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DEVELOPMENT OF LUNG FLUKE, *Paragonimus heterotremus*, IN RAT AND MICE, AND THE ROLE OF PARATENIC HOST IN ITS LIFE CYCLE

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ABSTRACT: Lung fluke, *Paragonimus heterotremus*, has been identified as the important pathogen for human paragonimiasis in Vietnam. Eating under cooked mountain crabs, which are contaminated with *P. heterotremus* metacercariae, is confirmed as the route of infection. In this study, we identified the role of paratenic host in the life cycle of *P. heterotremus* by experimental infection to house rat (*Rattus tanizumi*) and mice BALB/c, and then transferred to cats. The results showed that *P. heterotremus* metacercariae developed to adults in the lungs of rats. In contrast, they remain as juvenile worms in liver and muscles of mice. These juveniles developed to adults when they were transferred to cats, confirming that mice serve as the role of paratenic hosts in the life cycle of *P. heterotremus* in Vietnam. Thus, investigation for natural paratenic hosts of *P. heterotremus* is necessary, and not eating uncooked/undercooked meat of other animals in addition to mountain crabs should be added to prevention of paragonimus infection. Development of different size metacercariae of *P. heterotremus* in rats and mice were also discussed herein.

Keywords: *Paragonimus heterotremus*, development, paratenic host, rat and mice.

INTRODUCTION

Lung fluke of the genus *Paragonimus*, which parasite the lungs of human and animals, cause serious affection on the health of infected individuals [1, 2]. Infection occurs by eating uncooked/undercooked mountain crabs (the second intermediate hosts) infected with metacercariae or by consumption of raw/undercooked meat of paratenic hosts that harbor juvenile worms [2]. Paratenic hosts have been reported in the life cycle of some *Paragonimus* species, such as, *P. westermani*, *P. heterotremus*, *P. kellicotti*, *P. mexicanus* and *P. skrjabini* [2]. Paratenic hosts of *Paragonimus* species are usually mammals and rodents are common paratenic hosts in experiments.

In Vietnam, seven *Paragonimus* species have been detected in Northern and Central provinces so far [4]. Of these, only *P. heterotremus* has been proved to infect humans in Northern provinces [4, 5, 12]. The habit of eating raw/undercooked mountain crab hosts was identified as the way of infection. To date, there has been no a study on the role of paratenic hosts in the life cycle of *P. heterotremus* in Vietnam. Moreover, morphological studies showed variation of *P. heterotremus* metacercariae [6], including metacercariae as small as those of *P. pseudoheterotremus* which can be matured in rats [21]. This raises a question of whether there are differences among the development of different-size metacercariae of *P. heterotremus* in rats. Above mentioned issues will be tested in this study.

MATERIALS AND METHODS

Metacercariae of *P. heterotremus* were isolated from mountain crabs, *Potamiscus tannanti*, caught from An Lac commune, Luc Yen district, Yen Bai province and from Cam Ngoc commune, Cam Thuy district, Thanh Hoa province. Morphologically, metacercariae from Thanh Hoa province was oval in shape, and union in size (187-218 × 164-180 µm) with the width < 200 µm (fig. 1a); while metacecariae from Yen Bai was more round in shape with larger variation of size (167-300 × 156-297 µm), thus they were divided into 2 groups: >200 µm (fig. 1b) and <200 µm (equal as *P. pseudoheterotremus*, fig. 1c).

House rats (*Rattus tanezumi*) caught at

Ha Noi, where there is no source of *Paragonimus* infection, BALB/c mice and domestic cats (*Felis catus*) were used for experimental infection. Experimental animals were shown to be free from *Paragonimus* by stool examination before experiments.

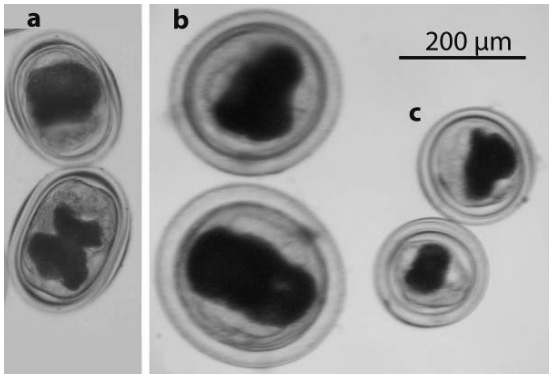

Figure 1. Metacercariae of *P. heterotremus* collected from Thanh Hoa and Yen Bai provinces

a. Metacercariae collected from Thanh Hoa; b-c. Metacercariae collected from Yen Bai showing variation of size.

Infection to animals: metacercariae of each group was counted and given to animals (rats and mice) via oral way after anesthesia.

Five mice and two rats were infected with 20 and 5 metacercariae/animal, respectively, of each metacercaria groups. From day 30th post infection, the feces of experimental animals were checked by sediment technique to find *Paragonimus* eggs, and one infected mouse of each group was fed to a cat.

After one and two months post infection, two mice of each group were autopsied to identify the development of worms, and experimental animals were autopsied after finding *Paragonimus* eggs in fecal sample. Flukes were collected from liver, lungs and muscles of the animals. Juvenile worms in muscles and liver were collected by digestion method with pepsin 1% at 36^{o}C for 6-8 hours to release flukes from the tissues. The flukes were washed by saline 0.9%, then pressed between two glasses, and preserved in 70% ethanol for pemernant slide by staining with carmine 1%, covered on slide by Canada balsam. The flukes were observed and measured under light microscopic. The data was statistically analyzed using SPSS (Statistical Package for the Social Sciences).

RESULTS AND DISCUSSION

The result of infection for BALB/c mice and transfer of juvenile *P. heterotremus* to cats

The result of autopsy of mice infected with *P. heterotremus* metacercariae showed that all experimental mice become infected with *P. heterotremus*. However, none of the flukes are mature after 60 days; they remain as juveniles in muscles and liver of mice. There was no difference of developmental rates among metacercaria groups. The recovery percentage of metacercariae collected from Thanh Hoa was 56.3% (including 38.8% in muscles and 17.5% in liver). These data are compatible to those (55.0% including 41.3% in muscle and 13.7% in liver) of metacercariae >200 µm and those (52.5% including 40.0% in muscle and 12.5% in liver) of metacercariae <200 µm from Yen Bai province (table 1).

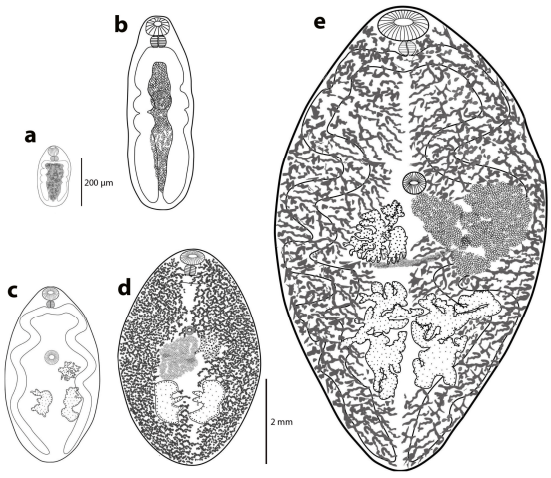

Figure 2. Development of *P. heterotremus* in experimental animals

a. Newly excysted metacercaria; b. Juvenile worm from muscle of mice at 1 month post infection; c. Young worm from liver of mice at 1 month post infection (showing testes and ovary); d. Adult worm collected from lung of rat; e. Adult worm collected from lung of cat. Fig. 2a. 2b share the same scale bar (in µm); Fig. 2c-e share the same scale bar (in mm).

Juvenile worms recovered from muscles (fig. 2b) were morphologically similar to the newly excysted metacercariae (fig. 2a) with the exception that they were slightly larger in size. There was no statistically significant difference among the size of juvenile worms derived from different metacercaria groups (p>0.05, table 2).

Table 1. The result of infection of *P. heterotremus* metacercaria to mice BALB/c

Metacercaria collected from	No. of mice*	No. of Mc/ mouse	Number (%) of juveniles recovered from		
			Liver	Muscles	Total
Thanh Hoa	5	20	14 (17.5)	31 (38.8)	45 (56.3)
Yen Bai, > 200 µm	5	20	11 (13.7)	33 (41.3)	44 (55.0)
Yen Bai, < 200 µm	5	20	10 (12.5)	32 (40.0)	42 (52.5)

* 1 mouse of each group was fed to cat 1 month post infection; Mc=Metacercariae.

Table 2. The measurement of worms collected from muscles and liver of mice infected with different metacercaria groups

Size	Worms derived from metacercaria collected in Thanh Hoa	Worms derived from metacercaria >200 µm collected in Yen Bai	Worms derived from metacercaria <200 µm collected in Yen Bai
Juvenile from muscles			
Body	760-1000 × 320-520 (827.5 × 449.5)	704-960 × 360-536 (846.5 × 463.8)	640-960 × 320-496 (822.5 × 446)
Oral sucker	96-112 (108)	96-120 (110)	96-112 (109)
Ventral sucker	104-128 (123)	104-136 (124.5)	104-128 (123.5)
Young worm from liver			
Body	2.4-4.2 × 1.3-2.0 (3.4 × 1.8)	2.8-4.3 × 1.2-2.1 (3.4 × 1.7)	2.0-4.6 × 1.2-2.2 (3.3 × 1.7)
Oral sucker	220-320 × 340-400 (262 × 375)	220-330 × 340-400 (269 × 379)	200-320 × 300-420 (265 × 368)
Ventral sucker	220-320 (266)	220-300 (271)	200-360 (280)
Testes	200-500 × 280-700 (382 × 541)	200-500 × 300-700 (389 × 559)	200-480 × 260-720 (386 × 551)
Ovary	260-400 × 300-500 (349 × 414)	280-400 × 320-510 (360 × 428)	260-420 × 300-540 (351 × 408)

Table 3. The result of infection to rat with different metacercaria groups

Metacercaria groups	Number of rat	Number of mc/rat	Egg release (day)	Recovery rate (%)
From Thanh Hoa	2	5	35-40	80-100
From Yen Bai >200 µm	2	5	34-36	40-100
From Yen Bai <200 µm	2	5	35-41	60-80

Flukes collected from liver (fig. 2c) were bigger than juvenile worms from muscles, and reproduction organs (testes and ovary) were observed. There was no statistically significant difference among the size of young flukes derived from different metacercaria groups (p>0.05, table 3).

The result of re-infection to cats with juvenile worms from mice of the previous infection showed that all three cats fed the mice,

which were previously infected with different metacercaria groups from Thanh Hoa and Yen Bai, became infected with adults of *P. heterotremus*. The time of releasing egg was 45-60 days post infection, and the developmental rates were 10-20%. The flukes are oval in shape, body size 10-15 mm, ovary and testes branched, vitelline well-developed and uterus is full of eggs (fig. 2e).

Table 4. Measurement of worms collected from lung of rats infected with different metaceracria groups

Size	Worms derived from metacercariae from Thanh Hoa	Worms derived from metacercariae >200 µm from Yen Bai	Worms derived from metacercariae <200 µm from Yen Bai
Body	4.5-5.3 × 3-3.5	4.8-5.5 × 3-3.5	5-5.4 × 2.8-3.6
	(4.9 × 3.3)	(5.1 × 3.3)	(5.2 × 3.4)
Oral sucker	360-420 × 500-700	360-400 × 520-720	380-400 × 520-700
	(388 × 648)	(388 × 668)	(396 × 664)
Ventral sucker	360-400 (380)	360-400 (384)	380-400 (396)
Testes	960-1100 × 720-980	980-1200 × 720-1000	1000-1200 × 700-1000
	(1052 × 812)	(1120 × 840)	(1100 × 820)
Ovary	500-660 × 560-680	580-720 × 580-680	600-700 × 600-700
	(588 × 604)	(620 × 612)	(620 × 620)

The result of infection for rats (*Rattus tanezumi*)

Paragonimus eggs were detected from fecal samples of all six experimental rats infected with *P. heterotremus* metacercariae. Two ratsinfected with metacercariae from Thanh Hoa released *Paragonimus* eggs at 35-40 days post infection, and the rates of metacercariae developed to adult were 80-100%. These data were similar to those (produced eggs after 34-36 days with developmental rate of 40-100%) of metacercariae >200 µm and those (produced eggs after 35-41 days and developmental rate of 60-80%) of metacercariae < 200 µm from Yen Bai (table 4). There was no statistically significant difference among the size of flukes derived from different metacercaria groups (p>0.05, table 4). Adults collected from rats (fig. 2d) are smaller than those of adults recovered from cats (fig. 2e).

DISCUSSION

Paragonimus heterotremus is the pathogen for human paragonimiasis from South to Southeast Asia and Southern China [2]. Among species of the genus *Paragonimus*, *P. heterotremus* is typical by the smallest metacercariae (<300 µm) in comparison with other reported species. Recently, a new species, *P. pseudoheterotremus*, was described from Thailand having metacercariae (about 200 µm) slightly smaller than that of *P. heterotremus*, although they showed similarity in morphology of adults and ITS2 sequence to each other [20, 21]. Biologically, *P. pseudoheterotremus* and *P. heterotremus* from Thailand are considered to be different from each other in susceptibility to rodent hosts. The former species can develop to adults in rats, but the latter one can not [21]. Since, the range of size of *P. heterotremus* metacercariae from Vietnam cover the size of *P. pseudoheterotremus*, it is important to clarify if there is *P. pseudoheterotremus* in Vietnam, and if there is any difference among development of different-size group of metacercariae in rats. The result of present study clearly showed that there is no difference among the development of different-size *P. heterotremus* metacercaria groups in rats and mice, although they are various in morphology and size.

The size of *P. pseudoheterotemus* metacercariae was considered to be slightly smaller than that of *P. heterotremus*. However, this comparison was made from specimen within Thailand only. When gathering all available data from various geographical locations, Doanh et al. (2013) [6] found that the size of *P. pseudoheterotremus* metacercariae is almost equal to that of *P. heterotremus* from China [11], India [17] and from Thanh Hoa, Vietnam [6]. Moreover, the variations of susceptibility of *P. heterotremus* to rats have been recorded. Sugiyama et al. (1990) [18] reported that Wistar rat was not sensitive to *P. heterotremus* from Thailand; they did not develop to adults. In contrast, Hu (1998) [11] and Yan et al. (1998) [19] found the sensitivity of Wistar rat to *P. heterotremus* from China. In India, *P. heterotremus* collected from Manipur did not mature in rats [16], while metacercariae from Arunachal Pradesh developed to adults [15]. The difference in host specificity among geographical populations was also seen in *P. westermani* [8-10, 14]. Thus, the slight variation of the size of metacercariae and susceptibility to experimental rats cannot be employed to separate *P. pseudoheterotremus* as a valid species; it should be a geographical population of *P. heterotremus* as confirmed by molecular analyses [6].

In this study, all metacercaria groups developed to adults in rats with high rates (up to 100%), and the time required for *P. heterotremus* to be mature in rats is shorter than that in dog and cats [7], confirming that rats can play the role as definitive host of *P. heterotremus* in Vietnam. In contrast, *P. heterotremus* metacercariae did not develop to adults in mice; they remain as juvenile worms in muscles and liver. When these juveniles were transferred from mice to cats, they develop to adults, indicating that mice play the role as paratenic hosts in the life cycle of *P. heterotremus* in Vietnam. More extensive survey for natural paratenic hosts of *P. heterotremus* is, therefore, necessary.

To date, *P. heterotremus* has been detected in Northern Vietnam, and proved to be pathogen for human paragonimiasis. The number of detected cases have been increased, especially in Lai Chau (Sin Ho), Son La, Lao Cai, Yen Bai [3]. Previous studies in Vietnam just propagated not eating undercooked mountain crabs to avoid paragonimus infection. The results of this study suggests that not eating uncooked meat of other animals in addition to mountain crabs should be added to prevent of paragonimus infection.

CONCLUSION

Metacercariae of *P. heterotremus* showed variation in morphology and size, but similarity in their development in house rats and mice.

In experiments, house rats play as definitive hosts of *P. heterotremus*. In contrast, mice play the role as paratenic host of this parasite.

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SỰ PHÁT TRIỂN CỦA SÁN LÁ PHỔI, *Paragonimus heterotremus*, Ở CHUỘT THÍ NGHIỆM VÀ VAI TRÒ VẬT CHỦ DỰ TRỮ TRONG VÒNG ĐỜI PHÁT TRIỂN CỦA CHÚNG

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TÓM TẮT

Loài sán lá phổi, *Paragonimus heterotremus,* được khẳng định là nguyên nhân gây bệnh sán lá phổi cho người và động vật. Ở các tỉnh miền Bắc Việt Nam, người nhiễm bệnh đã được xác định là do ăn cua núi (vật chủ trung gian 2) bị nhiễm ấu trùng chưa được nấu chín. Trong nghiên cứu này, chúng tôi xác định vai trò vật chủ dự trữ trong vòng đời phát triển của sán lá phổi *P. heterotremus* bằng cách gây nhiễm cho chuột, sau đó gây nhiễm chuyển tiếp cho mèo. Kết quả cho thấy, *P. heterotremus* phát triển đến trưởng thành ở chuột nhà, nhưng tồn tại ở dạng sán non ở cơ và gan chuột bạch. Khi gây nhiễm chuyển tiếp sán non từ chuột bạch cho mèo, chúng phát triển đến trưởng thành. Điều đó khẳng định vai trò vật chủ dự trữ trong vòng đời phát triển của sán lá phổi *P. heterotremus* ở Việt Nam, người và động vật có thể bị nhiễm bệnh do ăn phải vật chủ dự trữ mang mầm bệnh sán lá phổi. Vì vậy, điều tra xác định vật chủ dự trữ của sán lá phổi ngoài tự nhiên là việc cần thiết và để phòng tránh nhiễm sán lá phổi, ngoài việc không ăn cua núi chưa nấu chín kỹ, cần tránh ăn sống hoặc tái thịt các loài động vật khác. Nghiên cứu này cũng khẳng định không có khác biệt về sự phát triển của các nhóm metacercaria kích thước khác nhau của loài *P. heterotremus*.

Từ khóa: Paragonimus heterotremus, chuột bạch, chuột nhà, sán lá phổi, sự phát triển, vật chủ chứa.

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