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TWO-YEAR FOLLOW UP FROM BIRTH OF THAI CHILDREN FOR ROTAVIRUS INFECTION: SEROTYPES AND RNA ELECTROPHEROTYPES

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Abstract: Twenty-two group A rotavirus positive stool specimens that had been collected from 19 children in Chiang Mai, Thailand, followed up for two years from birth in 1988 to 1990 (Supawadee *et al.*, 1995), were examined for subgrouping (I and II), and VP7 serotyping (1, 2, 3 and 4) by enzyme-linked immunosorbent assay (ELISA) with specific monoclonal antibodies and RNA electropherotyping by polyacrylamide gel electrophoresis (PAGE). Of these, 18 (82%) could be subgrouped (10 subgroup I and eight subgroup II) and serotyped (five serotype 1, 10 serotype 2, one serotype 3 and two serotype 4). Serotype 1 virus was more predominant than type 2 during the period from January to June 1989 and type 2 was more predominant than type 1 from August 1989 to June 1990. Reinfection with rotavirus was observed in four children during the two years of the follow-up period after birth: one volunteer (No. 11) had an asymptomatic infection with serotype 2-RNA electropherotype S and a symptomatic infection with serotype 3-RNA electropherotype L. Each of the other three had asymptomatic infections twice, i.e.: one (No. 21) with serotype 1-RNA electropherotype L and undetermined serotype-RNA electropherotype L; one (No. 38) with serotype 2-RNA electropherotype S and undetermined serotype-RNA electroherotype L; and the other one (No. 23) with undetermined serotype-RNA electropherotype L and serotype 2-RNA electropherotype S. The results of 18 specimens from asymptomatic infections were compared with those of 202 specimens from symptomatic infections in the same geographical area in the same period of time and no definite relation was found between the VP7 serotype and severity of the illness.

INTRODUCTION

Group A rotavirus (RV) is known to be a common cause of severe diarrhea that occurs among children world wide. The rotavirus particle consists of outer and inner capsid layers and 11 viral RNA segments enclosed within them. The outer capsid consists of two structural proteins, VP4 and VP7, which are independent neutralization antigens and the inner capsid consists of VP6 which is a subgroup specific antigen. By these antigens, two subgroups, seven VP7 serotypes and three VP4 serotypes of human rotavirus (HRV) have so far been distinguished. Apart from the antigenic analysis, polyacrylamide gel electrophoresis of genomic RNA distinguishes long (L) and short (S) RNA electropherotypes of RV (Kapikian and Chanock, 1990). The prevalence of particular antigenic types and RNA electropherotypes of HRV in various parts of the world seems important not only for understanding the pathology and epidemiology of these infections but also for evaluating the efficacy of candidate rotavirus vaccines.

Our previous report (Supawadee *et al.*, 1995) described group A RV infection of children in Chiang Mai followed up for two years after birth, from October 1988 to November 1990. Among the 28 volunteer children, asymptomatic infection was observed in 15 (54%) and symptomatic infection in four (14%). We analyzed 202 group A RV-positive specimens collected from diarrheic children admitted to three hospitals in Chiang Mai during the period December 1988 to June 1990 (Urasawa et al., 1992 and unpublished data). In this report, 22 group A RV-positive specimens from the 19 volunteer children in the follow-up study in Chiang Mai were examined, and antigenic types of the virus from

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asyptomatic and symptomatic infections were also compared.

MATERIALS AND METHODS

Virus specimens

Stool specimens were obtained from 28 volunteers born in October and November 1988 in Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University. Specimens were collected every week during the follow-up period of two years after birth and were screened for the presence of group A RV as described in the previous report (Supawadee *et al.*, 1995).

Subgrouping and serotyping of human rotavirus

Direct subgrouping (I and II) and serotyping (VP7 1, 2, 3 and 4) of virus in feces were carried out by ELISA with monoclonal antibodies developed by Akatani and Ikegami (1987). Microplate wells were coated with dilutions of mouse ascites containing innercapsid and outercapsid-common monoclonal antibodies. Five to 10 per cent suspensions of fecal specimens were delivered to each well. After incubation at 4 °C overnight and washing, biotinylated subgroup- and type-specific monoclonal antibodies followed by streptavidin conjugated with horse radish peroxidase were given as detection reagents. OD was measured at 492 nm.

RNA electrophoresis

The method used to determine the RNA-pattern of RV genome by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was a minor modification of that described by Herring *et al.* (1982) as described previously (Hasegawa *et al.*, 1987). Fecal suspension diluted in Tris buffer containing EDTA, SDS and 2-mercaptoethanol was extracted with phenol and chloroform, and the supernatant was used as an RNA sample. The extracted RNA was analysed by SDS-PAGE following the method of Rodger *et al.* (1979); 10% polyacrylamide gels (0.75 mm thick) were used with the discontinuous buffer system as described by Laemli (1970). Electrophoresis was carried out at 20 mA at room temperature for four hours. After eletrophoresis, the gels were stained with silver nitrate.

RESULTS

Subgroups and serotypes

Subgroups and VP7 serotypes identified with ELISA by using monoclonal antibodies are shown in Table 1 and 2. Eighteen out of 22 specimens were identified as subgroup I or II and VP7 serotype 1, 2, 3 or 4. Specimens with subgroup II-serotype 1 specificity amounted to 4/6 (67%), those with subgroup I-serotype 2 specificity to 2/6 (33%) and neither serotype 3 nor 4 was found during the period from January to April 1989 (Table 1). In contrast, during the period from August 1989 to June 1990, subgroup I-serotype 2 viruses were found in 8/17 (47%), accompanied by subgroup II-serotype 1 (1/17, 5.9%), subgroup II-serotype 3 (1/17, 5.9%) and subgroup II-serotype 4 (2/17, 12%) (Table 2). Five specimens (29%) could not be assigned to either subgroup (I and II) or any serotype (1, 2, 3 and 4).

Serotypes in asymptomatic and symptomatic infection

Table 3 shows the distribution of subgroups and serotypes in asymptomatic and symptomatic infections in the same geographic area of Chiang Mai during the same period of time. Cases of symptomatic infection include volunteers No. 6, 7, 8 and 11 examined in this report and 198 hospitalized cases examined previously (Urasawa *et al.*, 1992 and unpublished data). Between December 1988 and June 1989, serotype 1 was predominant in 4/6 (67%) of asymptomatic and in 76/145 (52%)

Table 1 Subgroups, VP7 serotypes and RNA electropherotypes of rotaviruses in stool specimens collected between January 1989 and April 1989

Volunteer No.	Detection of virus		Symptom	Virus		
	Date	Age of weeks		Subgroup	Serotype	RNA
14	Jan. '89	11	−	II	1	L
34	Feb. '89	14	±	II	1	L
21	Feb. '89	15	−	II	1	L
11	Feb. '89	19	−	I	2	S
40	Apr. '89	18	±	II	1	L
38	Apr. '89	20	−	I	2	S

−, no diarrheic symptom. ±, diarhea at the successive week of the detection of rotavirus antigens. L, long. S, short.

Table 2 Subgroups, VP7 serotypes and RNA electropherotypes of rotaviruses in stool specimens collected between August 1989 and June 1990

Volunteer No.	Detection of virus		Symptom	Virus		
	Date	Age of weeks		Subgroup	Serotype	RNA
24	Aug. '89	40	±	I	2	S
16	Sep. '89	44	−	I	2	S
38	Sep. '89	44	−	NC	NC	L
5	Dec. '89	60	−	NC	NC	L
11	Jan. '90	64	++	II	3	L
35	Feb. '90	63	−	I	2	L
23	Feb. '90	66	−	NC	NC	L
21	Feb. '90	67	−	NC	NC	L
8	Feb. '90	69	+++	I	2	S
10	Feb. '90	70	−	I	2	S
7	Feb. '90	72	+	II	4	L
1	Feb. '90	74	−	II	1	L
39	Mar. '90	67	−	I	2	S
25	Mar. '90	70	−	NC	NC	S
23	Mar. '90	72	−	I	2	S
6	Apr. '90	80	++	I	2	S
2	June '90	90	±	II	4	L

−, no symptom. ±, diarrhea at the succesive week of the rotavirus-detection. +, mild diarrhea. ++, moderate diarrhea. +++, hospitalized with severe diarrhea. NC, not clear. L, long. S, short.

Table 3 Distribution of serotypes and subgroupa of human rotavirus in stools collected in Chiang Mai from December 1988 to June 1990

Period	Virus		Infection	
	Subgroup	Serotype	Asymptomatic	Symptomatic*
Dec. '88～ June '89	II	1	4 (67%)	29 (51%)
	NC	1	0	1 (1.8%)
	I	2	2 (3.3%)	6 (11%)
	II	4	0	3 (5.3%)
	II	NC	0	3 (5.3%)
	I	NC	0	8 (14%)
	NC	NC	0	7 (12%)
	Total		6 (10%)	57 (100%)
Aug. '89～ June '90	II	1	1 (7.7%)	16 (11%)
	I	1	0	1 (0.7%)
	NC	1	0	8 (5.5%)
	I	2	6 (46%)	76 (52%)
	II	3	0	3 (2.1%)
	II	4	1 (7.7%)	3 (2.1%)
	NC	4	0	1 (0.7%)
	II	NC	0	9 (6.2%)
	I	NC	0	18 (12%)
	NC	NC	5 (38%)	10 (6.9%)
	Total		13 (100%)	145 (100%)

*No. 11, 8, 7 and 6 and 198 cases reported by Urasawe *et al.* (1992)

of symptomatic infections. Between August 1989 and June 1990, type 2 was predominant in 6/13 (46%) of aymptomatic and in 76/145 (52%) of symptomatic infections. Thus, no significant difference was found between asymptomatic and symptomatic infections on the predominant serotype (1 or 2) in either period; i.e., $p>0.1$ during the period from December 1988 to June 1989 and $p>0.95$ during the period from August 1989 to June 1990 by the x^2 test.

RNA electropherotype

Viral RNAs from group A rotavirus-positive stool specimens were examined in PAGE and classified into two groups: long (L) electropherotype, in which RNA segment 11 migrates rapidly, and short (S) electropherotype, in which the same segment migrates slowly. Among the 18 samples whose subgroup and VP7 serotype was known, nine strains of S type were identified as subgroup I-serotype 2 and eight strains of L type were subgroup II-serotype 1, 3 or 4, but a strain from volunteer No. 35 with asymptomatic infection was subgroup I-serotype 2 possessing a long RNA electropherotype. Fig 1 shows RNA-PAGE of RV from three volunteers.

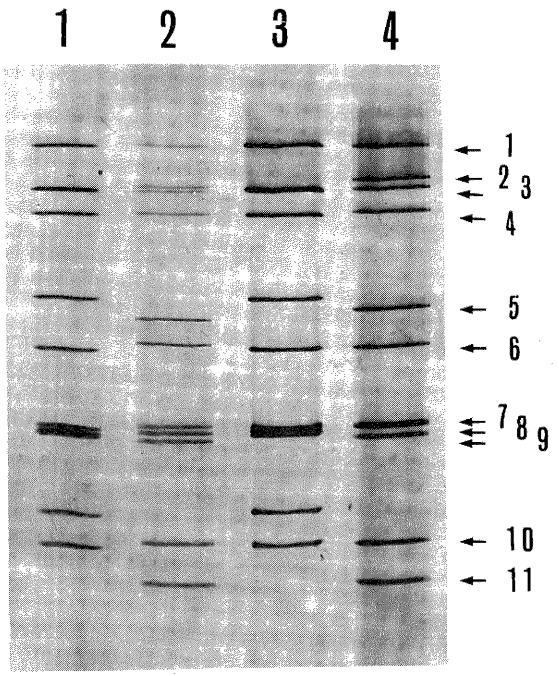

Figure 1 Electrophoretic migration patterns of double-stranded RNAs from stool specimens of volunteers No. 24 (1), 34 (2) and 39 (3). RNA pattern of (4) is a control representing the long pattern.

Reinfection

Four volunteers excreted RV antigens in their fecal specimens twice during the two years after birth as shown in Tables 1 and 2. No. 21 experienced asymptomatic infections twice with subgroup II-serotype 1-long RNA in February 1989 and with an unidentified subgroup and serotype-long RNA of HRV in February 1990; No. 11 suffered an asymptomatic infection with subgroup I-serotype 2-short RNA pattern in February 1989 and a symptomatic infection with subgroup II-serotype 3-long RNA pattern in January 1990; No. 38 had asymptomatic infections twice with subgroup I-serotype 2-short RNA pattern in April 1989 and with an unidentified subgroup and serotype-long RNA pattern in September 1989; No. 23 had asymptomatic infections twice with an unidentified subgroup and serotype-long RNA pattern in February 1990 and with subgroup I-serotype 2-short RNA pattern in March 1990.

DISCUSSION

Twenty-three HRV-positive stool specimens collected during a two-year follow-up study of 28 Thai children in Chiang Mai (Supawadee et al., 1995) were analyzed for subgrouping (I and II), serotyping (VP7 1, 2, 3 and 4) and RNA electropherotyping. Among the 23, six specimens were obtained from children aged from 11 to 20 weeks between January and April 1989 (1st period), and 17 were aged from 40 to 90 weeks between August 1989 and June 1990 (2nd period). All the volunteer children were born in October and November 1988, and they should have had fewer contacts with the virus and higher levels of maternal immunity against the virus in the 1st period than in the 2nd period. From many surveillance reports of rotavirus infection in various countries (Birch *et al.*, 1988; Flores *et al.*, 1988; George-Courbot *et al.*, 1988; Nakagomi *et al.*, 1988; Akatani *et al.*, 1989; Bishop *et al.*, 1989; Pongsuwanne *et al.*, 1989; Unicomb *et al.*, 1989; Urasawa *et al.*, 1989; Gouvea *et al.*, 1990; Kawamoto *et al.*, 1990; Matson *et al.*, 1990; Nakagomi *et al.*, 1990; Sethabutr *et al.*, 1990; Pipittajan *et al.*, 1991; White *et al.*, 1991; Urasawa *et al.*, 1992; Woods *et al.*, 1992; Ikegami and Akatani., 1993; Rassool *et al.*, 1993; Steele, *et al.*, 1995), it is considered that despite the overall predominance of serotype 1 virus, the frequency of individual serotypes varies considerably from place to place and year to year.

We had serotyped 198 group A rotavirus-positive fecal specimens collected from diarrheic children hospitalized in the same area of Chiang Mai from

December 1988 to June 1990 (Urasawa *et al.*, 1992 and unpublished data). In the present report 18 out of 23 rotavirus-positive specimens were collected from children with asymptomatic inapparent infections, and the serotypes of the asymptomatic and symptomatic infections were compared. As a result, no significant difference between asymptomatic and symptomatic infections was found on the predominant serotype (1 or 2) in two periods of December 1988 to June 1989 and of August 1989 to June 1990. This result indicates that viral factors alone are unlikely to account for attenuated infection. Previously, the P6 genotype (with M37-like VP4 allele) was reported to occur only in strains of the virus recovered from asymptomatically infected neonates (Hoshino, *et al.*, 1985; Flores *et al.*, 1988; Gorziglia *et al.*, 1988). However, strains carrying the P6 genotype were recovered from symptomatically infected neonates and older infants (Gerna *et al.*, 1990; Steele *et al.*, 1993 and 1995).

Urasawa *et al.* (1992) isolated three unusual HRV strains (Mc35, Mc323 and Mc345) by using primary African green monkey kidney cells from stool specimens collected in Chiang Mai during the period from 1987 to 1989, assigned them to VP7 serotype 9 and 10 and stated that they are closely related to bovine and porcine RVs. They also studied the VP4 gene of strain Mc35 and presumed that it represents subtypes of type 3 human rotavus Vp4 type 3B (Urasawa *et al.*, 1993). Four specimens (No. 11, 21, 23 and 25) untypable in this report could contain unusual RVs, but only direct ELISA for VP7 serotypes 1 to 4 was done this time.

It has been demonstrated that there is usually a definite relationship between the subgroup and serotype specificities of HRVs and the pattern of segmented RNA of the virus in PAGE: RVs with subgroup I specificity usually have serotype 2 or 8 specificity and a "short" or "supershort" RNA pattern, whereas RVs with subgroup II specificity generally have serotype 1, 3, 4 or 9 antigen and a "long" RNA electropherotype (Kapikian and Chanock, 1990). This was reconfirmed in this study, except in one strain from volunteer No. 35 with asymptomatic infection, which contained RV with subgroup I-serotype 2 antigen and a long RNA pattern which deviated from the correlation. Similar strains have been reported as Mc129 in Chiang Mai previously (Urasawa *et al.*, 1992) and some strains with antigenic and genetic properties different from the usual ones have been discovered among HRV isolates (Kobayashi *et al.*, 1989; Nakagomi *et al.*, 1987).

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A SEROLOGICAL STUDY OF DENGUE VIRUS INFECTION AMONG JAPANESE RESIDENTS IN MANILA.

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Abstract: A total of 84 serum specimens were collected from 67 Japanese residents in Manila, the Philippines, at a clinic during the periods from September 1992 through February 1993 and November 1993 through March 1994, during which time dengue viruses were actively circulating, and tested for antibodies to dengue (DEN) viruses. Fifteen (22.4%) individuals were confirmed as recent DEN infection by either the detection of anti-DEN IgM or a four-fold or more rise in neutralizing antibody titer in acute-convalescent paired sera. Major serotypes of current DEN epidemic appeared to be DEN type 1 and type 2 viruses. There was a case in which all three members of a family had been infected with DEN simultaneously, and their symptoms varied from DEN fever to DEN hemorrhagic fever.

INTRODUCTION

With a rapid increase in the number of Japanese people who visit tropical areas, a problem of contracting tropical diseases is becoming remarkably increased. Dengue (DEN)/dengue hemorrhagic fever (DHF) is one of the serious problems in various tropical areas. The Philippines has been a dengue epidemic area for years. Mean incidence rate of DEN infection in all ages among Filipinos is 3.0 per 100,000 population, with the highest being 10.5 in the children under one year old (Okabe, 1993). Manila area has shown the highest incidence in the country (Piad, 1994). There are many Japanese people living in and around Manila. However, the situation of DEN infection among these Japanese residents was not clear. We, therefore, became interested to find out the incidence rate among the Japanese living in Manila. During or at the end of DEN epidemic season from 1992 through 1994, we collected sera from the Japanese residents and tested for antibodies to DEN in order to give appropriate guide for preventive and therapeutic measures. This paper describes the results of the serological test.

MATERIALS AND METHODS

Serum specimens:

A total of 84 serum specimens were collected from 67 Japanese living in Manila, at the Manila Japanese Club Clinic during the periods from September 1992 through February 1993, and November 1993 through March 1994, during which time, DEN viruses were actively circulating. Among them, 10 came to the clinic with DEN-like symptoms, while other 57 came for a routine medical checkup or with some ailments or complaints, not available to us right now (non-DEN). The information on the individual vaccination history and early medical history were obtained through questionnaire from 37 individuals.

Serology:

IgM-class antibody to DEN virus was assayed by IgM-capture ELISA (Bundo and Igarashi, 1985). A mixture of DEN-1, 2, 3 and 4-infected cell culture fluid was used as antigen. Anti-dengue IgG was purified from a pool of dengue patient sera and conjugated with horseradish peroxidase (Wilson and Nakane, 1978). Positive ELISA reaction at a serum dilution of 1: 100 was considered IgM-positive.

Neutralizing antibody was assayed by 50 percent

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Table 1 Antibody-positive rate to dengue virus among Japanese residents

Clinical symptom*	No. patients	No. patients positive by	
		IgM-ELISA	NT
Dengue*	10	6(60.0)†	6(60.0)
Non-dengue*	57	8(14.0)	9(15.8)
Total	67	14(20.9)	15(22.4)

* Details are described in text.
† Number in parenthesis indicates percent.

focus-reduction neutralization (N) test in BHK21 cells with the use of peroxidase-anti-peroxidase (PAP) staining method (Okuno *et al.*, 1985). N titers over 10 were considered positive. Individuals showing four-fold or greater rise in titer in acute-convalescent paired sera were considered as recent infection.

Results and Discussion

In order to know the incidence of DEN infection among Japanese residents in Manila, sera were collected from Japanese residents who came to the clinic during DEN epidemic season and tested for the antibodies to DEN viruses. Fourteen (20.9%) individuals were positive for anti-DEN IgM (Table 1). Among them, eight were from non-DEN individuals. In adults, since the ratio of clinical disease to dengue infection is thought to be nearly one (Halstead, 1993), these non-DEN individuals might have had some symptoms of DEN infection, such as undifferentiated fever and non-specific constitutional symptoms. Unfortunately, the detailed clinical data were not available. Fifteen (22.4%) individuals were positive for DEN N antibody. Among them, eight had anti-DEN IgM. Monotypic DEN N antibody was detected in 8 individuals suggesting that they might have had primary DEN infection (W.H.O., 1986). Major monotypic antibodies were those to DEN-1 and DEN-2 viruses. Seven individuals possessed multiple serotypic N antibodies. Thus, 15 (22.4%) of 67 individuals were confirmed to have had recent DEN infection by either DEN-IgM ELISA or N test.

A brief history and serological information of the 10

Table 2 Serological data on patients with dengue-like symptom

Serum code	Age (Year)	Sex	Residence in the Philippines*	Days after onset	Anti-DEN IgM	N titers to				
						DEN1	DEN2	DEN3	DEN4	JEV
1A	34	F	6/12	3	+	<10	<10	<10	<10	520
1C1				23	+	390	110	50	35	560
1C2				82	+	330	64	17	18	660
2A	5	F	6/12	4	+	12	<10	<10	<10	<10
3A	37	M	6/12	3	+	<10	<10	<10	<10	12
3C				77	+	200	12	<10	<10	13
4A	27	M	2	4	−	<10	<10	<10	<10	60
4C				56	−	130	70	35	33	56
5A	31	F	2	5	−	<10	<10	<10	<10	800
5C				8	+	180	<10	<10	<10	700
6A	13	F	3/12	6	+	105	410	<10	<10	2600
7A	39	M	3	1	+	<10	<10	<10	<10	25
8A	6	F	4/12	5	−	<10	<10	<10	<10	38
8C				34	−	<10	<10	<10	<10	78
9A	9	M	4/12	5	−	<10	<10	<10	<10	1350
10A	36	F	4/12	5	−	<10	<10	<10	<10	39

*Duration in years of staying in the Philippines. 1, 2 and 3; and 8, 9 and 10 are two families.

DEN-like patients are shown in Table 2. Patients # 1, # 2, and # 3 were the members of a family (parents and a daughter). They appeared to have been infected with DEN (possibly DEN-1) at the same time. They developed the symptoms of DEN such as high fever, positive Tourniquet test, subcutaneous or gingival bleeding, and thrombocytopenia. The parents developed more severe symptoms than their child. Mother (# 1) showed DHF symptom and developed alopecia soon after the acute phase was subsided. It took her about one and a half years for a complete recovery. Father (# 3) had altered taste sensation when a wide area of his oral mucosa had come off on the fifth day, while their child (# 2) showed the symptoms of DEN fever. In the paired sera from patient # 4, anti-DEN IgM was not detected on the 4th and 56th day, although anti-DEN IgG rose significantly. Poor response of IgM antibody has also been observed, especially in the secondary infection (Ruechusatsawat *et al* ., 1994). Patient # 7 went back to Japan soon after taking the first blood sample on the first day of illness, which showed positive for anti-DEN IgM, but not for anti-DEN N antibody.

It should be noted that patients # 8, # 9, and # 10 were the members of a family (mother and two children) and they simultaneously developed DEN-like symptoms, such as high fever, pain behind the eyes, joint pain, sore throat and lymphadenopathy. However, DEN antibody was not detected. The patient # 9 (9 year old boy) had relatively high Japanese encephalitis virus (JEV) N antibody (N titer of 1350). This child had a history of receiving JEV vaccination twice. Since chikungunya (CHIK) virus, which is also transmitted by the same vector *Ae. aegypti,* induces DEN-like symptom, we screened N antibody to CHIK virus as well. Although, one CHIK antibody-positive case was detected, this family turned out to be negative. Further study is being undertaken.

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GROWTH OF MALAYSIAN *PARAGONIMUS WESTERMANI* IN MAMMALS AND THE MODE OF TRANSMISSION OF THE FLUKE AMONG MAMMALS

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ABSTRACT: The host susceptibility of Malaysian *Paragonimus westermani* was observed in cats, dogs and rats, infected with metacercariae. In rats, worms were harboured in the muscles and the flukes were morphologically similar to the excysted metacercariae except for their slightly larger body size. In cats, about a half number of the flukes were recovered from muscles and 40% from cysts found in the lungs. Majority of the flukes from these cysts were mature. In dogs, few mature flukes were obtained from cysts in the lungs and about 90% of flukes were recovered from muscles. The flukes from the muscles of cats and dogs were of the same juvenile stage as those from rats. The juvenile flukes recovered from muscles of the animals were orally given to dogs and cats. Majority of the flukes were found in cysts in the lungs and most of them were fully mature. However, some juvenile flukes still remained in the muscles of cats and dogs. Therefore, the Malaysian *P. westermani* has a higher final host susceptibility than those from other localities. The present study suggests that many species of animals living in Malaysian jungle serve as paratenic hosts and may play an important role in the completion of the life cycle of *P. westermani* in Malaysia.

INTRODUCTION

Japanese *Paragonimus westermani* (Kerbert, 1878) usually remains in the muscles of rat, mouse, rabbit, pig and wild boar and for long period without development, and such animals could play a role as paratenic hosts of the fluke (Habe, 1978, 1983; Miyazaki and Habe, 1976; Shibahara and Nishida, 1986). Ingestion of raw wild boar meat is an important source of human infection of *P. westermani* in Japan (Miyazaki *et al.*, 1978; Norimatu *et al.* 1975; Tokudome *et al.*, 1977). In contrast *P. westermani* in Philippines does not remain in the muscles of mammals as a juvenile fluke (Miyazaki and Habe, 1979; Yokogawa *et al.*, 1979). The study of lung flukes in mammals provide knowledge on the life cycle and these information would be useful for prevention and control of paragonimiasis. In Malaysia, only one species of lung fluke, *P. westermani* has been recorded from various animals belonging to the family Felidae and from the crab-eating monkey, and a few experimental infections of Malaysian *P. westermani* in cats have been done only to get adult worms specifically for identification. The growth of Malaysian *P. westermani* was not clear in the mammalian hosts. The present paper reports the sites and development of Malaysian *P. westermani* in cat, dog and rat and discusses the role of mammals as paratenic hosts, refering to the presumptive life cycle of this fluke.

MATERIALS AND METHODS

1) Experimental Animals and *P. westermani* Metacercariae

Seven adult dogs and 11 cats were used for investigation of site and growth of Malaysian *P. westermani* in mammalian hosts. Before experimental infection, the dogs and cats were shown to be free from lung fluke and intestinal parasites by stool examination. Thirty-two female albino rats, *Rattus norvegicus*, SD strain about 9 weeks old were also used.

Malaysian *P. westermani* metacercariae were recovered from fresh water crabs, *Parathelphusa maculata* and

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Parathelphusa malaysiana which were collected from Kampong Langkap near Kuala Pilah and Sungai Wa at Taman Negara, Penisular Malaysia,respectively. They were inoculated into dogs, cats and rats. The metacercariae from *P. maculata* collected at Ulu Langat near Kuala Lumpur were inoculated into cats.

2) Inoculation of Metacercariae

The experimental animals were infected with 25 to 150 metacercariae (Tables 1-3). Five cats and a dog were orally infected using a pipette and 29 rats were also orally infected with metacercariae in a little water using a syringe connected to a slender tube inserted into the stomach. Other animals, 6 cats, 6 dogs and 3 rats, were injected with the metacercariae into the peritoneal cavity with a little Ringer's solution using a syringe connected to a slender vinyl tube.

3) Recovery of the Flukes

Dogs, cats and rats were bled to death under anesthesia on days 208 - 258, 155 - 249 and 10 - 150 after infections, respectively, and flukes were recovered. Postmortem examinations were carried out on each animal immediately. Gross lesions which might be attributable to parasitic invasion were recorded. Visceral organs and cavities were examined for lung fluke infection and worms were recovered. Subsequently, the lungs, liver and muscles of the whole body were cut into slices, 3 to 4 mm thick, and kept in Ringer's solution at 36 - 38 °C for 6 - 8 hours to release flukes from the tissues. The sediment was poured into a petri dish and examined for flukes under a stereomicroscope. Most of the juvenile flukes recovered were inoculated into dogs and cats again. The remaining worms were used for morphological observations; they were pressed between two slide glasses in 70% alcohol and mounted with Canada balsam after staining with carmine.

4) Transfer of Juvenile *P. westermani* to Cats and Dogs

Most of the juvenile worms recovered from the muscles of cats, dogs and rats were fed again to 5 cats and 2 dogs. Numbers and ages of the juvenile flukes and animals used are shown in Table 4. The animals were autopsied at 161-212 days after inoculation. The same procedures mentioned above for fluke recovery and examination were employed.

Table 1 Results of experimental infection of cats with Malaysian *Paragonimus westermani* metacercariae (Mc.)

Cat No.	Body weight at necropsy (kg)	Sex	No. of Mc. given	Method of infection	Autopsy days after infection	No. of worms recovered (%)	No. of worms recovered from			
							cyst in lungs	lungs	pleural cavity	muscles
1	2.0	F	30	oral infection	220	14 (46.7)	10*	0	3†	1‡
2	2.9	F	30	oral infection	229	3 (10.0)	2*	0	0	1‡
3	2.6	F	30	oral infection	234	5 (16.7)	4*	0	0	1‡
4	2.0	M	30	oral infection	242	4 (13.3)	2*	0	1†	1‡
5	2.7	M	30	oral infection	249	5 (16.7)	4*	0	0	1‡
6	2.8	F	50	peritoneal inoculation	155	26 (52.0)	4*	14†	0	8‡
7	3.7	M	50	peritoneal inoculation	192	32 (64.0)	18*	0	1†	13‡
8	3.2	F	50	peritoneal inoculation	192	37 (74.0)	16*	0	2†	19‡
9	2.9	M	40	peritoneal inoculation	208	26 (65.0)	14*	0	0	12‡
10	2.9	F	47	peritoneal inoculation	237	30 (63.8)	10($^{8*}_{2\dagger}$)	0	2†	18‡
11	3.2	M	30	peritoneal inoculation	242	25 (83.3)	12*	0	1†	12($^{1‡}_{11‡}$)

F: female, M: male, * Adult worm (with eggs in uterus), † Immature worm (without eggs in uterus),
‡ Juvenile worm (similar to excysted metacercariae),
Cat Nos. 2, 4, 5, 7 and 8 were infected with metacercariae from Kuala Pilah.
Cat Nos. 1, 3, 6 and 9 from Sungai Wa and Cat Nos. 10 and 11 from Ulu Langet.

RESULTS

Experimental Infection of Cats with Metacercariae
Distribution of flukes:

The results of the experimental infection in cats with the metacercariae are shown in Table 1. The average recovery rate of five cats (Nos. 1-5), orally infected with metacercariae, was 20.7% ranging from 10.0 to 46.7%. Out of 31 flukes recovered, 22 were found in cysts in the lungs, 5 in the muscles and 4 in the pleural cavity. A total of 176 worms was recovered from the cats (Nos. 6 - 11) inoculated with metacercariae intraperitoneally and the average recovery rate of worms was 57.0% (52.0 - 83.3%). Eighty-two worms were recovered in the muscles and 88 in lungs or cysts in the lungs and a small number of flukes were found in the pleural cavity.

Postmortem examinations:

On gross examination of the infected cats, in which 1 to 9 cyst formations were recognized in the pulmonary parencyma, membraneous rust coloured fibrinous exudate was present in the cavity, and adhesions between the lung lobes were present. In the cats, in which migrating flukes were found in the pleural cavity or lungs, collections of stagnant thoracic fluid were present and small number of haemorrhagic spots, 4 - 6 mm in diameter, were recognized in the lungs. In cat No.6, a large amount of stagnant thoracic fluid with blood cells was present, and about 30 haemorrhagic spots or nodules, 1-16 mm in diameter, were present in the lungs.

Development of the flukes:

In cat No. 6, autopsied 155 days after infection, fourteen flukes recovered from nodules of the lungs were immature, while four flukes from cysts in the lungs were mature (Table 1). The sizes of the mature flukes were variable, 5.8 - 7.4 mm long and 3.1 - 4.8mm wide, with a few eggs in the uteri. In cats autopsied 192 days later, all flukes recovered from cysts in the lung were fully mature. The sizes were 8.2 - 9.1 mm in length and 5.1 - 6.0 mm in width and the worms had numerous eggs in the uteri. Flukes recovered from muscles were morphologically similar to excysted metacercariae except for their slightly larger body size (0.7 - 1.4 mm in length and 0.4 - 0.7 mm in width). The worms had numerous granules in their excretory bladder. The flukes from the pleural cavity were immature and their sizes were about 3.5 mm in length and 2.1 - 3.2 mm in width. Their ovary and testes were divided into 5 or 6 lobes but vitelline glands were less developed. No remarkable differences were observed in the development of the flukes from the three different localities in Malaysia.

Experimental Infection of Dogs with Metacercariae
Distribution of flukes:

Table 2 shows the results of experimental infection of 7 dogs with metacercariae from Kuala Pilah and Sungai wa. In dog No.1, orally infected with 150 metacercariae and necropsied 234 days after infection, only 3 (2%) worms were recovered from muscles. The remaining six dogs were infected intraperitoneally with 80 or 100 metacercariae and were necropsied 208 to 258

Table 2 Results of experimental infection of dogs with Malaysian *Paragonimus westermani* metacercariae (Mc.)

Dog No.	Body weight at necropsy (kg)	Sex	No. of Mc. given	Method of infection	Autopsy days after infection	No. of worms recovered (%)	No. of worms recovered from				
							cyst in lungs	lungs	pleural cavity	peritoneal cavity	muscles
1	17.7	F	150	oral infection	234	3 (2.0)	0	0	0	0	3‡
2	14.7	F	80	peritoneal inoculation	208	52 (65.0)	2*	0	1†	0	49‡
3	12.0	F	100	peritoneal inoculation	227	29 (29.0)	0	2†	1†	0	26(3†, 23‡)
4	12.2	F	100	peritoneal inoculation	240	9 (9.0)	0	0	0	0	9‡
5	10.3	M	100	peritoneal inoculation	245	35 (35.0)	4(2*, 2†)	0	0	1†	30‡
6	11.2	M	80	peritoneal inoculation	252	23 (28.8)	0	0	2†	0	21‡
7	15.6	F	80	peritoneal inoculation	258	23 (28.8)	2(1*, 1†)	0	2†	0	19‡

F: female, M: male, *, †, ‡: Cf. the foot in Table 1,
Dog Nos. 1, 3 and 4 were infected with metacercariae from Kuala Pilah.
Dog Nos. 2, 5, 6 and 7 were infected with metacercariae from Sungai Wa.

days after inoculation. The average recovery rates of worms was 32.6% (9.0 - 65.0%) and a total of 171 worms were recovered. Majority of the flukes (154 out of 171; 90.1%) were recovered from muscles and 6 and 8 flukes were found in cavities and cysts in lungs, respectively.

Postmortem examinations:

On gross examination, no abnormality was observed in any organs of 2 infected dogs (Nos. 1 and 4), from which flukes were only recovered from muscles. In the other dogs, some haemorrhagic lesions, 1 - 10 mm in diameter, were present in the lungs and flukes were found in the pleural cavity or in the lungs. In these dogs, stagnant haemorrhagic exudates were present in the pleural cavity, and adhesions of the lungs to the thoracic wall and between lung lobes were also observed. One or 2 typical worm-cysts were present in the lungs of dogs Nos. 2, 5 and 7; and 2 flukes were found in each cyst.

Development of flukes:

A total of 157 flukes were recovered from muscles of the dogs. Three of them measured 1.9 - 3.1 mm in length, and primordial cells of ovary, testis and uterus were recognized. The remaining 154 flukes were very stunted and they had granules in the excretory bladder, and a small stylet was still visible on the oral sucker. Their sizes ranged from 0.7 to 1.2mm in length and 0.4 to 0.6 mm in width. Five out of 8 flukes recovered from cysts of lung were 4.5 - 6.7 mm in length and 2.8 - 3.4 mm in width, and eggs were present in their uteri. Three other flukes from cysts in lungs and all the flukes from cavities and lungs were immature. Their sizes were 1.9 - 4.2mm in length and 1.4 - 3.3 mm in width, though their ovary and testes were developed slightly.

Experimental Infection of Rats with Metacercariae

Distribution of flukes:

Table 3 shows the results of oral infection of 29 albino rats with metacercariae from Kuala Pilah. The average recovery rate of flukes from the rats was 42.8 %. Almost all of the worms, 307 out of 310, were recovered from the muscles and only a few worms were found in the lungs or abdominal cavity. The 3 intraperitoneally inoculated rats with 50 metacercariae each from Taman Negara were necropsied 100 days after infection. The recovery rate of the flukes from these rats was 48.7% (46.0 - 52.0%) and all of them were recovered from muscles.

Postmortem examination:

On the gross examination of the infected rats, no pathological changes were generally observed except those examined 10 to 20 days after infection. In these rats, haemorrhagic tracks were found in the muscles of abdominal walls and legs, presumably due to migration of juvenile flukes.

Development of flukes in rats:

All the flukes recovered from rats were juvenile and morpho-logically similar to the excysted metacercariae with the exception that they were slightly larger in size. They were 0.7 - 0.9 mm in length and 0.3 to 0.5 mm in width.

Transfer of Juvenile *P. westermani* to Cats and Dogs

The results of oral infection in cats and dogs with juvenile worms recovered from muscles of cats, dogs and rats of the previous experiments are shown in Table 4. The average recovery rate of worms in five infected cats was 68.2%, ranging from 48.0 to 84.0%. Out of 99 flukes obtained, 77, 4, 11 and 7 flukes were recovered from cysts in the lungs, tissues of lungs, pleural cavity and muscles, respectively. Sixty-four out of 77 flukes recovered from cysts in the lungs were mature while 1 out of 11 from pleural cavity and five out of 7 flukes from muscles were still juvenile worms with granules in the excretory bladder.

In dog No. 8, 2 and 3 flukes were recovered from the pleural cavity and muscles, respectively. The recovery

Table 3 Results of experimental oral infection of albino rats with 25 *Paragonimus westermani* metacercariae from Kuala Pilah

Days after infection	No. of rats used	No. of worms* recovered (%)	No. of worms recovered from		
			lungs	peritoneal cavity	muscles
10	5	48 (38.4)	0	0	48
20	5	54 (43.2)	0	1	53
30	5	55 (44.0)	0	0	55
60	5	62 (49.6)	0	1	61
100	4	57 (57.0)	1	0	56
150	5	34 (27.2)	0	0	34

* All worms recovered were juvenile.

Table 4 Results of experimental infection of dogs and cats with juvenile *Paragonimus westermani* recovered from some paratenic host animals

Host animal	Body weight at autopsy (kg)	Sex	Juvenile worms recovered from (days after postinfection)		No. of juvenile worms fed	Autopsy days after infection	No. of worms recovered (%)	No. of worms recovered from			
								cyst in lungs	lungs	pleural cavity	muscles
Dog No.8	14.5	F	Rat	(150)	40	185	5 (12.5)	0	0	2†	3(2†, 1‡)
Dog No.9	16.4	F	Rat	(100)	35	209	10 (28.6)	8*	0	2†	not examined
Cat No.12	2.8	F	Cat	(192)	25	200	12 (48.0)	4*	0	5†	3(2†, 1‡)
Cat No.13	4.3	M	Cat	(237, 242)	23(12.11)	166, 161	19 (82.6)	16(11*, 5†)	0	2†	1‡
Cat No.14	3.6	M	Dog	(227)	17	172	12 (70.6)	12*	0	0	0
Cat No.15	4.3	M	Dog	(245, 252)	50(30, 20)	195, 202	42 (84.0)	33(25*, 8†)	4†	2†	3‡
Cat No.16	3.9	M	Rat	(100)	25	212	14 (56.0)	12*	0	2(1†, 1‡)	0

F: female, M: male, *, †, ‡: Cf. the foot in Table 1,

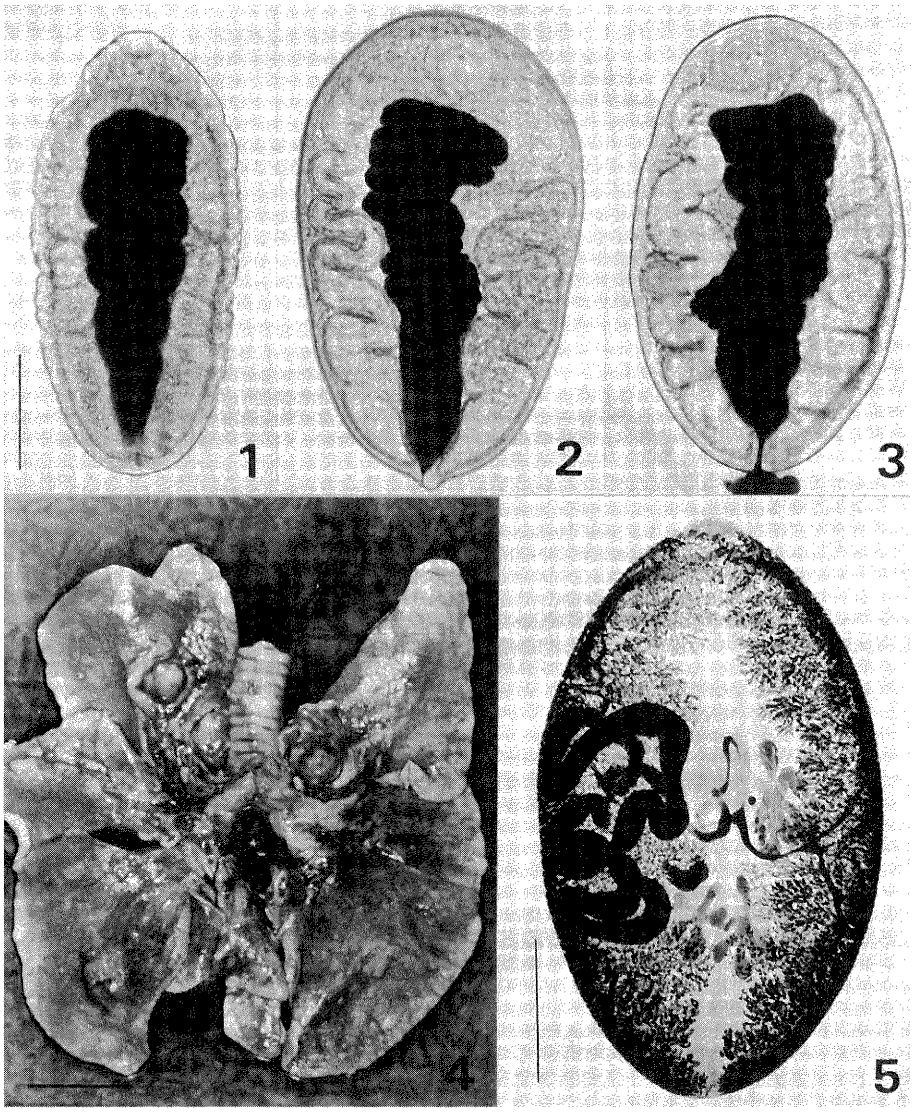
rate was 12.5%. All of the flukes recovered from the dog were immature, especially a single fluke from muscle having the same characteristics of the metacercarial stage. In dog No.9, typical cyst formation was recognized in the lungs, 8 fully matured flukes which measured 7.9 - 8.5 mm long by 3.8 - 4.4 mm wide were recovered. Two immature flukes were recovered from pleural cavity but the muscles of this dog were not examined.

DISCUSSION

It is well known that the distribution and development of *P. westermani* in favorable definitive hosts, like cats and dogs and in unfavorable hosts, such as rodents are quite different. In the former hosts, worms are found in cysts in the lungs and are mature, but in the latter hosts, majority of worms remain in the muscle with little development. In dogs and cats infected with *P. westermani* from Japan, the triploid fluke takes about 2 months to reach maturity, and the diploid fluke takes almost two and a half months (Takizawa, 1964; Habe, 1978; Shibahara, 1983). *P. westermani* from Korea, China and the Philippine also shows similar growth in dogs and cats (Fan, 1966; Miyazaki and Habe, 1979; Unpublish data, S. Habe). In general the worm recovery rates in cats and dogs infected with metacercariae from these countries were about 60 to 80% by oral inoculation and 90% more by peritoneal inoculation.

In our present study the average worm recovery rates of cats and dogs, orally infected with Malaysian *P. westermani*, were 20.7% and 2%, respectively. Kim (1969) infected six cats with 109 metacercariae from freshwater crabs, *Parathelphusa maculata* and *Johora johorense*, and recovered 31 worms from 61 to 108 days after infection. These rates are very low compared to those of *P. westermani* from other localities. The results of experimental intraperitoneal inoculation also shows a similar tendency. The Malaysian *P. westermani* has a lower infectivity to mammalian hosts than those from other localities. Furthermore, in spite of keeping the infections for 5 or more months after feeding with metacercariae, about 50% and 90% of the flukes were still recovered from the muscles of the cats and dogs respectively. The worms were very stunted and morphologically similar to the excysted metacercariae with the exception that their body sizes were slightly larger. The rates of mature flukes out of total worms recovered were only 40% from infected cats and 3% from infected dogs.

All the flukes recovered from albino rats were juvenile just like those that parasitized the muscles of dogs and cats. Such stagnated worms have been collected from other animals, such as pig, wild boar, mouse, hamster, guinea pig, rabbit, monkey and hen infected with *P. westermani* from Japan (Habe, 1978, 1982, 1983; Shibahara and Nishida, 1986; Takizawa, 1964). This is the first report where juvenile worms were recovered from cats and dogs infected with *P. westermani*. The development and distribution of Malaysian *P. westermani* in cat and dog are very different from the flukes of other countries but no significant differences are recognized in rat. However, Philippine *P. westermani* does not remain in the muscle of rats and migrates into the lungs and then mature in relatively high rate. This suggests that Malaysian *P. westermani* has a higher



Figs. 1-3. Living juvenile worms. Bar=0.2mm.
1. Worm recovered from muscle of rat 150 days after infection with metacercariae.
2. Worm recovered from muscle of dog 240 days after infection with metacercariae.
3. Worm recovered from muscle of cat 237 days after infection with metacercariae.

Fig. 4 Lungs of dog; 209 days after inoculation with 35 juvenile worms from muscles of rat. Bar=3cm.

Fig. 5 Mounted adult worm from worm cyst in lungs of cat; 172 days after inoculation with juvenile worms from muscles of dog. Bar=2mm.

final host susceptivility than the Japanese, Chinese and Korean flukes while the Philippine *P. westermani* has the lowest.

The final host of *P. westermani* is widely distributed among mammals, especially of the families Felidae and Canidae. In Malaysia, only one species of lung fluke, *P. westermani* has been recorded from tiger, *Felis tigris*, domestic cat, *Felis domestica*, clouded leopard, *Neofelis nebulosa diardithe*, wild cats, *Felis bengalensis*, *F. planiceps* and *F. temminckii*, black panther, *Felis pardus*, and crab-eating monkey, *Macaca irus irus* (Groves et al., 1967; Lee and Miyazaki, 1965; Lim and Betterton, 1977; Rohde, 1963, 1967; Miyazaki and Kwo, 1969). Most of the natural final hosts belong to Felidae. In spite of the juvenile worms recovered from muscles of experimental cats in the present study, and it is without doubt

that the animals of Felidae are the most important and suitable final hosts for Malaysian *P. westermani*. However, our results showed Canidae to be unsuitable hosts for Malaysian *P. westermani*.

It was experimentally proved that juvenile flukes remaining in the muscles of dogs, cats and albino rats were able to reinfect other carnivores. In the case of cat infections, the recovery rate of flukes was 68.2% on the average, while in dogs, it was 20.7%. In the animals inoculated with juvenile flukes, the worm recovery and maturity rate were also higher than those infected with the metacercariae. Carnivorous animals are more readily infected by the Malaysian *P. westermani* via feeding of juvenile worms from paratenic hosts than metacercariae from crabs. Therefore, the present study suggests that many wild mammals living in jungle must serve as paratenic hosts and they play a very important role in the completion of the life cycle of the flukes in Malaysia and its neighborhood. In experiments the same mode of infection has been shown in the triploid and diploid types of *P. westermani, P. miyazakii* Kamo et al., *P. mexicanus* Miyazaki et Ishii and *P. heterotremus* Chen et Hsia (Fan and Khaw, 1964; Fan and Hsu, 1965; Habe, 1978, 1983; Miyazaki and Habe, 1976; Shibahara and Nishida, 1986; Sugiyama et al., 1990). For example, it was proven that the wild boar, *Sus scrofa leucomystax*, plays an important role as the paratenic host for human paragonimiasis westermani (triploid type) in the southern part of Kyushu, Japan (Norimatsu et al., 1975; Miyazaki et al., 1978; Tokudome et al., 1977). Kwo and Miyazaki (1968) reported heavy infections of *P. westermani* in all the tigers examined in North Sumatra, Indonesia, and Miyazaki and Habe (1976) postulated that those tigers might have been infected due to feeding on animals harboring immature worms and serving as paratenic hosts. Lim and Betterton (1977) reported that civet cats have the potential of being paratenic hosts from feeding experiments and suggested that wild boar and a number of small mammals were also potential paratenic hosts. Although locally acquired paragonimiasis has not been observed in man in Malaysia, *P. westermani* has been found in a number of animals and freshwater crabs. Lim and Betterton (1977) gave the reason that freshwater crab is not generally used as a source of food and wild boar meat is infrequently eaten. In addition to that, the development of *P. westermani* in man could be considered to be similar to that seen in the present experiments where mature worms may not be found.

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Short communication

SEROEPIDEMIOLOGICAL STUDIES OF HEPATITIS VIRUSES IN THE DOMINICAN REPUBLIC II. THE PREVALENCE OF HEPATITIS D AND E VIRUS INFECTIONS

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Abstract: A total of 841 serum samples collected and stored at the Center of Gastroenterological Disease, Aybar Hospital, Santo Domingo, Dominican Republic were tested for antibodies against hepatitis E virus (anti-HEV). Sera of 60 hepatitis B virus (HBV) asymptomatic carriers selected from 2,000 apparently healthy individuals in the Dominican Republic were tested for antibodies against hepatitis D virus (anti-HDV). The study demonstrated that the prevalence of anti-HEV was 19.0% in individuals under 15 years of age as compared with the lower prevalence of 4.5% in individuals older than 16 years. The prevalence of anti-HDV in HBV carriers was 6.7%, demonstrating that HDV and HEV are prevailing in this country.

Among five hepatitis viruses of hepatitis A(HAV), B(HBV), C(HCV), D(HDV), and E(HEV), a seroepidemiological study of the former three viruses in the Dominican Republic was described previously (Shichijo *et al.*, 1995).

HEV belongs to the *Togaviridae* family and is an enterically transmitted agent that causes epidemic and sporadic forms of hepatitis in tropical countries. Clinical symptoms due to HEV infection are nearly the same as those caused by HAV infection (Bradley, 1990). It has been found, however, that young adults are more frequently infected with HEV than children; HEV infection rarely prevails within a family and that infection of pregnant women with HEV produces higher maternal and fetal mortality. HEV is known to distribute widely in the world, and India, Indonesia, Nepal, Hong Kong and Egypt have been reported to be the areas endemic for HEV (Goldsmith *et al.*, 1992, Lok *et al.*, 1992, Corwin *et al.*, 1994, Longer *et al.*, 1994).

HDV is a defective RNA virus, and it can be transmitted only when HBV infection occurs simultaneously, or only when a chronic HBV carrier is infected with HDV. HDV and HBV share the same route of infection (Rizzetto *et al.*, 1983, Rizzetto *et al.*, 1990). In our previous study (Shichijo *et al.*, 1995), it was found that HBV infection is still prevalent in the Dominican Republic. The purpose of the present study is to investigate whether and to what extent HEV and HDV infections exist in the Dominican Republic.

Anti-HEV antibodies were tested by ELISA. Since HEV cannot be grown in tissue culture, thirty amino acids corresponding to from nucleotides(nt) No. 6,965 to 7,054 of the open reading frame 2 of the HEV genome were synthesized and used as a coated antigen. The synthetic HEV peptide (5μg) was pipetted into each well of 96 microtiter plates. After incubation for 1h at 37°C or overnight at 4°C, the wells were washed three times with phosphate-buffer saline containing 0.05% Tween 20 (PBS-T). The plates then received 100μl per well of PBS-T containing 5% skim milk and 10μl of test serum and incubated for 1h at 37°C. The wells were washed 5 times and further incubated at 37°C for 1h with 100μl of horseradish peroxidase conjugated goat anti-human-IgG $F(ab')_2$ (diluted to 1/10,000 in PBS-T-5% skim milk). After washing 5 times, o-phenylenediamine•2HCl (OPD) was added and the absorbance was read at

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492nm after 30 min incubation at room temperature. In the experiments, anti-HEV antibody-positive and -negative sera were simultaneously tested as controls.

When the reaction was positive, the presence of anti-HEV antibody was confirmed by the following two methods. One method was a simple repeat of the experiment to check the reproducibility of positive reaction, and the other was an absorption experiment. In the latter experiment, 10μg/well of antigen peptide were simultaneously added with test serum to the plates which was already coated with antigen peptide. Only when an anti-HEV-positive serum became negative after the absorption test, the test serum was taken as antibody-positive. The RIA kit (Abbott, Lab., North Chicago, Illinois, USA) was used for screening anti-HDV antibodies.

The prevalence of anti-HEV antibody by age and sex was shown in Table 1 and Figure 1. Each age group consists of more than 80 samples. The highest antibody positive rate was 30% for female in an age group from 6 to 10 years and 19.1% for male in an age group from 11 to 15 years old. The overall prevalence of anti-HEV was much higher (19.0%) in individuals under 15 years of age than in individuals older than 16 years (4.5%). This prevalence by age appears slightly different from the higher infection rates in young-to-middle-age adults reported in India, Indonesia and Egypt (Goldsmith *et al.*, 1992, Corwin *et al.*, 1994, Arankalle *et al.*, 1995). The

Table 1 Prevalence of anti-HEV by age and sex in the Dominican individuals

Sex	Age group	0-5	6-10	11-15	16-20	21-30	31-40	41-50	51-	Total
Male	No. of tested	44	41	42	45	49	65	57	54	397
	No. of anti-HEV positive	7	7	8	1	3	3	2	3	36
	% positive	15.9	17.1	19.1	2.3	6.1	4.6	3.5	9.3	9.1
Female	No. of tested	40	40	40	46	69	80	69	60	444
	No. of anti-HEV positive	7	12	6	2	2	5	5	1	40
	% positive	17.5	30.0	15.0	4.3	2.9	6.3	7.2	1.7	9.0
Total	No. of tested	84	81	82	91	118	145	126	114	841
	No. of anti-HEV positive	14	19	14	3	5	8	7	4	76
	% positive	16.6	23.4	17.1	3.3	4.2	5.5	5.6	5.3	9.0

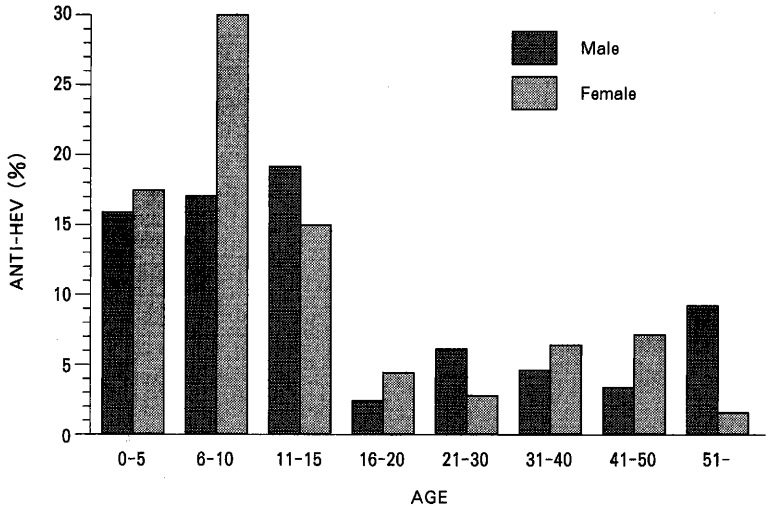

Figure 1 Prevalence of anti-HEV by age and sex in the Dominican Republic

Table 2 Prevalence of HBsAg and anti-HDV in Healthy Individuals in the Dominican Republic

HBsAg		Anti-HDV	
No. of test	No. (%) of HBsAg-positive	No. of test	No. (%) of Anti-HDV positive
2,000	63 (3.1)	60	4 (6.7)

reason for the difference was unknown, however, one possibility is that HEV may have been introduced to the Dominican Republic sometime in the past 15 years and sporadic but continuous infection has been taking place since then. Higher seroprevalence rates under 15 years of age could be explained as the consequence of very active behaviors of the children of these age groups, that facilitate frequent infection with HEV via fecally contaminated water or by person-to-person contact. The HEV infection in the Dominican Republic, however, was not highly widespread and its incidence was considerably much lower than that of similarly transmitted HAV, indicating that epidemic or sporadic HEV infection is possible to occur in this country in the future. To our knowledge, the present paper is the first description of the existence of HEV infection in the Carribean countries.

Various seroepidemiologic studies on HDV infection have demonstrated that the prevalence is high in some parts of Africa, south Americas and southern Italy (Fonseca *et al.*, 1988, Lalla *et al.*, 1990, Aceti *et al.*, 1991, Sjogren & Colichon, 1991, Hadler *et al.*, 1992, Sagnelli *et al.*, 1992, Torres & Machado, 1994, Cenac *et al.*, 1995). On the other hand, in the United States and western Europe, it was shown that HDV infection is restricted to the groups with frequent percutaneus exposure to blood and its components, primarily i.v. drug users, hemodialyzed patients (Hoy *et al.*, 1984, Lettau *et al.*, 1987).

Sixty three out of 2,000 healthy Dominicans were HBsAg-positive as shown in Table 2. Sixty of those sera were tested for anti-HDV and four of them (6.7%) were found to be positive. This rate was higher than that observed in the United States or in Japan where it has been reported to be about 1% or less (Iwanami *et al.*, 1993), but it was much lower than those of highly endemic countries as described above.

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Research Note

REACTIVITY OF SERA FROM PATIENTS WITH ACUTE *PLASMODIUM FALCIPARUM* AND *P. VIVAX* INFECTIONS WITH AN ANTIGEN PREPARATION FROM A *P. FALCIPARUM* ISOLATE: ——MUTUALLY EXCLUSIVE REACTIVITY WITH A 47kD AND A 29kD BAND RESPECTIVELY.

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Abstract: Western blot analysis was carried out on sera obtained from patients with *P. falciparum* (Pf) and *P. vivax* (Pv) on an antigen preparation of a laboratory propagated isolate of Pf (# 40) cultured *in vitro*. The reactivity to a 47kD band was sought based on the results from a previous study (Kano *et al.*, 1990a). Pooled Pf sera (n = 15) reacted with the 47kD band whereas pooled Pv sera (n = 13) as well as pooled normal human sera (n = 45) were not reactive with the 47kD band. An interesting finding was that the pooled Pv sera reacted with a 29kD band instead, while the pooled Pf sera was non-reactive to the 29kD band. In addition, two of four Pf-and three of four Pv- individual sera reacted with the 47kD and 29kD bands respectively. All of these sera had high IFAT (indirect fluorescent antibody test) titer. One of the sera obtained reacted with both the 47kD and 29kD bands but was later found to be from a patient with mixed Pf and Pv infection. The potential of these findings in diagnosis of Pf and Pv infections is discussed.

Key Words: *P. falciparum*, *P. vivax*, malaria, diagnosis

INTRODUCTION

The presence of high antibody levels against the malaria parasite can be found in the sera of most adult individuals in malaria endemic areas regardless of whether these individuals have clinical malaria or not. These antibodies react specifically with various antigenic bands prepared from the parasite when analyzed using Western Blotting. However, Kano *et al.* (1990a) reported that in a longitudinal study on the serum taken from a Japanese man with imported Pf infection, a 47kD band was strongly reactive only during the acute phase of the infection with progressively decreasing reactivity and was barely detectable in convalescence (about 2 months after clinical presentation). A pilot study carried out in various endemic locations in the Sudan by the same researchers showed that individuals positive for malaria parasites have antibody titers (against crude malaria antigen preparation) as well as reactivity to the 47kD band, but individuals negative for malaria parasites but with high antibody titers did not recognize the 47kD band. The latter group probably represent those with cumulative past malaria infections. They concluded that the 47kD molecule may be useful in determining present or recent infections in serological surveys of malaria. The present study attempted to extend above findings to the Malaysian situation and thus to determine whether sera taken from patients with falciparum malaria infection react with an antigen preparation of a Malaysian Pf isolate. An unexpected finding of the reactivity of Malaysian sera taken from Pv infection to a 29kD of the same antigen preparation was noted. This finding provided interesting information regarding the possible use of both the

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47kD and 29kD epitopes in diagnosis of acute infections by both species.

METERIALS AND METHODS

Sera were obtained from Malaysian Pf-and Pv-infected symptomatic and parasitemic patients prior to drug treatments, from the District Hospital of Kuala Krai, Kelantan, Malaysia. The IFAT was carried out as described by Kano *et al.* (1990b). Briefly, four-fold dilutions of sera (1:4 to 1:4096) were incubated for 30 min onto the antigen spots from Pf cultured isolate (SGE1 strain) and Pv clinical isolate placed onto glass slides. After washing, diluted fluorescein conjugated rabbit anti-human IgG (1:30, Behring, Germany) was then applied and incubated for 30 min. Following final washing, the fluorescence was read under an incident light illuminating type fluorescent microscope (Olympus BH-RFC, Japan) to determine titers.

The Pf isolate (designated # 40) was obtained from a patient with acute Pf infection during the 1990/92 transmission season and cultured for more than 20 passages *in vitro* using a modified method of Trager (Zolg *et al.*, 1982) in RPMI 1640 supplemented with 5% washed human red blood cells, 10% heat-inactivated human serum and maintained in an atmosphere of 5% oxygen, 5% carbon dioxide, and 90% nitrogen.

Parasite antigen preparation for SDS-PAGE was carried as described previously (Kano *et al.*, 1990a). Briefly, asynchronous cultured isolate # 40 (at least 5% parasitemia) was obtained, centrifuged and the supernatant discarded. The parasitized packed cells were resuspended and incubated in 1.5 volume of 0.15% saponin at 37°C for 20 min, after which the lysed cells were washed in 10 volume PBS and the dark-brown parasite pellet was collected. The sediment was then dissolved in the Laemmli's antigen solution (Laemmli, 1970). The SDS-PAGE was conducted in 10% acrylamide gel under reducing conditions. The electrophoresed proteins were transferred onto a nitrocellulose membrane. The membrane was cut into strips and Western blotting was carried out under reducing conditions using standard procedures (Laemmli, 1970). Briefly 1:100 dilution of sera were reacted separately to each membrane strip and the reactivity of the sera was revealed by reaction with a secondary goat anti-human IgG (peroxidase conjugated) (Cappel, USA) and 4-chloro-1-naphthol as substrate.

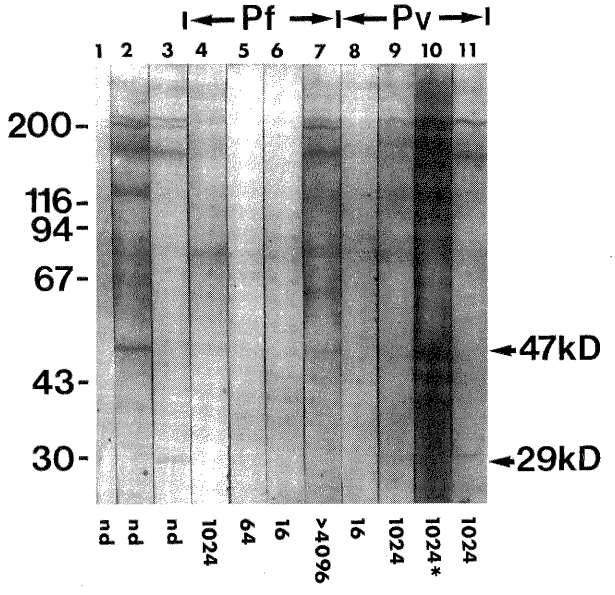

Figure 1 Western blot analysis of sera obtained from patients with Pf and Pv infections against a Malaysian Pf antigen preparation (isolate #40). Strip 1 = pooled normal human sera (n=45), strip 2 = pooled Pf sera (n=15), strip 3 = pooled Pv sera (n =13); and individual sera taken from Pf (strip 4 to 7) and Pv (strips 8 to 11) infections. Individual Pf sera reacted with the 47kD band but not the 29kD band whereas individual Pv sera reacted with the 29kD band not the 47kD band. The serum used in strip 10 was obtained from a patient with mixed Pf and Pv and infections. (nd = not determined; *= based on Pv titer, the Pf titer was > 4096).

RESULTS AND DISCUSSION

Figure 1 shows the Western blot profile of the Malaysian Pf-isolate (# 40) reacted with pooled and individual Pf and Pv sera of Malaysian patients. The IFAT titers are indicated at the bottom of the figure. Pooled normal human sera (Strip 1, n = 45) did not show any reactivity. Pooled Pf sera (Strip 2, n = 15) reacted strongly with the 47kD band but not with the 29kD band while pooled Pv sera (Strip 3, n = 13) reacted with the 29kD band but not the 47kD band. Two of four individual sera from Pf-infected patients (Strips 4 and 7) reacted with the 47kD band, both of which had high IFAT titers. Three of four individual sera with high IFAT titers from Pv-infected patients (strips 9, 10 and 11) reacted with the 29kD band. One of these (Strip 10) reacted with both the 47kD and 29kD bands. This serum was later found to have been obtained from a mixed Pf-, Pv-infected patient. Sera with relatively low IFAT titer (Strip 5, 6 and 8) did not produce any bands following

Western blotting. The obtained result employing individual sera from Pf infected patients reacted with SDS-PAGE antigen from the Malaysian isolate (# 40), was consistent with a previous finding (Kano *et al.*, 1990a) that the sera from patients with acute Pf infection contain antibodies to a 47kD band. On the other hand, it was interesting to note that sera taken from patients with acute Pv infections contain antibodies to a 29kD band of the same antigen preparation. This pattern of reactivity was found to be mutually exclusive, in that Pf sera reacted with the 47kD but not 29kD and Pv sera reacted with the 29kD but not the 47kD band. The reactivity towards the 47kD band by Pf not Pv sera suggests that this epitope is unique in Pf. However, the reactivity pattern towards the 29kD was quite unexpected. This epitope may share a common antigenic structure in Pf and Pv. Whereas it may be "hidden" in erythrocytic stages of Pf, and sera taken from patients with acute Pf infection did not contain measurable amounts of antibodies towards this masked Pf antigenic polypeptide. However, the 29kD epitope may have been exposed in Pv, and patients with acute Pv infections could be ready to produce antibodies against it. Under the denaturing and reducing conditions of SDS-PAGE, this particular epitope was then exposed, and Pv sera which contain the corresponding anti-29kD antibodies would thus produce a band during Western blotting. Future work should determine whether the reactivity was a unique feature of the isolate used or whether this phenomenon could be substantiated with other isolates and sera taken from other geographical locations. It would also be interesting to determine whether the reactivity to the 29kD band could only be seen in acute Pv infections but not in convalescence as reported for the 47kD band. Further characterization of the 47kD and 29kD molecule would prove valuable for the diagnosis or epidemiological survey of Pf and Pv infections.

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