Animals

Cats and dogs used for experimental infections were purchased from markets in the Shanghai city area. A badger of unknown geographic origin was kindly donated by the Shanghai Zoo. Deworming with praziquantel was carried out one week before infection with metacercariae isolated from crabs collected from Yongjia County.

Adult worm specimen preparation

The adult worms were fixed with 70% alcohol after being lightly compressed. After staining with carmine hydrochloride the specimens were mounted on a slide for microscopic examination.

Karyotype study

Chromosome slides were prepared according to Yao *et al* (1996) with some modifications. Adult worms of *P. westermani* were collected from the lungs of dogs infected approximately 300 days earlier. The worms were washed three times with warm normal saline and put into a solution containing 0.01% colchicine and then incubated at 37°C for two hours. Testis tissue of each worm was dissected and put into 0.075 mol/l potassium chloride for 30 minutes. The tissue was then fixed/rinsed in methyl alcohol/glacial acetic acid (3:1) (three changes of 15 minutes each). The tissue was macerated and spread onto a slide, placed at 60°C and a drop of 60% glacial acetic acid was added. The slide was dried by being passed quickly through a flame. Giemsa's stain was used before microscopic examination.

Patients

Patients' records were collected from the departments of infectious diseases of three hospitals (*ie*, Yongjia County People's Hospital, Wenzhou City No. 5 People's Hospital and Wenzhou City No. 8 People's Hospital). About half of the records were from these hospital patients during the present project (1996-2000), and the other half from the period of 1980 to 1995.

Immunological tests

Intradermal test: Adult *P. westermani* antigen at a dilution of 1:2000 was prepared for the intradermal test. A papule equal to or greater than 13 mm in diameter, 20 minutes after injection with the antigen, was scored as positive.

Indirect hemagglutination test (IHA) and ELISA: Both techniques were used for antibody detection. Adult worms were used as antigen. ELISA kits were purchased from Department of Parasitology, Nanjing Medical University. For IHA, when the sensitized erythrocytes coagulated together in an antigen dilution of 1:10 or more, the test was considered to be positive.

Metacercaria membrane reaction (MMR): Cysts of *P. westermani* were isolated from fresh water crabs and incubated in 10% pigs' bile at 40°C for 4-6 hours to yield freshly excysted metacercariae. Five excysted metacercariae were put onto a cavity slide and 3 drops of serum added. A cover slide was put on, and its edges sealed with vaseline or paraffin wax. Slides were observed under a low-power microscope after incubation at 37°C for 24-28 hours. If a thin, colloid membrane, either focal or covering most or whole body of the metacercaria, was seen in the specimen, a positive reaction was recorded (Wang *et al*, 1988).

RESULTS

Parasitological survey

Measurements of metacercarial cysts isolated from crabs in Yongjia are shown in Table 1. The cysts are basically round with a two-layered wall and milky white in color. The diameters of the cysts were quite similar (average 331.8-338.3 μm; range 292.4-380.1 μm). The outer wall was thinner (average of 8.47 μm) and the inner wall thicker (average of 16.97 μm). Inside the cyst the metacercaria fills almost the entire space available and has a median, large black excretory sac flanked on both sides by an intestinal cecum (Fig 1).

Table 1
Sizes of cysts of *P. westermani* from crabs collected from different endemic localities in Yongjia County.

Endemic areas	No. measured	Diameter (μm) X±SD (Range)	Thickness of cyst wall (μm) X±SD (Range)	
			Inner wall	Outer wall
Sidu	100	335.6 ± 16.3 (292.4 - 380.1)	17.1 ± 1.8 (11.6 - 21.6)	9.1 ± 1.1 (8.3 - 11.6)
Zhangxi	100	334.5 ± 17.5 (299.7 - 365.5)	17.0 ± 2.6 (11.6 - 23.2)	7.8 ± 1.1 (5.0 - 10.0)
Doumen	50	338.3 ± 18.0 (292.4 - 365.5)	16.2 ± 2.2 (13.3 - 21.6)	8.3 ± 1.2 (6.6 - 11.6)
Daruoyan	60	331.8 ± 16.5 (292.4 - 365.5)	-	-

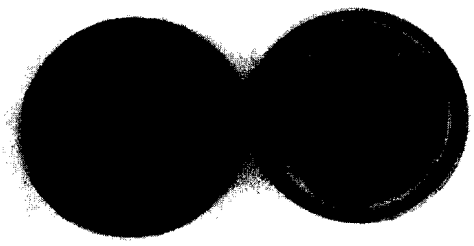

Fig 1–Cyst of *Paragonimus westermani* from a crab (*Sinopotamon sp*) from Yongjia County, Zhejiang Province (x 40).

Fig 2–Unsplit spines on the surface of a juvenile worm aged 38 days from an infected cat (SEM x 1,500).

P. westermani worms of different ages collected from experimentally infected animals (7 cats, 13 dogs and a badger) were measured as flattened whole-mount specimens (Table 2). Thirty days after infection, most worms were located in the thoracic and abdominal cavities and all were juvenile. In one cat, 44 days after infection, a single adult worm with eggs in its uterus was found in the lung; the remaining worms in the cat were small juveniles in the thoracic and abdominal cavities. In our series, adult worms were found in the lungs from day 58 onwards. After that time, only a very few small immature worms remained in the thoracic and abdominal cavities. The length and width of worms change with age, as shown in Table 2. The widest part of the body is slightly posterior to the ventral sucker. Scanning electron micrography (SEM) shows that undivided cuticular spines are dense on the body surface of the worm below 116 days of age from both infected cats and dogs (Fig 2). In older worms, some spines become partially or completely split longitudinally and may appear as pairs of spines (Fig 3). From day 378 on, unsplit (single), paired and grouped spines can be seen on the surface of the worms (Fig 4). Other features of adult morphology (size, ratio of suckers, size and shapes of ovary and testes) correspond exactly with *P. westermani* as described by Fan (1994) (Fig 5).

Fig 3–Splitting and paired spines on an adult worm aged 125 days from a dog (SEM x 1,500).

Fig 4–Grouped spines on an adult worm aged 378 days from a dog (SEM x 1,500).

Fig 5–An adult worm of *P. westermani*, aged 96 days, collected from a dog (x 8).

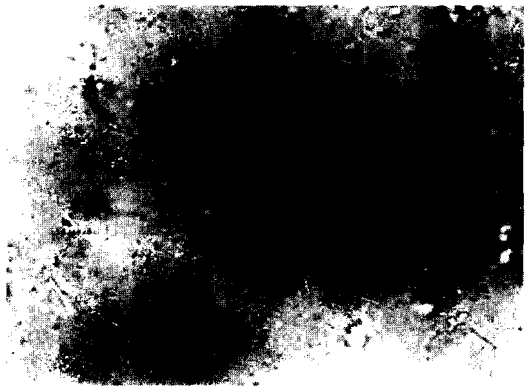

Fig 6–An eggs of *P. westermani* from the lung cyst of a dog, preserved in formalin (x 40).

One hundred eggs were measured from adult worms from each endemic locality (Table 3). Eggs from different localities differ little in size. The average length and width of the eggs are 76.2 μm and 45.6 μm respectively (ranges 66.4-92.9 μm and 38.2-54.8 μm). The egg is elliptical, yellow in color, and has an operculum at one end. The eggshell is often thickened at the non-operculated end (Fig 6). The characteristics of the eggs are similar to those reported in Chinese literature (Fan, 1994).

Clinical presentations

A total of 94 patients were included in this paper for clinical analysis: 63 were males and 31 females. Their ages ranged from 18 months to 53 years with an average age of 15.4 years; seventy-two (76.6%) were below the age of 20. Almost all of them were either children (pre-school and school age), or farmers. All, with the exception of one who had a history of drinking only stream water, had eaten raw or undercooked fresh-water crabs.

The earliest symptoms included coughing (42), tiredness (37), fever (34), bloody sputum (31), loss of appetite (29) chest pain (23) and headache (17). Less common were night sweating, nausea, vomiting, pallor, emaciation and chest tightness. Five patients showed pleural effusion identified by pleuracentesis and/or chest X-ray; three had ascites.

Table 2
Measurement of the sizes of different ages of *P. westermani* in animals infected with the cysts collected from different endemic localities.

Days after infection	Animal	Endemic areas	No. worms exam	Length (mm) $\bar{X}$±SD	Width (mm) $\bar{X}$±SD	Length/width
30	Cat	Sidu	20	2.0±0.4	1.1±0.1	(1.2-2.7):1
44	Cat	Sidu	22	3.8±0.5	2.1±0.5	(1.3-2.4):1
54	Cat	Zhangxi	5	3.2±0.3	2.3±0.5	(1.2-1.6):1
58	Cat	Zhangxi	11	3.2±0.7	2.4±0.5	(1.2-1.6):1
87	Dog	Sidu	20	7.5±1.4	3.8±0.7	(1.4-2.4):1
105	Dog	Sidu	10	6.2±1.0	3.0±0.6	(1.8-2.5):1
115	Dog	Doumen	10	11.2±1.2	5.8±0.6	(1.6-2.4):1
116	Dog	Daruoyan	8	9.6±2.2	5.0±1.4	(1.7-2.4):1
120	Dog	Sidu	30	8.0±1.9	4.7±1.0	(1.2-2.1):1
125	Cat	Doumen	26	9.3±1.3	5.2±0.7	(1.1-2.1):1
125	Cat	Sidu	5	5.6±1.6	3.4±0.8	(1.2-2.0):1
125	Dog	Sidu	20	7.5±1.3	4.1±0.7	(1.4-2.1):1
166	Dog	Sidu	16	7.9±0.5	3.8±0.5	(1.7-2.6):1
169	Dog	Sidu	5	6.5±1.3	4.1±0.7	(1.2-2.1):1
227	Dog	Daruoyan	5	11.1±1.6	6.0±0.9	(1.6-2.0):1
246	Badger	Unknown	14	7.4±1.3	4.5±0.7	(1.2-2.1):1
305	Dog	Daruoyan	6	8.8±2.0	4.5±1.4	(1.7-2.3):1
315	Dog	Sidu	30	10.2±1.3	6.0±0.6	(1.3-2.1):1
378	Dog	Daruoyan	7	6.2±0.9	3.3±0.5	(1.6-2.4):1
410	Cat	Zhangxi	5	9.0±1.3	4.9±0.7	(1.6-2.3):1
455	Dog	Zhangxi	10	10.9±1.2	6.2±0.8	(1.5-2.1):1

Table 3
Sizes of eggs from adult worms raised from cysts in crabs collected from different endemic localities in Yongjia.

Endemic areas	No. measured	Length (μm) $\bar{X}$±SD	Width (μm) $\bar{X}$±SD	Length/width
Sidu	100	73.1±3.8	44.1±4.1	(1.04-1.96):1
Zhangxi	100	78.6±4.6	45.9±3.2	(1.41-1.96):1
Doumen	100	77.3±3.2	45.5±2.9	(1.39-1.88):1
Daruoyan	100	75.8±3.7	46.7±3.0	(1.41-1.96):1

Clinical types: 67 patients had pulmonary or pulmo-pleuritis symptoms. In 20 cases the noteworthy symptoms were of hepatic injury; there were five cerebral and two subcutaneous nodular presentations.

In the cases of pulmonary-type paragonimiasis, the main symptoms were coughing and brownish sputum with blood. On chest X-ray or computerized tomography (CT), a cavernous shadow, sometimes with tunnel in connection with the lung, and a mass of ring-like shadow were typical signs suggesting *P. westermani* infection (Fig 7). Other signs were atypical, including infiltration or inflammation in the lungs and pleural effusion. Hydrothorax

Fig 7–A cavernous shadow on CT image of the right lung in a patient with paragonimiasis westermani.

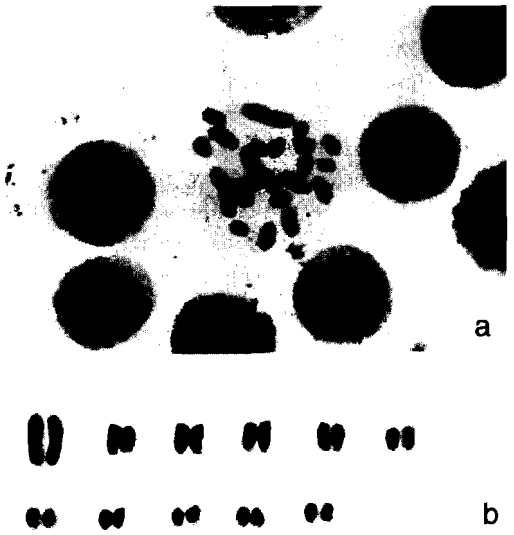

Fig 8–*P. westermani* in Yongjia from a dog a. Meiosis metaphase, b. Karyotype plate, 2n=22, n=11.

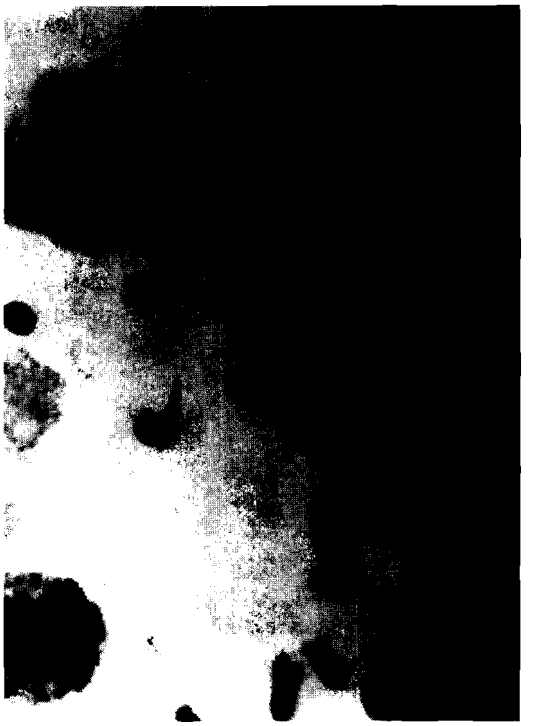

Fig 9–*P. westermani* in Yongjia from a dog showing mitosis metaphase of a testis cell and several sperms.

was found in five cases. Eosinophilia was moderate: in 44 cases the mean total leukocyte count was 17.7 x 10^9/l (range 6.4 to 54.6 x 10^9/l), the mean eosinophil direct count was 10.9 x 10^6/l (range 1.12 to 44.8 x 10^6/l) and mean percentage of eosinophil was 29.0±20.6 (X±SD) with a range of 2-78%. The mean erythrocyte sedimentation rate (ESR) in five cases measured was 26 mm at the end of one hour. The mean age was 17.1 years in this series (range 3 to 43 years).

In the series of 20 cases of the hepatic type, the clinical presentations were mainly fatigue, loss of appetite, abdominal flatulence, hepatomegaly and tenderness and eosinophilia. Impaired liver function was indicated by an increase of serum alanine aminotransferase (ALT) in a few cases and a significant increase in the percentage of serum γ-globulin upon electrophoresis in most of the cases (average 0.342±0.073). Eosinophilia was very distinct: among 19 cases examined, the mean total leukocyte count was 29.0 x 10^9/l (range 7.6 to 64.0 x 10^9/l), the mean eosinophil direct count was 17.8 x 10^6/l (range 4.30 to 52.8 x 10^6/l) and mean percentage of eosinophil was 58.2±19.5 (X±SD) with a range of 24-97%. Values for these three indices (leukocyte count, eosinophil percentage and eosinophil direct count), in the hepatic type of the disease were

statistically significantly different (all $p<0.01$) from values obtained in the series of the pulmonary type. The mean ESR in 13 cases was 66.8 mm at the end of one hour, a much higher value than seen in the pulmonary type. The hepatic type of the disease was mostly found in children below 10 years of age, and was usually accompanied by fever. According to their histories, infections in these cases were probably recent (1-3 months). The mean age in this series was 7.2 years. One patient was 34 years of age and the others all below 10 years old (18 months to 9 years).

In the five cases of cerebral paragonimiasis, the symptoms included vomiting (4 cases), headache (4), epilepsy (2), fainting attack (1), fecal and urine incontinence (1) and weakness of the lower limbs (1). The presence of many eosinophilic leukocytes in the cerebrospinal fluid was a useful aid to diagnosis, apart from the other symptoms of paragonimiasis.

Subcutaneous nodules were found in two cases and biopsy revealed granulomas with abundant eosinophils and Charcot-Leyden crystals. Serological tests for *P. westermani* were positive, although no pulmonary symptoms and signs were evident, nor were eggs seen in the sputa.

Diagnosis: Parasitological diagnosis was made by the finding of typical *P. westermani* eggs in the sputa after 24-hour specimens were digested using 10% sodium hydroxide (42 cases) and in feces (two child cases). Pathological examination of tumor-like brain tissue from an operative patient showed *Paragonimus* eggs in one case. In the remaining 49 cases, parasitological evidence was not found, and a clinical diagnosis was made according to: i) a history of living in an endemic area and eating raw or under-cooked fresh-water crabs; ii) clinical symptoms and/or signs suggesting the disease (*eg* pulmonary shadows on X-ray or CT and peripheral blood eosinophilia); iii) positive serological tests using *P. westermani* adult worm antigen. Tests included MMR (all of 30 cases tested were positive including 10 cases negative for eggs in the sputa), IHA (54 out of 56 tested were positive including 16 out of 18 cases negative for eggs) and ELISA (all of 57 tested were positive including 25 cases negative for eggs in the sputa). In the intradermal test, 90 out of 91 cases were positive; iv) disappearance of signs and symptoms after treatment with praziquantel and/or bithional (Bitin).

Treatment: Sixty-six cases were treated with praziquantel. The dosage-schedules included; i) a total dose of 100 mg/kg body weight divided into 4-6 doses spread over 2-3 days (22 cases); ii) a total of 150-250 mg/kg administered as above over 2-3 days (44 cases). Five cases were given two treatment courses owing to unsatisfactory results.

Treatment with Bitin was offered to a further 27 patients: a 50 mg/kg/day, divided into three doses, was given on alternate days for 15 treatment days (total course 29 days). Four patients with cerebral paragonimiasis were each given two courses of treatment, one week apart, with satisfactory results. Another cerebral case was treated with Bitin, followed by praziquantel. Twenty-two cases with other than cerebral paragonimiasis were treated with Bitin at a daily doses of 50 mg/kg on alternate days for 15 treatment days. One patient who was seriously ill left hospital for economic reasons, without treatment and died several days later.

The chemotherapeutic efficacy of both agents was high. Most patients were cured and return to normal life. For the 15 hospitalized cases treated with praziquantel, sputum was checked for eggs every 1-2 days. In 12 cases, eggs disappeared from the sputum after 11-15 days. In the three cases in which eggs were still present, the daily egg count was reduced by 89.9% (from an average of 1,388 eggs/day before treatment to an average of 140 eggs/day 25 days after chemotherapy). In all 15 patients, symptoms either disappeared or were significantly reduced. The cerebral cases made a full recovery. In hepatic cases, hepatomegaly either reduced in size, or could not be palpated. Appetite improved, flatulence diminished and ascites disappeared. The side-effects

of praziquantel treatment were mild and tolerable, even after larger total doses. One or two days after praziquantel treatment began, most of the patients felt much better. Although gastrointestinal disturbance was often complained of by Bitin-treated patients, it was tolerable and did not cause treatment to be discontinued.

At a two-month follow-up after praziquantel treatment, 18 of 33 cases who had been egg-positive before treatment showed no eggs in the sputum. To the remaining egg-positive patients, one or two additional courses of praziquantel were given. At a six-month follow-up, 17 out of 20 (85%) cases were negative for eggs. After repeated treatment, all the 20 cases followed five years after the last treatment were egg-negative with a final cure rate of 100%.

Karyotype study

Testes dissected from a single worm from each of 15 experimentally-infected animals (nine dogs and six cats) were used for karyotype studies. Worms originating from all four endemic townships in Yongjia were included. The adult flukes were collected after eggs appeared in the stool of the hosts. Karyotypes were successfully determined from worms from five of the dogs and from all six cats. In all cases, 22 chromosomes were seen. The haploid number for the genus *Paragonimus* is 11, therefore all worms were diploid (Fig 8). Sperm was seen in the seminal receptacles of parasites from 14 of the host animals (Fig 9), indicating that they had mated with diploid individuals. The chromosome number in the remaining specimens was determined to be 22.

DISCUSSION

Our work is in agreement with the many other published morphological studies of *P. westermani*. Our SEM observation on surface spines is noteworthy. The type of spine is one of the criteria used in the classification of the genus and hence ontogenetic changes in these must be considered. Spines on adults younger than 116 days of age were always single, lacking the longitudinal splitting seen in some spines on older adults. In old worms (*ie*, >378 days) Mixed spine types can be seen on the surface: single, paired and grouped forms.

The work of Miyazaki (1977) and Terasaki (1980) demonstrated that different ploidy forms of *P. westermani* exist and have different biological properties. Diploids occur throughout the range of the species. Triploids occur in Japan, Korea and China (Blair *et al*, 1999). Eggs, metacercariae and adults of triploid worms are larger than those of diploids (He *et al*, 1981; Yuan, 1984; Fan, 1994). Diploid worms produce normal sperm and must cross-fertilize another for viable eggs to be produced. The presence of sperm in the seminal receptacle of a worm shows that it has mated with a diploid specimen. Triploids do not produce sperm, are parthenogenetic and thus do not require a mate to produce viable eggs. A single triploid may form a lung cyst within which it can produce viable eggs. Unmated diploids, however, will not form lung cysts and generally remain in the thoracic cavity or cause atypical, mild symptoms by migrating in the viscera (Fan, 1994). It is a widely held view in China that diploid worms ('small strain') cause little disease in humans and that pulmonary symptoms, including the appearance of eggs in the sputum, are produced only by triploids ('large strain') (Fan, 1994; He *et al*, 1981; 1982; Li *et al*, 1987; Yuan, 1984; Wang *et al*, 1986; Zhang and Wang, 1986; Wang, 1998).

Many of our patients had eggs in the sputum and typical pulmonary symptoms despite the fact that all the worms we studied in Yongjia were of the diploid type. If triploid worms occur at all in Yongjia, they must be rare. We demonstrated ploidy directly by karyotyping and also indirectly by the sizes of eggs and metacercariae and by the presence of sperm in the seminal receptacle. Production of pulmonary symptoms by diploid worms has been noted by other authors. Wang *et al* (1986) and Wang (1998) found *P. westermani* from Shaoxing County, Zhejiang, to be diploid.

Shaoxing is a famous endemic area of paragonimiasis with high prevalence and severe morbidity due to pulmonary symptoms recorded during the 1930s to 1950s. The authors (Wang *et al*, 1986; Wang, 1998) suggested that a high prevalence of the diploid form can induce pulmonary symptoms probably because a number of worms would occur in each patient, mate and form lung cysts. In the 1990s, after an intensive health education and control effort, along with a rise in living standards, prevalence in Shaoxing has decreased sharply. However, bloody sputum is still seen in a few cases and the karyotypes of worms raised from experimental animals are still diploid in form. Lei *et al* (1987) collected infected crabs from the areas in which pulmonary paragonimiasis cases were diagnosed in Yueqing County, Zhejiang, adjacent to Yongjia County and found a sputum egg-positive rate of 10.9% (37/341 among those with a positive intradermal test, the positive intradermal test rate being 34.8% in a total population of 1,434). Sizes of metacercarial cysts suggested that these worms were diploid; karyotyping of adults from experimental hosts revealed 22 chromosomes, and sperm was found in the seminal receptacle. They suggested that diploid-form *P. westermani* might cause typical pulmonary manifestations. In our study, the level of metacercarial infection in the intermediate host crabs we collected was not high. However, pulmonary symptoms were common and eggs were found in sputa and/or feces in many cases. We agree with Lei *et al* (1987), who worked in the neighboring county, that diploid *P. westermani* can cause typical pulmonary manifestations, where human prevalence is high; however, even where intensity of metacercarial infection in crabs is low, and human prevalence nowadays is not high, as in our series, pulmonary symptoms can also be caused by diploid *P. westermani*.

Our clinical observations show that apart from well-documented pulmonary, cerebral and subcutaneous types of paragonimiasis westermani, there exists a distinct hepatic form of the disease: the first report of this form was published by Shao (1984). On analysis of these cases, several characteristics were found. Firstly, most (19/20) were children below 10 years of age with a relatively recent history of eating raw or under-cooked crabs; secondly, hepatic injury, shown by hepatomegaly, tenderness of the liver and related symptoms (*eg* loss of appetite, flatulence, fatigue, fever, and even ascites), as well as elevated serum ALT, were mild or moderate; thirdly, laboratory examinations revealed a very significant increase in peripheral eosinophils (24-97%; mean 58.2%), ESR and serum γ-globulin, compared with the pulmonary type. These observations suggest that the hepatic type of the disease may be induced by juvenile fluke penetration through the liver.

In conclusion: i) the flukes collected from many streams in four townships of Yongjia County belong to the 'small form' or diploid form of *P. westermani*. This was demonstrated by measurements of eggs and metacercariae, presence of sperm in the seminal receptacle and by karyotyping; ii) clinical studies showed many patients with pulmonary symptoms and eggs in the sputum; the diploid *P. westermani* can cause pulmonary symptoms; iii) the histories of a number of young (most <10 years of age) patients with a distinctive clinical picture of hepatic injury suggested that these patients only recently acquired their infections. The so-called 'hepatic form' of paragonimiasis might be induced by juvenile worms penetrating the liver. Small children may be hypersensitive to the worm antigens.

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PARAGONIMIASIS IN YONGJIA COUNTY, ZHEJIANG PROVINCE, CHINA: CLINICAL, PARASITOLOGICAL AND KARYOTYPIC STUDIES ON *PARAGONIMUS WESTERMANI*

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Abstracts. Paragonimiasis in Yongjia County, Zhejiang Province, China, has been of such public health concern that a specialized Institute for Paragonimiasis Control has been established there. The study reported here involved both parasitological research on *Paragonimus westermani* in the endemic areas and a clinical analysis of 94 cases of paragonimiasis. Eggs were found in sputum, feces and brain tissue in 45 cases. Twenty patients, 19 of whom where children under 10 years of age, showed hepatic injury. Treatment with praziquantel or bithionol had satisfactory results in all cases. Dimensions of the parasite eggs, metacercariae from freshwater crabs and adult flukes suggested that the form of *P. westermani* in Yongjia is the 'small type': a suggestion supported by karyotypic studies on gonad cells from adult flukes collected from experimentally infected animals as all were found to be diploid. In conclusion, the 'small type' or diploid form of *P. westermani* is the cause of paragonimiasis pulmonary symptoms in Yongjia.

INTRODUCTION

Paragonimiasis in Yongjia County, Zhejiang Province, China, has been of such public concern that a control team for the disease was set up in 1972 and a specialized Institute for Paragonimiasis Control was established there in 1989.

During the past five years, a comprehensive study on *Paragonimus westermani* and paragonimiasis has been conducted in Yongjia County. The work included a field survey and laboratory studies, clinical observation of hospitalized cases and their treatment and a retrospective review of some older cases in Yongjia and Wenzhou. One focus of the work has been to determine the ploidy of *P. westermani* in Yongjia: diploids, triploids and tetraploids have been reported in China (Blair *et al*, 1999). The relationship between ploidy and clinical manifestations has been debated in the Chinese literature for many years.

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MATERIALS AND METHODS

Locality studied

Four townships where paragonimiasis is endemic were selected for field studies: Sidu and Doumen in the eastern part of Yongjia County; Daruoyan in the center of the county; Zhangxi in the north.

Crabs

The second intermediate hosts, fresh water crabs of the genus *Sinopotamon*, were collected from many streams in the four townships.

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