感染,钉螺感染毛蚴比例分别为1∶0,1∶5,1∶10,1∶15,1∶20,1∶25;集体感染随机分6组(Ⅶ~Ⅻ组),每组200只钉螺,按组别集中感染,Ⅶ~Ⅻ组钉螺感染毛蚴比例分别同Ⅰ~Ⅵ组。然后对每组钉螺的感染数、死亡数及尾蚴逸出量进行比较,确定最佳感染方法和比例。以第1代人工感染性钉螺逸出的尾蚴感染实验动物,获取成熟虫卵并孵化毛蚴,然后采用个体感染方式,以1∶15的比例继续感染钉螺,获得第2代人工感染性钉螺。比较第1代与第2代人工感染性钉螺的感染数、死亡数及尾蚴逸出数。通过动物感染实验,比较人工第1代、第2代感染性钉螺与自然感染性钉螺日本血吸虫成虫发育率、每克粪卵数(fecal eggs per gram, FEPG)及每克肝卵数(liver eggs per gram, LEPG)。**结果**:个体感染Ⅰ~Ⅵ组的钉螺感染数分别为0±0,22.7±4.2,31.7±4.5,53.0±5.3,39.3±5.9,32.7±4.7;钉螺死亡数分别为21.7±3.1,25.0±3.6,31.3±4.9,44.7±6.5,78.3±9.5,89.7±13.6;钉螺平均逸蚴量为0±0,308.0±96.6,428.1±146.2,527.0±171.1,571.4±148.9,602.9±356.3。集体感染Ⅶ~Ⅻ组,钉螺感染数分别为0±0,12.3±2.5,18.7±4.7,28.3±4.2,33.3±4.7,29.3±5.5;钉螺死亡数分别为22.7±3.8,23.7±4.5,28.3±5.5,47.0±9.5,75.7±8.5,86.3±12.2;钉螺平均逸蚴量为0±0,244.5±57.3,292.3±74.8,347.1±100.8,477.2±142.1,447.3±161.4。用人工制备的第1代感染性钉螺对血吸虫进行人工传代研究,成功获得了人工第2代感染性钉螺,感染率为24.65%,钉螺死亡率为24.50%;与人工第1代钉螺26.65%的感染率及22.35%的死亡率差异均无统计学意义($P>0.05$)。在尾蚴感染动物试验中,人工第1代、第2代感染性钉螺与自然感染性钉螺的成

虫发育率分别为68.50%,73.50%,71.00%,3组间差异无统计学意义($P>0.05$);自然感染性钉螺和人工第1代、第2代感染性钉螺的FEPG分别为1 503±269,1 683±233,1 541±117;LEPG分别为6 641±1 819,6 272±1 419,7 263±1 643,3组间比较差异无统计学意义($P>0.05$)。**结论**:通过用人工感染的方法可以获得日本血吸虫感染性钉螺。个体感染方式优于集体感染方式,感染时钉螺与毛蚴的最佳比例为1:15。人工感染性钉螺经传代后,第1代与第2代钉螺在感染数、死亡数及尾蚴逸出数等方面无明显差别。比较人工第1代、第2代感染性钉螺与自然感染性钉螺的成虫发育率、FEPG及LEPG,差异也无统计学意义,证明人工传代的血吸虫尾蚴(室内株)能达到自然野生株尾蚴的感染效果。

[**关键词**] 日本血吸虫; 湖北钉螺; 毛蚴; 人工感染; 传代
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Schistosomiasis is an important parasitic zoonosis, which distributes in 76 tropical and sub-tropical countries and regions. Over 200 million people are infected [1] and 600 million people are threatened by the disease. Schistosomiasis not only endangers the health of domestic, but also hinders the development of society and economy. China is one of the 4 countries with the most severe prevalence of this dissease in the world. The *Schistosoma Japonicum* (*S. Japonicum*) is the only schistosomiasis that exists in China, which is mainly epidemic in the lake district of Hunan, Hubei, Jiangsu, Jiangxi, and Anhui province, and the mountainous district of Yunnan and Sichuan province[2]. Thanks to the effort of more than half a century, China has made great achievements in the prevention and treatment of schistosomiasis. However, because of the large number of definitive hosts of *S. Japonicum*. and the frequent movement of human and livestock, the disease spreads rather easily. In recent years, there are 0.5 million schistosomiasis patients in China, among which about 28 000 patients are in the advanced stage. Worse still, more and more advanced-stage patients come out every year[3]. Besides, because of the drug resistance due to the long-term usage of praziquantel in chemotherapy and the widely existed *Oncomelania hupensis*, the intermediate host, it is still a great challenge to control and obstruct schistosomiasis in China[4-6].

The common antigens used in laboratory today, no matter ovum or adult antigens, are mainly from animals infected by cercariae of *S. Japonicum*. In fact, the infected snails plays a key part in the process of the infection, which determines its importance and restriction in the study of the prevention and treatment of schistosomiasis. Preliminary studies[7-12] on the death rate and infection rate in the infected snails and the optimal proportion of infection between snails and miracidia have been carried out. However continuous systemic research, especially researches on laboratory breed of schistosome are rare. In this research, artificially infected snails were prepared and indoor schistosome strains were established, providing the basis for further studies on the comparative analysis of schistosome's hereditary variation under selection pressure of indoor artificial breed and in wild circumstances.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Schistosome-negative snails

A total of 7 800 lakeshore snails (Oncomelania hupensis) was collected from Junshan in Yueyang city, Hunan province (of which 7 200 were used in artificial infection, 600 in subculture). The snails were fed in laboratory for 2 months. Cercariae in snails were determined by the method of cercariae shedding every 2 weeks so as to ensure that the snails had not been infected naturally.

1.1.2 Infected snails

A total of 100 naturally infected snails was provided by Professor ZHUO Shangjiong in the Institute of Schistosomiasis Prevention and Treatment in Hunan province. One hundred of artificially infected snails were prepared in our laboratory. All infected snails

were vigorous and produced large amount of cercariae.

1.1.3 Laboratory animal

A total of 60 clean female Kunming mice weighing 25 – 30 gram bought from the Department of Laboratory Animal of Central South University served as the laboratory animal.

1.1.4 Main equipments

JJ-2B tissue homogenizer was made in Ronghua Equipment Manufacture Co. Ltd. (Jintan, Jiangsu); anatomical lens were from Beijing Taike Equipment Manufacture Co. Ltd. (Beijing); and sample injector was from Shanghai Qiujing Biochemical Reagents and Equipment Co. Ltd.; perforated plates for snails infection were 100-well plates made by organic glass plates and cylindrical containers.

1.2 Methods

1.2.1 Preparation of wild *S. Japonicum* miracidia

Thirty naturally infected snails were placed in dechlorinated water of 20 – 25 ℃, pH 6.6 – 7.8, for 1 – 2 h in sunlight to obtain the cercariae. Thirty Kunming mice were infected through abdominal skin, 20 – 30 cercariae each mouse. Forty-five days after the infection, the livers of the mice were taken out and smashed in homogenizer. Then the mature eggs were collected with 260-pore nylon sieve, and placed in dechlorinated water of 25 – 30 ℃, pH7.5 – 7.8. The egg incubated in the water of hyposmolality and sunlight. And 5 min later, the miracidia began to come out; 20 min later the incubation reached the peak.

1.2.2 Infection of snails with *S. Japonicum* miracidia

Two thousand four hundred negative snails were divided randomly, 200 in each group. Groups Ⅰ-Ⅵ were for individual infection, and Groups Ⅶ-Ⅻ for collective infection. The infection were performed in a room of 25℃. The same experiment was repeated 3 times.

1.2.2.1 Individual infection

One snail was added in each well of the 100-well plate. Dechlorinated water (25℃) was added into the well until the well was full. Every group contains 200 wells. The proportions of snail to miracidiae in Group Ⅰ – Ⅵ were 1∶0, 1∶5, 1∶10, 1∶15, 1∶20, 1∶25, respectively. The number of the miracidiae was counted under the anatomical lens, and determined amount of miracidiae were added with sample injector. After the injecting, the wells were covered so that the snail do not come out. The infection was performed for 4 h in 25℃, pH 7.5 – 7.8 under sunlight.

1.2.2.2 Collective infection

Six square plates (Group Ⅶ-Ⅻ) were prepared and 200 negative snails were placed in each plate. Dechlorinated water was added into the plates until the water covers all snails. The proportions of snails to miracidiae in Group Ⅶ-Ⅻ were the same as that in Group Ⅰ-Ⅵ.

1.2.3 Subculture of schistosome in laboratory

Six hundred negative snails were prepared. We used the first-generation artificially infected snails to infect the laboratory animals. Miracidiae hatched from the mature eggs were obtained. Two hundred negative snails were infected individually; the proportion of snails to miracidia was 1∶15. The same experiment was repeated 3 times.

1.2.4 Feeding and observation of infected snails

Twelve square plates of 30 cm × 25 cm were prepared and cushioned with sponge and straw paper. The infected snails were placed in these plates, labeled with their group numbers, and fed in room temperature[13]. During the observation period, the dead snails were picked out and counted accumulatively.

1.2.5 Screening of artificially infected snails and counting of cercariae

The mean temperature was recorded every day. When the accumulated temperature reached 1 489.43 daydegree[14-15], the screening of infected snails was carried out using the method of cercariae shedding. The screening should be done 3 times, and a 10-day interval between 2 screening was needed so as to

ensure that the miracidia had grown to cercariae. The screened infected snails were numbered and fed individually in small dishes, and cercariae were tested every 10 days until the snails died. The feeding, screening, and cercariae counting of the second-generation infected snails were performed with the above-mentioned method.

1.2.6 Infecting laboratory animals with the cercariae from naturally and artificially infected snails

Thirty Kunming mice were randomly divided into 3 groups, 10 in each group: the group of naturally infected snails, the first-generation artificially infected snails, and the second-generation artificially infected snails. The Kunming mice were infected with cercariae through abdominal skin. Each mouse was infected with exactly 20 cercariae which were counted with anatomical lens. Forty-five days after the infection, the mice were dissected and schistosomes were taken out of hepatic portal veins using heart perfusion. Schistosomes adult were accurately counted after the mesentery vessel was surveyed to make sure that all the adult worms were out. One gram liver tissue was taken from each mouse, added with 1.2% NaCl to make the volume of homogenate reach 10 mL. The homogenized suspension (500 μL) was taken out to smear 10 slides. The total number of egg was counted, and the liver egg per gram (LEPG) was calculated. The excrements of each mouse in 24 hours before being killed were collected, and fecal eggs per gram (FEPG) was calculated with Kato-Katz Method[12].

2 RESULTS

2.1 Hatching of *S. Japonicum* miracidia

The cercaria from naturally infected snails has good vitality. The Kunming mice infected by it through abdominal skin were found to have many nodules of eggs in the liver. The liver was grinded and filtered with sieve, and the mature eggs were obtained. The miracidia hatched from these eggs were of good vitality and high density.

2.2 Mortality and infection rate of artificially infected snails

When the accumulated temperature reached 1 449.43 daydegree, the infected snails were screened under biological microscope by the method of cercariae shedding. The number of died snails and infected ones in Group Ⅰ-Ⅻ were counted, and the results were listed in Tab. 1.

Tab. 1 Comparison of the quantity of infected and fatal snails in Group Ⅰ-Ⅻ ($\bar{x} \pm s$, $n = 200$)

Infection ratio	Groups	Infected sanils	Fatal snails	Cercariae shedding
1:0	Ⅰ	0.0 ± 0.0	21.7 ± 3.1	0.0 ± 0.0
	Ⅶ	0.0 ± 0.0	22.7 ± 3.8	0.0 ± 0.0
1:5	Ⅱ	22.7 ± 4.2	25.0 ± 3.6	308.0 ± 96.6
	Ⅷ	12.3 ± 2.5	23.7 ± 4.5	244.5 ± 57.3
1:10	Ⅲ	31.7 ± 4.5	31.3 ± 4.9	428.1 ± 146.2
	Ⅸ	18.7 ± 4.7	28.3 ± 5.5	292.3 ± 74.8
1:15	Ⅳ	53.0 ± 5.3	44.7 ± 6.5	527.0 ± 171.1
	Ⅹ	28.3 ± 4.2	47.0 ± 9.5	347.1 ± 100.8
1:20	Ⅴ	39.3 ± 5.9	78.3 ± 9.5	571.4 ± 148.9
	Ⅺ	33.3 ± 4.7	75.7 ± 8.5	477.2 ± 142.1
1:25	Ⅵ	32.7 ± 4.7	89.7 ± 13.6	602.9 ± 356.3
	Ⅻ	29.3 ± 5.5	86.3 ± 12.2	447.3 ± 161.4

2.3 Infection rate, mortality, and amount of cercariae of the second-generation artificially infected snails

The eggs from the animals infected by the first-generation artificially infected snails successfully grew into miracidia which were of large amount and good vitality. The infected negative snails with miracidia were continued breeding and observation until the second-generation infective snails were screen out. The number of infected snails, dead snails, and the number of cercariae of the second-generation artificially infected snails had no statistical difference from that of the first generation ($P > 0.05$, Tab. 2).

Tab. 2 Comparison of the quantity of infected snails, dead snails, and cercariae shedding between the first and the second generation snails infected by artificial method ($\bar{x} \pm s$, $n = 200$)

Groups	Infected snails	Fatal snails	Cercariae shedding	Infection rate
First generation	53.0 ± 5.3	44.7 ± 6.5	527.0 ± 171.1	26.65%
Second generation	49.3 ± 7.8 *	49.0 ± 0.0 *	568.3 ± 178.2 *	24.65% *

Compared with the first generation snails, * $P > 0.05$.

2.4 Comparison of the rate of cercariae growing into adult schistosomes between artificially and naturally infected snails

The Kunming mice were dissected 45 days after they were infected, and the adult schistosomes in them were washed out by way of heart perfusion. No significant difference was found in the worm burden among the group of first-generation artificially infected snails, the group of naturally infected snails, and the group of second-generation artificially infected snails ($P > 0.05$, Tab. 3).

Tab. 3 Comparison of worm burden between artificially and naturally infected oncomelania group ($\bar{x} \pm s$, $n = 10$)

Groups	Worm burden	Developmental rate
Naturally infected snails	14.20 ± 1.93	71.00%
The first generation of artificially infected snails	13.70 ± 2.31 *	68.50% *#
The second generation of artificially infected snails	14.70 ± 2.45 *	73.50% *

Compared with the naturally infected snails, * $P > 0.05$; compared with the second generation of artificially infected snails, # $P > 0.05$.

2.5 Experiment of infecting animals with cercariae from artificially and naturally infected snails

No significant difference was found between the FEPGs and LEPGs of the group of naturally infected snails, the group of first-generation artificially infected snails, and the group of second-generation artificially infected snails ($P > 0.05$, Tab. 4).

Tab. 4 Comparison of FEPGs and LEPGs between artificially and naturally infected snails ($\bar{x} \pm s$, $n = 10$)

Groups	FEPG	LEPG
Naturally infected snails	1 503 ± 269	6 641 ± 1 819
The first generation of artificially infected snails	1 683 ± 233 *#	6 272 ± 1 419 *#
The second generation of artificially infected snails	1 541 ± 117 *	7 263 ± 1 643 *

Compared with the naturally infected snails, * $P > 0.05$; compared with the second generation of artificially infected snails, # $P > 0.05$.

3 DISCUSSION

With the strengthening of the work of prevention and cure of schistosomiasis, the amount of naturally infected snails is controlled by drugs, and the positive rate of natural schistosome infection is lowered by controlling the source of infection. Otherwise, with the influence of regionalism and seasonality, it is difficult to get the positive snails for study in time. The collection of infected snails is an hard and lengthy work, and becomes more and more difficult. Therefore, it is necessary to carry out the study of artificial subculture of positive snails, so as to support the teaching and research of schistosomiasis. In term of intermediate host, *Oncomelania hupensis* is also victim of schistosomiasis. Damages caused to snails by *S. Japonicum* include mechanical damage due to miracidia migration, immunopathological lesion and metabolic disturbance caused

by nutrient loss[16-17]. These damages affect the growth, development, reproduction, and survival of the snails. Studies have shown that when *Oncomelania hupensis* is infected by miracidia of *S. Japonicum*, the following changes will occur: arrest of growth, atrophy of generative organs, decreased ova and hatchability[18]. As a result, the survival time of the infected snails dependent upon the schistosome number they were infected. The larger the number is, the shorter they survive. In this research, we infected the snails in Group Ⅰ - Ⅻ with different amount of miracidia, and observed and compared the infection rate, death rate, amount of cercaria shedding of each group. The results showed that the best proportion of snails and miracidia was 1∶15. It was indicated in the present study that as the amount of miracidia increased, the cercaria shedding from the infected snails increased, but the number of dead snails increased as well. So the infection rate decreases after the proportion rises to a certain level. We should consider both the amount of cercariae and the number of death and survival time of the snails when perusing higher infection rate.

At present, studies on the artificial infection and passage of *S. Japonicum* are rare in China. HU Zhongqin, et al.[19] used the schistosome in Sichuan and Zhejiang province to infect the snails in Guichi of Anhui province and compared their infection rate after subculture. Our laboratory has carried out a systemic study on the artificial infection of schistosome to snails. In this study, we studied the subculture of schistosome using the prepared first-generation artificially infected snails, and obtained the second-generation artificially infected snails, with an infection rate of 24.65% and a snails death rate of 24.50%, which was not significantly different from those of the first generation (infection rate 26.65%, snails death rate 23.35%). We compared several indexes between artificially and naturally infected snails. In the experiment of infecting animals by cercariae, the rate of cercariae growing into adult schistosomes was 68.50%, 73.50%, and 71.00% in the groups of first-generation artificially infected snails, naturally infected oncomelania, and second-generation artificially infected snails, respectively. And there was no statistical difference between the 3 groups in cercariae infection and rate of cercariae growing into adult worm. And the FEPG and LEPG of the 3 groups were not statistically different either. The above results indicate that cercariae from the artificially bred snails (indoor strain) have the same infection ability as that from the wild ones. Though it is still not sure whether the artificial bred indoor snails can outlive the test of long-term artificial circumstance, whether the invasion ability or even the genetics of the cercariae will change. So it is necessary to carry out further study on the artificially bred snails. In this research, we produced infected snails through artificial method, and established a preliminary indoor schistosome strain by artificial subculture of positive snails, providing material for further studies on the anti-fecundity vaccine and genetic variation of *S. Japonicum*.

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