[Jpn. J. Parasitol., Vol. 45, No. 3, 207–214, June, 1996]

Epidemiological Survey for Parasitic Diseases, Especially for Paragonimiasis, in Guatemala

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(Accepted May 28, 1996)

Abstract

An epidemiological survey for parasitic diseases, especially for paragonimiasis, by immunoserological method was carried out in Parcelamiento 46, Plan de Los Amates and Los Cerritos, state of Santa Rosa, Guatemala, in 1994. Out of 412 of children and adults, 10 (2.4%) were positive by intradermal test for *Paragonimus mexicanus* antigen. The number of positives by enzyme linked immunosorbent assay, latex agglutination test and agar double diffusion test performed on intradermal test positives were 6, 6 and 2, respectively. Among them, 2 reacted positively to every test, and were strongly suspected to be patients of paragonimiasis. Stool examinations for helminth eggs were performed on 401 samples with both Kato-Katz and formalin-ether centrifugation (MGL) techniques. *Ascaris lumbricoides* (28%), *Trichuris trichiura* (53%) and Hookworm (43%) were detected at high proportions. *Hymenolepis nana* and *Taenia* sp. were also detected, but no *Paragonimus* sp. egg was found from either stool or sputum. Results of the examination for intestinal protozoan cysts showed the presence of *Entamoeba histolytica* (6%), *Entamoeba coli* (33%), *Iodamoeba buetschlii* (9%) and *Giardia intestinalis* (6%).

Key words: *Paragonimus mexicanus*; paragonimiasis; stool examination; enzyme-linked immunosorbent assay; agar double diffusion test; Guatemala.

Introduction

In central and south America the causative agent of human paragonimiasis has long been regarded as *Paragonimus westermanii* which had been imported by immigrants from the Orient. Recently, however, many new species of *Paragonimus* have been reported in Mexico, Colombia, Peru and Ecuador. These species have been described as *P. rudis* (Diesing, 1850), *P. mexicanus* (Miyazaki and Ishii, 1968), *P. caliensis* (Little, 1968), *P. peruvianus* (Miyazaki *et al.*, 1969a, b; Miyazaki, 1974; Lamothe-Argmedo *et al.*, 1979), *P. amazonicus* (Miyazaki *et al.*, 1973), *P. inca* (Miyazaki *et al.*, 1975) and *P. ecuadoriensis* (Voelker and Arzube, 1979). Furthermore, paragonimiasis was reported from Guatemala (Caballero, 1946, 1955) and Venezuela (Alarcón de Noya *et al.*, 1985). However, there was much controversy concerning the independence of these species. More recently, Brenes and others (1980) reported that *P. mexicanus* and *P. peruvianus* were the same species and Miyazaki (1982) also reported that *P. peruvianus* and *P. ecuadoriensis* were synonymous with *P. mexicanus*. These opinions were mainly based on the morphological characteristics of the adult worms. It is generally accepted that the shape of the ovary and testis, the shape and size of the eggs, and the relative size of the

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The investigation was supported by a Grant-in-aid for the International Scientific Research Program of the Ministry of Education, Science, Sports and Culture in Japan in 1994 (No. 06041099).

oral and ventral suckers are good criteria for differentiating adult *Paragonimus* species. The authors have carried out not only morphological studies on cercariae, metacercariae and adult flukes of the parasite, but also epidemiological, pathological and immunoserological studies of the disease in several endemics in order to clarify the features of Latin American *Paragonimus* and paragonimiasis (Yokogawa *et al.*, 1983; Ito *et al.*, 1985; Tongu *et al.*, 1985; Tongu *et al.*, 1987; Kobayashi *et al.*, 1988; Tongu *et al.*, 1990). The present study deals with the results of parasitological surveys on inhabitants in Guatemala. The morphological features of the flukes and data on the infection rate of metacercariae in crabs, the second intermediate host, obtained simultaneously with this study, have been published elsewhere (Tongu *et al.*, 1995).

Materials and Methods

Study areas

This study was conducted in state of Santa Rosa, Guatemala in August, 1994. Three villages located along the Los Esclavos River basin were chosen: Parcelamiento 46, Plan de Los Amates and Los Cerritos (Fig. 1). The former two are situated about 50 km inland from the Pacific Ocean and 1,200 m above sea level, and the other one is located 20 km from the seashore and 100 m above sea level. The test subjects were students of primary and secondary schools, and inhabitants who were mostly agricultural workers, employed at local farms and ranches.

Immunoserological survey

The following tests were selected for immunoserological survey: intradermal test (IDT) as a screening device, followed by a latex agglutination test (LAT), enzyme linked immunosorbent assay (ELISA) and agar double diffusion test (DDT) if IDT was positive. All of the IDT positives and as many as possible of the IDT negatives had stool examinations by both cellophane thick smear technique (modified Kato-Katz; Katz *et al.*, 1972) and formalin-ether centrifugation technique (MGL; Ritchie, 1948). Furthermore, sputum examinations were also performed on all IDT positives using the centrifugation technique with 2% NaOH solution.

Fig. 1 Map of Guatemala.

The intradermal test

The veronal-buffered saline extracts (VBS antigen) of lyophilized and delipidized adult worms from infected with metacercariae of *Paragonimus mexicanus* (*P. mex*) were used as the antigen (Chaffee *et al.*, 1954; Yokogawa *et al.*, 1955). The antigen contained 30 μg protein/ml. A new, sterile tuberculin syringe was used to inject 10–20 μl of antigen into the skin of the flexor surface of the forearm of each individual. This amount was usually sufficient to raise a wheal 3–4 mm in diameter. A positive reaction was one in which the original 3–4 mm increased by more than 5 mm within 15 minutes after injection. The wheal was frequently accompanied by erythema. If the IDT was positive, then 5 ml of blood was drawn by venipuncture for use in the immunoserological tests. The sera separated from blood samples were stored at –70°C, and were then delivered to Kyorin University under dry ice storage.

Latex agglutination test

The latex agglutination test (LAT) was carried out according to the procedure of Tsubota and

Ozawa (1977) and Tsubota and others (1977). Antigen corted latex particles were prepared as described previously (Kobayashi *et al.*, 1988).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was carried out as described previously (Voller, 1976; Tanaka *et al.*, 1979) with minor modifications. The carbonate buffer extract of lyophilized adult worm of *P. mex* was used as the antigen. Peroxidase conjugated antihuman IgG (Jackson Immunoresearch Lab. Inc., West Grove, PA, USA) in a dilution of 1:30,000 and 0.03% of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS; Nacalai Tesque, Kyoto, Japan) in citrate buffer pH 4.0 as a substrate (Matsuda *et al.*, 1984) were used in this study. The reaction of ELISA was observed in 0.3 ml flat bottom wells of polystyrene microtiter plates. Color development was read at 450–550 nm using a MTP-12 micro-ELISA auto reader (Corona, Tokyo, Japan). The titer was expressed as the reciprocal of the highest sample dilution in which the optical density (OD) was greater than the mean OD of 20 normal Japanese sera at a dilution of 1:20 plus 2 SD.

Agar double diffusion test (DDT)

For the Agar double diffusion test (DDT), 0.1% saline extracts of adult worms of *P. mex.*, *P. miyazakii* (*P. m*) and *P. westermanii* (*P. w*) were used as the antigens (8 mg protein/ml), and 0.9% agarose in veronal buffered saline (VBS), pH 8.2, was employed for the plate. The size of the wells for both antigen and serum was 2 mm in diameter and the distance between them was 3 mm; 5 μl of the antigen or serum was added to the wells (Ouchterlony, 1949).

Statistical analysis

All statistical analyses were carried out using the HALB System (Ver.3.33), a software system for data analysis (Gendaisugaku Inc., Tokyo, Japan).

Results

Intradermal test

IDTs were performed at Parcelamiento 46, Plan de Los Amates and Los Cerritos in Santa Rosa. The former two villages are located 1,200 m above sea level and the latter at 100 m. Parcelamiento 46 and Plan de Los Amates face each other across the Los Escravos River. At Parcelamiento 46, a total of 37 primary school children, all aged below 14, 21 males and 16 females, were examined. In addition, 2 females in their thirties living beside the school were also examined. Of the total examined, 4 (10%) were positive, 3 (14%) males and 1 (2.6%) female, as shown in Table 1. The positive female was 38 years old. At Plan de Los Amates, a total of 42 primary school children, 20 male and 22 female aged from 7 to 15, were examined. In addition, 15 females aged over 18 living in the village and 6 preschool children (4 males and 2 females) were also examined. Of the total examined, 4 (6%) were

Table 1 Results of immunoserological tests for paragonimiasis

No.	Village	Age	Sex	IDT	ELISA	LAT	DDT
4	Parcelamiento 46	8	M	+	–	–	–
6	Parcelamiento 46	9	M	+	>1:1280	>1:1024	+
9	Parcelamiento 46	8	M	+	1:80	1:64	–
38	Parcelamiento 46	38	F	+	>1:1280	>1:1024	++
55	Plan de Los Amates	15	F	+	–	–	–
63	Plan de Los Amates	13	M	+	–	1:32	–
66	Plan de Los Amates	15	M	+	1:20	1:16	–
103	Plan de Los Amates	5	M	+	1:320	1:32	–
170	Plan de Los Amates	9	M	+	1:40	–	–
286	Los Cerritos	12	F	+	–	–	–

IDT, Intradermal test; ELISA, Enzyme-linked immunosorbent assay; LAT, Latex agglutination test; DDT, Agar double diffusion test.

positive, 3 (14%) male and 1 (4.5%) female. At Los Cerritos, 203 primary school children aged from 7 to 14 and 48 secondary school students aged from 15 to 23 were examined. In addition, 57 other inhabitants were also examined. A positive reaction was observed in a 12-year-old girl. It is noteworthy that more than 95% of the test subjects from Parcelamiento 46 and Plan de Los Amates, and about 50% of those from Los Cerritos confessed to the ingestion of crabs captured in nearby streams.

Stool examination

Stool examination for helminth eggs was performed by both cellophane thick smear technique (modified Kato-Katz method) and formalin-ether centrifugation technique (MGL). With the MGL technique, using iodine staining, protozoan cysts were also examined. A total of 401 stools from both children and adults in Parcelamiento 46, Plan de Los Amates and Los Cerritos were examined by both techniques or one, 399 by Kato-Katz technique and 362 by MGL. The results of the stool examinations for helminth eggs employing these two techniques are summarized in Table 2.

Out of 401 stools, 114 (29.1%) were positive for *Ascaris lumbricoides* eggs, 211 (54.0%) for *Trichuris trichiura*, 172 (44.0%) for hookworm, 15 (4.3%) for *Hymenolepis nana* and 2 (0.5%) for *Taenia sp.* Furthermore, larvae of *Strongyloides stercoralis* were detected in seventeen cases in Los Cerritos. However, in Parcelamient 46 and Plan de Los Amates they were not counted because of insufficient time for their identification; nevertheless, a few nematode larvae were found. There were no significant differences among the 3 villages in the ratio of helminth infection. Additionally, no correlation was observed between the positive rate and age or sex.

Results of the examination for intestinal protozoan cysts by MGL are shown in Table 3. Out of 362 stools, the cyst of *Entamoeba histolytica* was found in 23 (6.4%), *Entamoeba coli* in 120 (33.1%), *Iodoamoeba buetschlii* in 39 (10.8%) and *Giardia intestinalis* in 33 (9.1%). It was remarkable that protozoan cyst positive was very common below 15 years old, as follows: *E. histolytica*, 7.6%; *E. coli*, 32.7%; *I. buetschlii*, 12.7%; *G. intestinalis*, 7.6%. Each infection rate of this age group was signifi-

Table 2 Results of stool examination for helminth infections with cellophane thick smear technique (Kato-Katz) and formalin-ether centrifugation technique (MGL)

	Parcelamiento 46			Plan de Los Amates			Los Cerritos			Total		
	M	F	Total	M	F	Total	M	F	Total	M	F	Total
As	12	4	16	7	11	18	42	38	80	61	53	114
	(57)	(25)	(43)	(39)	(28)	(32)	(33)	(21)	(26)	(37)	(23)	(28)
Tt	10	4	14	11	18	29	84	84	168	105	106	211
	(48)	(25)	(38)	(61)	(46)	(52)	(66)	(47)	(55)	(63)	(45)	(53)
Hw	17	8	25	9	18	27	67	53	120	93	79	172
	(81)	(50)	(68)	(50)	(46)	(48)	(52)	(30)	(39)	(56)	(34)	(43)
Ss	ND	ND	ND	ND	ND	ND	9	8	17	9	8	17
							(7)	(5)	(6)	(7)	(5)	(6)
Hn	2	3	5	1	1	2	4	4	8	7	8	15
	(10)	(19)	(14)	(6)	(3)	(4)	(3)	(2)	(3)	(4)	(3)	(4)
T.sp	2	0	2	0	0	0	0	0	0	2	0	2
	(10)		(5)							(1)		(0.5)
No. Exam.	21	16	37	18	38	56	128	179	307	167	234	401

( ): Percent infection.

As, *Ascaris lumbricoides*; Tt, *Trichuris trichiura*; Hw, hookworm; Ss, *Strongyloides stercoralis*; Hn, *Hymenolepis nana*; T.sp, *Taenia* sp.; ND, Not determined.

Table 3 Results of stool examination for intestinal protozoan cyst with formalin-ether centrifugation technique (MGL)

	Parcelamiento 46			Plan de Los Amates			Los Cerritos			Total		
	M	F	Total	M	F	Total	M	F	Total	M	F	Total
Eh	1	1	2	0	1	1	9	11	20	10	13	23
	(5)	(6)	(5)		(3)	(20)	(7)	(7)	(7)	(6)	(7)	(6)
Ec	14	11	25	7	17	24	25	46	71	46	74	120
	(67)	(69)	(68)	(39)	(44)	(43)	(20)	(28)	(25)	(28)	(37)	(33)
Ib	2	1	3	1	7	8	9	14	23	12	22	34
	(10)	(6)	(8)	(6)	(18)	(14)	(7)	(8)	(8)	(7)	(11)	(9)
Gl	1	2	3	2	1	3	9	8	17	12	11	23
	(5)	(13)	(8)	(11)	(3)	(5)	(7)	(5)	(6)	(7)	(6)	(6)
No. Exam.	21	16	37	18	38	56	123	166	289	162	200	362

( ): Percent infection.

Eh, *Entamoeba histolytica*; Ec, *Entamoeba coli*; Ib, *Iodamoeba buetschlii*; Gl, *Giardia intestinalis*.

cantly higher than that of corresponding group 15 years or older ($p<0.01$). However, there was no significant difference between males and females.

Immunoserological tests

ELISA, LAT and DDT were carried out on 10 IDT positive sera. A comparison of the results is summarized in Table 1. Out of the 10 IDT positives, 7 (70%) were positive by ELISA, LAT and/or DDT, 2 (20%) by all three examinations with remarkably high titers in ELISA and LAT, 3 (30%) by both ELISA and LAT, 1 by ELISA only and 1 by LAT only. Both DDT positive sera showed stronger precipitin band formation against *P. mex* antigen than against *P. m* antigen or *P. w* antigen (Fig. 2). In order to clarify the cross reactivity among other trematode infections, ELISA and DDT using *Fasciola hepatica* and *Schistosoma mansoni* antigens were carried out on IDT positive sera. Among them, case No. 9 showed positive reaction against *F. hepatica* antigen by ELISA with a titer of 1:640, and also by DDT (data not shown). This case was strongly suspected to be a fascioliasis.

Discussion

A parasitological survey was performed on inhabitants of Parcelamiento 46, Plan de Los Amates

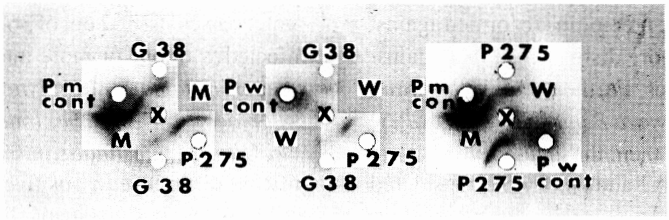

Fig. 2 Agar double diffusion test (DDT).
X, *Paragonimus mexicanus* antigen; M, *P. miyazakii* antigen; W, *P. westermanii* antigen; G38, Serum of No. 38; P275, Serum of a patient with *P. mexicanus* obtained in Peru; Pm cont, Serum of a patient with *P. miyazakii*; Pw cont, Serum of a patient with *P. westermanii*.

and Los Cerritos, State of Santa Rosa, Guatemala. The former two villages were revealed to be endemic areas of paragonimiasis by the investigation of crabs belong to *Pseudothelphusa* spp., the second intermediate host of *Paragonimus*. The infection rate of metacercariae of *Paragonimus* in the crabs was very high (Tongu *et al.*, 1995). Furthermore, the fact that inhabitants were accustomed to ingesting the crabs suggested the existence of human paragonimiasis. IDT using *P. mex* antigen was carried out on a total of 412 inhabitants. However, we could get only a small number of positives in this study, the reason being that the subjects of IDT in Parcelamiento 46 and Plan de Los Amates were mainly school children. It has been widely accepted that the positive ratio of IDT in paragonimiasis is correlated to age (Yokogawa *et al.*, 1955; Yokogawa *et al.*, 1983). It was the grain harvesting season in this district when this survey was carried out, and adult inhabitants could not be gathered for the examinations.

Among the total of 10 IDT positives, the number of positives of each immunoserological examination was 6 by ELISA, 6 by LAT and 2 by DDT, (Table 1). The results of ELISA and LAT were in good agreement. However, it is well known that these methods are so sensitive that false positive reactions often occur. The evaluations, we employed in the examinations whether positive or negative, had been determined for use in Japan. However, we could not agree with the argument that in the tropics where there is a high prevalence of parasitic infections, the threshold should be set at higher dilution, since low titers of ELISA and LAT had been observed in a few cases of egg positives (Kobayashi *et al.*, 1988). There were 2 IDT positives with not only *P. mex* antigen but also *P. m* and *P. w* antigens. However, the precipitin arc formed against *P. mex* antigen was more distinct than that against the others in both cases. Furthermore, spar formation was observed between *P. mex* and *P. m* or *P. w* (Fig. 2). In this study, then, the 2 cases which were positive for ELISA, LAT and DDT were considered to be paragonimiasis.

Stool examination for helminth eggs was carried out on 401 samples with both the Kato-Katz technique and MGL or one of them. The prevalence rate of soil-transmitted nematode, that is, *Ascaris lumbricoides* (28%), *Trichuris trichiura* (53%) and hookworm (43%), was very high. Other helminth infections proved by stool examination were *Strongyloides stercoralis* (6%), *Hymenolepis nana* (4%) and *Taenia sp.* (0.5%). But no eggs of *Paragonimus* were found from either stool or sputum.

In our survey in 1981 in Peru, out of 290 stools 25.2% were positive for *Ascaris lumbricoides* eggs, 17.2% for *Trichuris trichiura*, 2.1% for hookworm, 1.4% for *Enterobius vermicularis*, 2.1% for *Trichostrongylus* sp., 2.1% for *Fasciola hepatica*, 16.9% for *Hymenolepis diminuta*, 1.4% for *Taenia sp.*, 1.0% for larvae of *Strongyloides stercoralis* and 1 case (0.3%) for *Paragonimus* eggs, and one additional case of paragonimiasis was found by sputum examination. In Ecuador in 1981, *Ascaris lumbricoides* eggs were found in 66.0% out of 294 stools examined, 88.1% *Trichuris trichiura*, 69.4% hookworm, 2.7% *Hymenolepis nana* and 1 case each of *Paragonimus, Fasciola hepatica, Enterobius vermicularis, Trichostrongyloides sp.* and 3 cases of *Strongyloides* larvae. Further 18 cases of *Paragonimus* eggs were detected by sputum examination. The same surveys were carried out in Mexico (1983), Costa Rica (1986), Venezuela (1989) and Brazil (1991), but no eggs of *Paragonimus* were found from either stool or sputum. The results we obtained showed little difference among these countries with regard to infection rates of helminths. The detection rate of nematode eggs was not different between Kato-Katz and MGL techniques in this study, similar to previous studies. In Guatemala, the same results were obtained, and also, no difference in the prevalence rate was found between males and females; however, these helminths were commonly observed in the younger age groups.

As for the results of the MGL examination for protozoan cysts, 162 out of 362 stools (44.8%) were infected with one or more intestinal protozoa. The cysts of *Entamoeba histolytica* were found in 6%, *Entamoeba coli* in 33%, *Iodamoeba buetschlii* in 9%, *Giardia intestinalis* in 6%. There was no significant difference in positive ratio between males and females, but a statistically high infection rate was seen in the age group of 14 years and below ($P<0.01$). Primitive living conditions, lack of a clean water supply and a poor sewage system may well account for the high infection rate of intestinal protozoa among the inhabitants.

In Guatemala, Caballero first reported *P. rudis* from skunk, *Mephilis macrora macrora* (Caballero, 1946, 1956). However, he simply used the oldest name, *P. rudis*, which is regarded as *nomen nudum* at present (Voelker *et al.*, 1981), without any taxonomical discussion. Miyazaki (1955) determined the specimen which had been reported as *P. rudis* by Caballero to be *P. kellicottii* by the typical shape of the spines and ovary. Subsequently, Miyazaki and Ishii (1968) corrected the Guatemalan *Paragonimus* to *P. mexicanus*. Nevertheless, these descriptions were based on the flukes from naturally infected animals, and no human paragonimiasis had been mentioned yet. In our survey, we found strongly suspected human cases of paragonimiasis. Although absolute reliance can be placed upon immunoserological diagnosis in paragonimiasis (Yokogawa and Tsuji, 1962, Yokogawa *et al.*, 1974; Kobayashi *et al.*, 1988), further investigations are required in order to clarify the endemic nature of the infection and to establish a system of prevention.

Acknowledgments

The authors thank all staff members of Laboratorio Multidisciplinario, Facultado de Ciencias Medicas, Universidad de San Carlos de Guatemala for all the facilities provided field work. We also thank Professor Rafael Lamothe Argmedo, Instituto de Biología, Universidad Nacional Autonoma de México for helpful discussions.

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