HUMORAL IMMUNE RESPONSE OF CATS TO *PARAGONIMUS* INFECTION

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INTRODUCTION

Paragonimus siamensis was originally reported by Miyazaki and Wykoff in 1965 from Udorn Thani province, Northeast Thailand. The complete life cycle has not yet been fully determined. Species of crabs such as *Parathelphusa diagasti*, *Parathelphusa germaini* were found to be intermediate host of paragonimiasis in Thailand and the definitive hosts being cats, bandicoots and rattus species.

The diagnosis of paragonimiasis is currently based only on the finding of the *Paragonimus* eggs in the sputum and/or faeces. The sero-immunological techniques will be of value in the diagnosis of paragonimiasis especially extrapulmonary paragonimiasis, if the sputum and stool are negative and in the evaluation of the chemotherapy of paragonimiasis. The immunological tests that have been used in the diagnosis of paragonimiasis include:- complement fixation test (Ando, 1917; Yokogawa and Awano, 1956; Sadun *et al.*, 1958; Yokogawa *et al.*, 1962), gel diffusion (Phillipson, *et al.*, 1962; Yogore *et al.*, 1965) intradermal test (Yokogawa and Awano, 1956), flocculation test (Takano, 1960) and immunoelectrophoresis (Yogore *et al.*, 1965; Tsuji *et al.*, 1967). This study was carried out to observe the humoral immune response of cats infected by *P. siamensis*, and assess the comparative sensitivity and specificity of the tests eg. the enzyme linked immunosorbent assay (ELISA), Complement fixation test (CFT) and the immunoelectrophoresis (IEP) test.

This work was supported in part by SEAMEO-TROPMED Project and in part by Faculty of Tropical Medicine, Mahidol University.

MATERIALS AND METHODS

Experimental animals : Twenty domestic cats weighing 2 to 4 kilograms, of both sex were divided into 2 groups; each group consisted of 10 cats. Cats in the first group (group A and B) were rendered worm free prior to infection and used for studying humoral immune response. Stool examination was done using formalin-ether concentration technique (Ritchie *et al.*, 1948). Those animals habouring parasites such as hookworm, *Toxocara cati*, and *Spirometra mansoni* were treated with ancylol (4.5 mg/kg body weight), piperazine (200 mg/kg body weight) and Yomesan (100 mg/kg body weight). Both group A and B were then infected with 30 and 60 metacercariae respectively. Cats in the second group without previous deworming were infected with *Paragonimus* in order to obtain adult worms for the preparation of the antigens.

Briefly, five cats in the first group (group A) were fed through a stomach tube orally each with 30 *P. siamensis* infective stage metacercariae obtained from the hearts and livers of the rice field crab intermediate host, *Parathelphusa germaini*, while the other 5 cats (group B) were given 60 metacercariae from the same source. In the second group, 9 cats were infected with 30-50 *P. siamensis* infective stage metacercariae, while one cat was

infected with 16 *P. heterotremus* infective stage metacercariae obtained from the leg muscles of the mountain crab intermediate host, *Tiwaripotamon beusekomae.*

Worm recovery : The animals were sacrificed when *Paragonimus* eggs appeared in the faeces usually 2 to 3 months after infection. The adult worms were recovered from the lung cyst and pleural cavity. The worms were washed several times with normal saline and finally suspended in distilled water. They were then lyophilized and kept at -60°C until extraction.

Paragonimus antigens : Two crude soluble antigens (Chaffee-type antigen) were prepared by delipidatio prior to extraction of *P. siamensis* and *P. heterotremus* adult worms with veronal bicarbonate buffered salt solution according to the method of Chaffee *et al.*, (1954). The protein content was assayed by the method of Lowry *et al.*, (1951).

Sera obtained from the experimental cats were used for the serological tests:-

10 sera from the first group (A & B) prior to infection;

40 sera from the first group (A & B) at 2, 4, 8 and 12 weeks after infecting with 30 and 60 *P. siamensis* metacercariae;

Sera from 5 cats positive for hookworm, 2 *Toxocara cati*, 1 hookworm and *Spirometra mansoni*, 1 hookworm and *Toxocara cati*, 1 *Spirometra mansoni;*

2 sera from cats of the second group, one positive for *P. heterotremus* at 5 months while another positive for *P. siamensis* at 4 months post infection.

Enzyme-conjugated anti-immunoglobulin: Rabbit anti-cat IgG serum was obtained by immunizing 2 rabbits with cat IgG. The IgG fraction of rabbit anti-cat IgG serum was isolated by means of ion-exchange column chromatography on diethylaminoethyl cellulose (DE-52) in 0.01 M phosphate buffer, pH 8.0. Cat IgG was purified by the above method using normal cat serum. The IgG fraction of rabbit anti-cat IgG (4.125 mg) was conjugated with 10 mg of peroxidase enzyme (Horseradish, type VI) by the method described by Avrameas *et al.*, (1978).

Three serological tests used were the enzyme linked immunosorbent assay (ELISA), the complement fixation test (CFT) and the immunoelectrophoresis (IEP) test.

The ELISA was performed as described by Ambroise-Thomas and Desgeorges (1978). Prior titration showed that the protein antigen concentration optimum for plate sensitization was 50 μg per ml. The peroxidase-labelled IgG fraction of rabbit anti-cat IgG (H+L) was used at a dilution of 1: 200. All sera were tested singly. The absorbance was measured at 492 nm in an ELISA Reader (Dynatech). The dilution which gave reading higher than 0.04 was considered as an end-point.

The CFT was carried out according to a microtechnique adapted from the LBCF method of complement fixation test (Thompson *et al.*, 1968). Prior titrations showed that the optimal dilutions of the antigen was 0.5 mg/ml. The well which gave reading of 30% hemolysis was considered as an end-point.

The immunoelectrophoresis (IEP) test was performed as previously described (Scheidegger, 1955). The concentration of antigen used was 20 mg per ml.

RESULTS

The number of adult worms recovered from lungs and pleural cavities of ten experimental cats 13 weeks after infection with *P. siamensis* is shown in Table 1. Only 7-14 worm (mean= 10) were recovered from cats infected with 60 metacercariae, whereas 2-27 worms (mean= 14) were recovered from those infected with 30 metacercariae. The percentage worm recovery of group A and group B ranged from 6.8 to 90.0 and 11.7 to 23.3 respectively.

Table 1

Worms recovery from Group 1 (A and B) cats experimentally infected with 30 and 60 *P. siamensis* metacercariae respectively.

Group 1	Worms recovered from		Total (%)
	Lungs	Pleural Cavities	
A	13	3	16 (53.3)
	26	1	27 (90.0)
	16	3	19 (63.3)
	1	1	2 (6.8)
	7	1	8 (26.7)
B	8	2	10 (16.7)
	7	1	8 (13.3)
	6	2	8 (13.3)
	13	1	14 (23.3)
	2	5	7 (11.7)

There was no correlation between the numbers of worm recovered and the number of metacercariae infected ($p > 0.05$).

Geometric mean values of ELISA and CFT titers from cats of group A and group B and geometric mean values and 95% confidence intervals of the combined group throughout the 12 week period of observation are shown in Fig. 1 and Fig. 2. There was variability in titers among cats infected with equal numbers of metacercariae. However, there was no significant difference in both ELISA and CF titers between the two groups. With both tests, antibodies could be detected as early as 2nd week after infection and the cats remained positive throughout the 12 week period of observation. In contrast, IEP

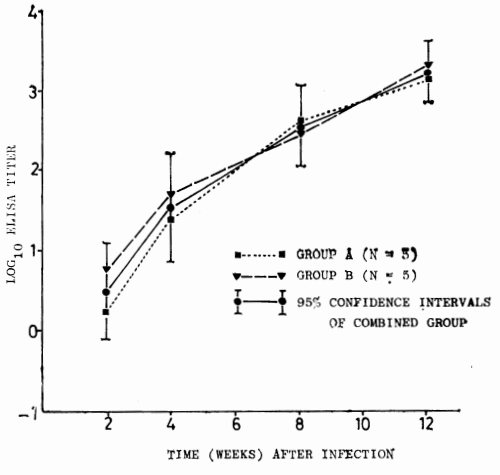

Fig. 1—Geometric mean values of ELISA titers from cats infected experimentally with 30 (Group A) and 60 (Group B) metacercariae of *P. siamensis* and geometric mean values and 95% confidence intervals of the combined group.

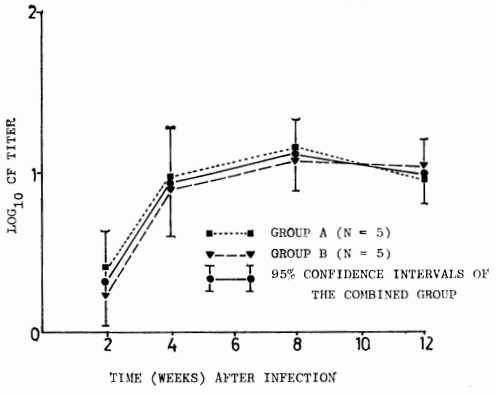

Fig. 2—Geometric mean values of CF titers from cats infected experimentally with 30 (Group A) and 60 (Group B) metacercariae of *P. siamensis* and geometric mean values and 95% confidence intervals of the combined group.

Table 2

Comparison of results obtained by ELISA and CFT in ten cats infected with *P, siamensis* metacercariae.

Duration of infection	ELISA	CFT	Number
2nd week	+	+	3
	−	−	5
	+	−	0
	−	+	2
4th week	+	+	8
	−	−	0
	+	−	0
	−	+	2
8th week	+	+	10
12th week	+	+	10

test was persistently negative. With ELISA, the mean values and 95% confidence interval of the the combined group were consistently increasing at each subsequent time up to 12 week period of observation ($p < 0.01$). The pattern of rising in titer of CFT was different from that of ELISA in that a significant rise was observed only from 2nd week to 4th after infection ($p < 0.01$) and then reached the plateau. There was no correlation between the infective dose and the antibody response. After drug treatment and before experimental infection, all cats were negative by both ELISA and CF test.

Sensitivity of the two tests were compared (Table 2). Three (30%) and 5 (50%) gave positive results in ELISA and CFT respectively at 2nd week after infection. Eight (80%) and 10 (100%) were positive in ELISA and CFT at 4th week. These differences were not statistically significant indicating that both are equally sensitive ($p > 0.05$). At 8th and 12th week, all ten cats (100%) showed positive with both tests.

Fig. 3 shows comparison of ELISA and CF titers. There was no significant difference

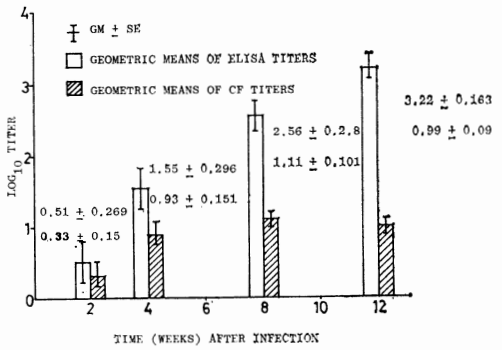

Fig. 3—Comparison of ELISA and CF titers showing the geometric mean and standard error.

between the titers of the two tests at 2nd week after infection ($p > 0.05$). However, at 4th, 8th and 12th week after infection, ELISA titers were significantly higher than those of CFT ($p < 0.05$).

The specificity of ELISA and CFT was further evaluated by testing 10 sera from cats naturally infected with hookworm, *Toxocara cati*, *Spirometra mansoni* or mixed infected with *Spirometra mansoni* and hookworm, *Toxocara cati* and hookworm, and one serum from cat infected with *P. heterotre-*

Table 3

Specificity of ELISA and CFT.

Stool Examination (FECT)*	No. of sera tested	Antigen	Titer	
			ELISA	CFT
P. siamensis	1**	*P. siamensis*	5120	8
		P. heterotremus	2560	4
P. heterotremus	1+	*P. siamensis*	5120	0
		P. heterotremus	5120	0
Parasite free	10	*P. siamensis*	0	0
Hookworm	5	*P. siamensis*	0	0
Toxocara cati	2	*P. siamensis*	0	0
Spirometra mansoni	1	*P. siamensis*	0	0
Spirometra mansoni and hookworm	1	*P. siamensis*	0	0
Toxocara cati and hookworm	1	*P. siamensis*	0	0

* Formalin ether-concentration technique.

** Serum obtained from one cat 4 months after infection with *P. siamensis* metacercariae.

+ Serum obtained from one cat 5 months after infection with *P. heterotremus* metacercariae.

mus. The results are shown in Table 3. Normal control sera and sera from cats naturally infected with parasites other than *Paragonimus* gave no cross reaction against *P. siamensis* antigen with both tests. However, serum from one cat at 4 months after infection with *P. siamensis* and from another cat at 5 months after infection with *P. heterotremus* were positive against homologous and heterologous antigens with both tests in the former and only ELISA in the latter. These indicated that both ELISA and CFT could not be used for species differentiation between *P. siamensis* and *P. heterotremus* infection.

DISCUSSION

This present study showed that cats infected with different infective dose of *P. siamensis* metacercariae showed recovery rate of adult worms incommensurate with the infective dose, since only 2-27 worms (mean = 14) were recovered in cats infected with 30 metacercariae, whereas 7-14 worms (mean = 10) were recovered from those infected with 60 metacercariae. No explanation could be accounted for the relatively less worm recovery with the higher infective dose. Similar observation was made by Yogore *et al.*, (1965) who recovered 18 and 21 worms from 2 cats infected with 25 metacercariae of *P. westermani* and only 2 worms from one cat infected with 50 metacercariae.

The antibody response could be detected only by ELISA and CFT, but not by IEP test. Failure to detect precipitating antibody by the IEP test in this study was not known. It is possible that the level of circulating precipitating antibody is below the sensitivity of the test. Attempt to increase its sensitivity by applying secondary antibody according to the technique of Darcy (1972) was not successful. In contrast, Yogore *et al.*, (1965) showed that sera from cats infected with *P. westermani* produced as many as 2-5

precipitating bands upon testing with homologous antigen in the gel diffusion and IEP tests. The IEP test has been successfully used for species differentiation between *P. westermani*, *P. ohirai* and *P. miyazaki* (Tsuji *et al.*, 1967), and in the diagnosis of human paragonimiasis (Tsuji and Yokogawa, 1975).

The ELISA and CF antibody response was related to the duration of infection (Figs. 1, 2). The only difference was that ELISA titer was raised at each subsequent time of observation for 12 weeks whereas CF titer was raised significantly up to 4 weeks to reach a plateau at 8-12 weeks after infection.

Comparison between the sensitivity of ELISA and CFT showed that both tests were equally sensitive in the detection of the antibody response at various times after infection, but significantly higher antibody titer was obtained with ELISA at 4-12 weeks after infection. ELISA would therefore be preferable to CFT for use in the diagnosis of *Paragonimus* infection. As regards the specificity, both ELISA and CFT were fairly specific for paragonimiasis, since the tests were negative in the cats prior to challenge with *Paragonimus* metacercariae eventhough they were infected by various parasites including hookworm, *Toxocara cati*, and *Spirometra mansoni*. However, the test could not be used for differential diagnosis of infection by different species of *Paragonimus* as evident by the cross reaction between sera from cat infected with *P. heterotremus* and the antigen from *P. siamensis* and vice versa. It would be of interest to extend this study to other genus of trematode infection in order to determine the magnitude of cross reaction.

It was apparent from this study that serum from one cat infected with 16 metacercariae of *P. heterotremus* was negative by CFT but positive by ELISA at 5 months after infection. This finding suggested that the infection was by *P. heterotremus* but not by *P. siamensis* in this cat, ELISA was more sensitive than CFT. To derive at a more definite conclusion, further studies are needed. Difficulty in obtaining metacercariae of *P. heterotremus* was a major obstacle in substantiating this chance observation.

SUMMARY

The time course of humoral immune response to *Paragonimus siamensis* was studied in 10 cats experimentally infected with either 30 or 60 matacercariae and the antibody produced was detected by enzyme-linked immunosorbent assay (ELISA), complement fixation test (CFT), and immunoelectrophoresis (IEP) test. With ELISA and CFT, antibodies was detected as early as 2nd week after infection and the cats remained positive throughout the 12 week period of observation. In contrast, the IEP test was persistently negative. With respect to sensitivity, both ELISA and CFT are equally sensitive but the mean ELISA titer was consistently higher than that of CFT. The magnitude of the antibody response appeared to be related to duration of the infection but not to the infective dose and the number of worms recovered. There was variability in titers among cats infected with equal numbers of metacercariae. The tests can not be used for differential diagnosis of infections by *P. siamensis* and *P. heterotremus* because of the cross-reaction. Such cross-reaction did not not occur against unrelated parasites including hookworm, *Toxocara cati* and *Spirometra mansoni*.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Malinee Anantaphruti for the animals and to Mrs. Surang Tantivanich who helped in developing the enzymatic technique.

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