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OPTIMIZATION OF AN EXPERIMENTAL MODEL FOR THE RECOVERY OF ADULT *HAPLORCHIS PUMILIO* (HETEROPHYIDAE: DIGENEA)

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ABSTRACT: Recent studies in Vietnam and other Asian countries have shown that fish-borne zoonotic intestinal trematodes (FZT) occur very frequently in humans. The dominant intestinal FZT in Vietnamese fish are species of *Haplorchis*, in particular *H. pumilio*. However, basic studies on the biology and pathology of adult *H. pumilio* are difficult because of the lack of a standardized experimental animal model. The objective of this study was to establish and optimize such an animal-infection model for *H. pumilio*. Using metacercariae isolated from naturally infected fish, experiments were conducted to identify a suitable experimental animal host species, as well as the optimum metacercariae infection dose, and to determine the post-infection interval for patency. In a series of experiments, mice (*Mus musculus*) and chickens (*Gallus gallus* dom.) were infected with different numbers of metacercariae, and worm recoveries were made at varying intervals post-infection (PI). Based on the mean number of adult flukes recovered/number of metacercariae inoculated and the percent of hosts infected, mice were significantly more susceptible to infection than were chickens. The proportion of metacercariae developing to the adult stage increased with dose size. The peak worm recovery (geometric mean) was found to be day 7, although not all recovered flukes were gravid until day 9 PI. These results describe a mouse infection model with good predictability for intestinal flukes, such as *H. pumilio*, results which could facilitate investigations on important biological and pathological aspects of intestinal fluke infections.

More than 40 million people are estimated to be infected by fish-borne zoonotic trematodes worldwide, most of them in Southeast Asia (WHO, 2004). Although the fish-borne liver flukes (Opisthorchidae) are receiving a deserved increase in attention, the food safety and health impacts of the larger group of intestinal fish-borne flukes are only beginning to emerge (WHO, 1995, 2004; Chai et al., 2005). These flukes, which include numerous species belonging mainly to the Heterophyidae and Echinostomatidae, are widely spread and often highly prevalent as multi-species complexes in humans, especially in south and east Asia and Africa (WHO, 1995; Chai et al., 2005; Waikagul and Radomyos 2005; Yu and Xu, 2005). Recent investigations have shown that zoonotic fish-borne intestinal trematodes (FZT) are highly endemic in both humans and fish in Vietnam (Dung et al., 2007; Thu et al., 2007). Among these, *Haplorchis pumilio* appears to be the most common species of metacercariae in Vietnam (Hop et al., 2007; Thien et al., 2007; Thu et al., 2007; Chi et al., 2008), although *H. taichui* and *H. yokogawai* are also present, as they are in other regions (Chai et al., 2005). Their potential importance to public health and aquaculture poses the need for more information on the biology, ecology, and epidemiology of these trematodes. For example, although there are several reports on pathology in humans due to infection with *H. taichui* and other heterophyid flukes (Chai et al., 2005; Sukontason et al., 2005), little is known regarding the effects of *H. pumilio* infection in humans, including the resulting pathology and immunology. Some studies on worm infectivity, and development in chickens and mice, have been reported for *H. taichui* and several other species of Heterophyidae, including *Heterophyopsis continua*, *Stellantchasmus falcatus*, and *Centrocestus armatus* (Hong et al., 1989, 1990; Wongsawad et al., 1998; Sukontason et al., 2001; Kumchoo et al., 2003). However, published information on *H. pumilio* is too limited to standardize a protocol for experimental investigations on host–parasite interactions for this species.

The range of potential hosts that have been experimentally infected with *H. pumilio* is broad and includes rats, hamsters, mice, chickens, pigeons, dogs, cats, and rhesus monkeys (Pearson and Ow-Yang, 1982). However, some studies have reported conflicting results with chickens and have involved mice to only a very limited extent, although these animal species are well suited for laboratory investigations (Sommerville, 1982; Umadevi and Madhavi, 2006). Because of the particular advantages associated with the use of mice as an experimental host (e.g., greater relevance to human biology and greater knowledge based on host genetics and immunology), it was desirable to compare these 2 hosts in a systematic manner to determine their suitability as an experimental infection model and to standardize an infection protocol. A reliable experimental model could facilitate host–parasite investigations including pathology and immune responses to infection, parasite longevity, and efficacy of various treatment methods. A reliable model could also provide a biological method to identify unknown fish metacercariae for both diagnostic and research purposes, as well providing fluke eggs for laboratory snail infections (Chai, 2004).

MATERIALS AND METHODS

Recovery of metacercariae from fish

Two freshwater fish, the grass carp (*Ctenopharyngodon idellus*) and the silver carp (*Hypothalmichthys molitrix*), were obtained from household ponds in northern Vietnam (Nam Dinh Province), where previous studies have found a high occurrence of *H. pumilio* in fish and humans (Dung et al., 2007). The fish were placed on ice and transported to the Research Institute for Aquaculture No. 1, Tu Son, Bac Ninh Province, Vietnam located about 120 km from the study site. There, the fish were kept in cold storage (4 C) until processed for recovery of metacercariae, usually within 36 hr of collection.

The metacercariae were recovered using the pepsin digestion procedure described in WHO (1995, Annex 6) and as modified by Thu et al. (2007). Fish weighing <100 g were digested whole, and fish weighing >100 g were divided into 5 sub-sections (fish head, gill, muscles, fin, and skin and scales; 10–20 g/section). The fish samples were minced

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by processing in a meat grinder, until paste-like, and subsequently digested in a solution of 1% pepsin and 0.06 M HCl in distilled water at 37 C for 2–3 hr. The digested sample was filtered through a 1 × 1-mm mesh brass sieve, and the sediment was allowed to settle in 0.85% saline. This washing step was repeated until the supernatant was almost clear. The supernatant was then carefully discarded and the sediment transferred into a Petri dish containing 6–7 ml 0.85% saline and examined under a stereomicroscope. Any metacercariae present were separated, counted, and identified by mounting on a glass slide under a cover slip and viewing with a compound microscope (×100) or a dissecting stereomicroscope. Metacercariae identified as *H. pumilio* (Yamaguti, 1971; Velasquez, 1973; Pearson and Ow-Yang 1982; Scholtz et al., 1991; Kaewkes, 2003) were separated into a Petri dish containing 0.85% saline until they were used for the animal infections, always within 12 hr of isolation from the fish.

Animal infections

All animal-experiment protocols followed the animal care guidelines of the Research Institute in Aquaculture No. 1. and were approved by the Research Director. Three-week-old out-bred Swiss mice (*Mus musculus*) were obtained from the Center of Research and Production of Experimental Animals at the National Institute for Hygiene and Epidemiology in Hanoi, Vietnam. Day-old chickens (*Gallus gallus* dom., strain: Tam Hoang) were obtained from the Department of Parasitology at the National Institute of Veterinary Research, Hanoi. Fecal parasitological examination of the mice, prior to use, revealed infections only with pinworms (species not determined). The metacercariae were gavaged using a 20-gauge feeding tube into animals lightly sedated with diethyl ether. For recovery of the adult flukes, the animals were killed either by cervical dislocation (mice) or administration of an overdose of diethyl ether (chickens). Their small intestine was removed, opened in 0.85% saline in a glass beaker, and the contents separated by swirling the intestine. The contents were allowed to settle, and the supernatant was poured off. This washing step was repeated until the supernatant was almost clear; it was then poured into a Petri dish containing 0.85% saline and examined using a stereomicroscope. The adult flukes were separated and pooled in a Petri dish containing 0.85% saline; the worms were fixed by pipetting into near-boiling water, then removed after approximately 10 sec and stored in 5% formalin until stained for identification. Adult flukes from each mouse or chicken group were pooled, and 10 flukes were randomly selected and stained for approximately 15 min in Meyer's hematoxylin and identified using the key developed by Pearson and Ow-Yang (1982). The length and width of the fluke and its oral and ventral sucker, along with the number and size of eggs present, were used as a measure of the development stage (age) of the adult flukes.

Comparisons of mice and chickens for their suitability as an experimental host, and the determination of the effect of metacercariae dosage on adult worm development, were investigated in Experiment 1, in which all adult flukes were recovered at day 5 post-infection (PI). Because mice proved to be a more-suitable experimental host for *H. pumilio* in Experiment 1, only mice were used in Experiment 2, which was designed to determine the optimal day PI for recovering the largest number of adult flukes. Mice were divided into 4 groups of 10 individuals, and each group was intubated with 100 metacercariae. One group was then necropsied for recovery of adult flukes at 5, 7, 9, and 11 days PI. One mouse died at day 9 PI, presumably due to trauma suffered by oral gavage.

Data analysis

Optimal metacercaria dosage size is defined as the number of metacercariae producing both the highest recovery (number) of adult flukes and the highest percentage of infections in an animal host group. Total fluke recovery, logarithmically transformed [ln(x + 1)], was compared between animal host species and dosage using a 1- or 2-way analysis of variance (ANOVA). Mean values of log-transformed fluke recovery, and their 95% confidence intervals (CI), were back-transformed to the original scale (for convenience, the back-transformed mean value is referred to as the geometric mean). The number of adult flukes recovered was divided by the actual metacercariae dosage to yield the proportion recovered. For comparison of these proportions, the fate of each metacercaria (i.e., found as an adult or not recovered) was compared between the 4 metacercariae dosages or animal host species using Chi-square values from cross-tabulations. Logistic regression (Hosmer and Lemeshow 1989) was used to analyze the 2 factors and possible interaction between them. Host animals were scored as infected (at least 1 adult fluke recovered) or negative, and the effect of dosage and host species on infection prevalence was analyzed using logistic regression. In Experiment 2, prevalence of infection in hosts was compared, over time, by Chi-square tests. Adult fluke counts (log-transformed) and measurements of flukes were compared, among days, using a 1-way ANOVA, and post hoc comparisons were done with the Scheffe test (Scheffé, 1953). *P*-values <0.05 were used to indicate significant differences.

RESULTS

Identification of adult flukes

Confirmation of metacercarie identification was made on adult worms recovered in Experiment 2. From each day of recovery PI, 10 adult flukes were randomly selected for staining to verify the identification of the metacercariae as *H. pumilio*. The identifications were made based on the general morphological traits typical for *H. pumilio*, i.e., the presence of a central group of 32–40 sclerites on the ventral sucker in the ventro-genital complex. Of the 40 randomly selected stained adult flukes recovered from experimentally infected mice, 33 could be clearly identified as *H. pumilio*. In the remaining 7 flukes, either the presence of eggs covering the ventro-genital complex, or the quality of the stained specimens, made it impossible to make a firm identification. These findings confirmed that the metacercariae used for the inoculation experiments were nearly all, if not completely, *H. pumilio*.

Optimal experimental host and infection dosage

In Experiment 1, from the number of flukes recovered at day 5 PI, the proportion of metacercariae recovered as adults and the proportion of animals with infections was higher in mice than in chickens ($P < 0.001$) at all metacercariae dose sizes (Table I). The proportion of metacercariae recovered as adults increased with metacercariae dose in mice ($P < 0.001$), although this recovery was very similar at dosages of 20 and 30 metacercariae. Recovery differed significantly between metacercariae dosages in the chicken, but the trend was less obvious; the adult worm recovery increased with metacercariae dose in both host species, but the pattern was different in mice and chickens, as indicated by a significant interaction term (2-way ANOVA $P < 0.001$)

Optimal time interval for worm recovery

In Experiment 2, the proportion of metacercariae recovered as adult flukes was significantly higher at day 7 PI than at days 5, 9, and 11 PI (Table II). At days 7 and 9 PI, all intubated mice were infected. The developmental stages of the adult flukes recovered at these different time intervals are shown in Table III. The sizes of the fluke lengths and widths, and the ventral and oral sucker diameters, were significantly smaller at day 7 PI as compared to day 9 PI. As early as day 5 PI, eggs were present in flukes in 9 of 10 mice and in 7 of 10 chickens at day PI. All flukes recovered at days 9 PI and 11 PI were gravid.

DISCUSSION

Our results from the experimental infections with *H. pumilio* clearly show that mice are superior to chickens in terms of high

TABLE I. Comparison of adult fluke-infection outcomes from inoculation of *Haplorchis pumilio* metacercariae (MC) in mice and chickens at 5 days post-infection.*

Host	No. MC inoculated	Mean† no. adults recovered (95% CI)	Median proportion of MC recovered as adults (range)	Proportion of animals with adult worms
Mice	10	0.34 (−0.05–0.88)	0.00 (0.00–0.20)	0.30
	20	2.79 (1.45–4.85)	0.21 (0.00–0.57)	0.80
	30	4.75 (3.01–7.24)	0.19 (0.00–0.52)	0.95
	40	9.90 (6.39–15.08)	0.35 (0.09–0.65)	1.00
P-value‡ (dose response)		<0.001	<0.001	<0.001
Chickens	10	0	0	0.00
	20	0.94 (0.35–1.78)	0.05 (0.00–0.38)	0.53
	30	1.81 (0.40–4.64)	0.04 (0.00–0.41)	0.70
	40	2.25 (0.91–4.53)	0.08 (0.00–0.16)	0.80
P-value‡ (dose response)		<0.01	<0.001	<0.01

* Worm burdens and proportion of experimental hosts infected were significantly higher in mice than in chickens ($P < 0.05$).
† Geometric mean.
‡ 1-way analysis of variance or Chi-square test.

recovery of adult worms and their development. Sommerville (1982) was the first to report infection of mice with *H. pumilio*, but was unsuccessful in infecting 3- to 7-day-old chickens. However, as in our investigation, Umadevi and Madhavi (2006) were able to infect day-old chickens, indicating host age may be an important factor. Infectivity to 2-wk-old chickens is also reported for *H. taichui*, indicating species differences within species of *Haplorchis* (Kumchoo et al., 2003).

At a dose of 100 metacercariae, adult worm recovery, as a proportion of the number of metacercariae inoculated, was maximal at day 7 PI and then declined, although gravid worms were still present at day 11 PI at the end point of the experiment. Sommerville (1982) recovered gravid flukes from mice beginning at days 3–4 PI, although only about 40% had eggs; the longevity of the flukes was not determined. Similarly, longevity studies on *H. pumilio* in chickens have not been done; however, the data from longevity studies in other species of the Haplorchinae (Pearson and Ow-Yang, 1982) are of interest. Martin (1958) reported that *Stellantchasmus falcatus* persisted in cats for at least 44 days, and *H. taichui* lasted up to 48 days in chickens (Kumchoo et al., 2003). This suggests that the turnover of intestinal heterophyid flukes may not be brief and that their persistence may be dependent on host species and worm infrapopulation size. The decline in the number of recovered adult flukes in this study after day 7 PI may be due to the initiation of immune-mediated expulsion (''self-cure''), as described for *H. pumilio* by Khalifa et al. (1977), a phenomenon observed in many intestinal trematode and nematode infections (Chai et al., 1984; Yu and Mott, 1994; Toledo et al., 2006; Chai, 2007; Maruyama and Nawa, 2007). Alternatively, the large number of worms occurring at day 7 PI may have compromised the survival of some of the worms through density-dependent competition for resources, as has been reported for other intestinal parasites (Keymer, 1982; Smith, 1987; Elkins et al., 1991). A crowding effect may also be responsible for the higher recovery of adults at a metacercariae dose of 40 than at 100 (0.35 compared to 0.17). The reduction in adult worm size we observed at day 7 PI, and the rebound in size apparent at day 9 PI, may reflect the impact of one, or both, of these conditions. Further refinement of this mouse model for intestinal fluke infections should address the longevity of adult flukes and the impact of infrapopulation intensity, especially on intestinal pathology and the initiation of a possible immune expulsion event. This aspect is relevant to human infections, in which large variations in intestinal worm burdens are common (Anderson and May, 1985; Wakelin, 1994).

TABLE II. Recovery of *Haplorchis pumilio* adults from mice at different days post-infection (PI) with 100 metacercariae (MC).

Day of fluke recovery PI	No. of mice	No. of flukes recovered (GM* and 95% CI)	Median proportion of MC recovered as adults (range)	Proportion of hosts with adult flukes
5	10	12.75 (4.10–36.06)	0.17 (0.00–0.53)	0.90
7	10	38.19 (31.69–45.99)	0.41 (0.25–0.54)	1.00
9	9	10.58 (4.81–22.1)	0.11 (0.01–0.33)	1.00
11	10	9.26 (3.13–24.50)	0.18 (0.00–0.43)	0.90
P-value		<0.05†	<0.001‡	NS‡

* GM = geometric mean.
† 1-way ANOVA.
‡ Chi-square test; NS = not significant.

The mouse model described in this report meets many of the requirements for laboratory investigations on the basic and clinical aspects of *Haplorchis* spp. infections and possibly for other heterophyid trematodes. However, this model can be further improved. For example, other strains of mice, including inbred strains, should be evaluated for susceptibility, worm development and fecundity, and longevity. Depending upon the nature of the host–parasite interaction of interest, some strains of mice may be more useful than others, as is often the case with investigations on host immunity (Chai et al., 1984; Wakelin, 1994). For the investigation of suitable snail hosts, snail infection, and cercariae emergence dynamics, this host model can provide the fluke eggs needed for snail infections. Methodologically, we also recommend (for animal welfare reasons) that diethyl ether be replaced by isoflurane for anaesthesia, which would be less irritating for the respiratory pathways of the mouse and have no affect on infection outcome.

TABLE III. Measurements (μm) of adult *Haplorchis pumilio* and their eggs recovered from mice at days 5, 7, 9, and 11 post-infection (PI).

Measurement	Day 5	Day 7	Day 9	Day 11	*P*-value*
Body length	506.9 ± 32.5	416.2 ± 65.8†	521.2 ± 30.0	502.9 ± 42.5	<0.01*
Body width	231.0 ± 68.6	144.3 ± 18.2†	241.2 ± 39.1	274.9 ± 87.0	<0.01*
Ventral sucker, length	119.8 ± 20.4	88.2 ± 13.5†	122.4 ± 14.4	131.8 ± 20.1	<0.01*
Ventral sucker, width	103.5 ± 17.1	75.5 ± 11.6	91.8 ± 19.6	106.3 ± 31.3	<0.05*
Oral sucker, length	95.4 ± 12.9	95.9 ± 13.3	101.5 ± 9.6	101.5 ± 7.8	NS*
Oral sucker, width	115.8 ± 11.3	115.8 ± 8.8	110.2 ± 9.6	136.7 ± 12.3†	<0.01*
Egg length	67.4 ± 1.7	64.8 ± 5.9	66.8 ± 3.2	67.3 ± 2.3	NS*
Egg width	41.9 ± 1.7	42.3 ± 4.5	45.4 ± 3.6	45.9 ± 3.4	NS*
Median no. of eggs (range)	40 (0–50)	15 (0–50)	100 (6–100)	100 (50–100)	<0.001‡

* 1-way ANOVA; NS = not significant.
† This group differs significantly from at least one of the others. On day 5 PI, 1 fluke had no eggs and on day 7 PI, 3 flukes had no eggs.
‡ Kruskal Wallis rank test.

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