[Jpn. J. Parasitol., Vol. 35, No. 5, 437–443, October, 1986]

Immunodiagnosis of Schistosomiasis Haematobia using Eggs Collected from Human Urine as Antigen

MIZUKI HIRATA[1], TOMOYUKI HIEDA[1], HIROSHI TSUTSUMI[1], MASAAKI SHIMADA[2], KATSUYUKI SATO[2], DAVY K. KOECH[3] AND EDITH WAMBAYI[3]

(Received for publication; September 12, 1985)

Key words: *Schistosoma haematobium, Schistosoma mansoni,* egg antigen, enzyme-linked immunosorbent assay, circumoval precipitin test, counter immunoelectrophoresis

Introduction

As antigen for immunodiagnosis of schistosomasis haematobia, the various evolutive stages of *Schistosoma mansoni* have been widely employed, since the life cycle of *S. haematobium* is difficult to control in the laboratory, compared with that of *S. mansoni.* In endemic area of schistosomiasis haematobia, it is easy to collect eggs of *S. haematobium* from the urine of the infected patients and then to use these eggs for immunoserological tests. Previously, we have examined such eggs and found them to be enveloped by immune precipitates prior to their incubation with human sera. Subsequently it has been suggested that these eggs be treated with pepsin-HCl solution to remove such precipitates (Koech *et al.,* 1984).

The present study deals with the evaluation of the enzyme-linked immunosorbent assay (ELISA), circumoval precipitin test (COPT) and counter immunoelectrophoresis (CIE) using eggs treated with pepsin-HCl solution. Since the geographical distributions of *S. haematobium* and *S. mansoni* are sometimes close to each other or overlap, sera were collected from both endemic areas for comparative studies. We also employed COPT to investigate the difference in human urine egg reactivity from hamster liver egg reactivity.

[1]*Department of Parasitology, Kurume University School of Medicine, Kurume 830, Japan.*
[2]*Department of Parasitology, Institute for Tropical Medicine, Nagasaki University, Nagasaki 852, Japan.*
[3]*Communicable Diseases Control and Research Project, Kenya Medical Research Institute, Nairobi, Kenya.*

Materials and Methods

Samples

Stools, urine and blood were collected from 109 outpatients who visited Kinango Hospital and Kwale Hospital in the Kinango Division, Kwale District, Kenya. The prevalence rate of *S. haematobium* in children of this area was around 97% according to our preliminary survey. No *S. mansoni* eggs were detected in parasitological examinations of stools. In a study on the cross-reactivity of egg antigen, specimens (stools and blood) were also collected from 105 residents living in an *S. mansoni*-endemic area, Mwea Division, Kirinyaga District – where no *S. haematobium* infection has been known. Sera of 46 Japanese consenting outpatients who had no history of schistosomiasis served as a negative control group. Cross-reactivity for sera of subjects with a parasitic disease other than schistosomiasis was studied using 26 serum samples from Japanese patients with anisakiasis (14), clonorchiasis (3), paragonimiasis (3), gnathostomiasis (3), ascariasis (1), amoebiasis (1) and toxocariasis (1).

Parasitological examination

Urine samples (10 ml) were concentrated by centrifugation (86 samples) or by the nucleopore filtration technique (18 samples). Stools were examined by the formalin-ethyl-ether concentration technique. For *S. mansoni* eggs, the stools were examined quantitatively using a sample of 20 mg by the "quick" Kato smear technique (Peters *et al.*, 1980).

Antigen preparations

We collected *S. haematobium* eggs from urine passed by elementary shcool children in Mwachinga Village, Kwale District, and other *S. haematobium* eggs from the livers of hamsters experimentally infected 12 weeks previously. Pooled urine was passed through a 300 mesh-sieve, and eggs trapped on the sieve were collected and washed several times with 1.7% saline solution. The eggs were subsequently treated with pepsin-HCl solution (pH 1.8) at 37°C for 2 hrs to remove immune precipitates and then washed with saline solution again (Koech *et al.*, 1984). Eggs from infected hamsters were obtained from the livers by the digestion method (Yokogawa and Sano, 1966).

The eggs isolated were fragmented in hypotonic saline (0.1%) using a motor-driven Teflon homogenizer and then subjected to repeated freeze-thawing (Tsuji, 1974). The homogenates were extracted over 2 days in a cold room and then centrifuged at 15,000 rpm for 1 hr. After sterilization by passing through a 0.45-μm Milipore filter, the supernatant was kept at 4°C. Lyophilized eggs were employed for COPT.

Serological tests

The method of COPT and classification of the reaction was performed according to Yokogawa *et al.* (1967).

CIE was carried out with the use of 1.0% agarose (Nakarai Chem. Co.) dissolved in veronal-HCl buffer (pH 8.2, 0.025 M). Egg antigen was used at a protein concentration of 200 μg/ml (Lowry *et al.*, 1951). Electrophoresis and reading of reactions were performed as previously reported (Hirata *et al.*, 1977).

ELISA was performed according to the method specified by the WHO (1976) and by Nakao *et al.* (1981). The egg filter-sterilized antigens were diluted in carbonate buffer (pH 9.6, 0.05 M) to give a protein concentration of 5 μg/ml, and 200 μl of each were poured into the wells of a Dynatech Microplate (M129A). The plate was incubated for 2 hrs at 37°C and them kept at 4°C until use. After washing by 0.15 M phosphate-buffered saline, pH 7.4 (PBS), containing 0.05% Tween 20, the wells were covered with 250 μl of 1% BSA. Two hundred microliters of sera (dilution 1:100) and the same volume of peroxidase-conjugated anti-human IgG (dilution 1:2000) (Cappel Laboratories) were used in the subsequent reactions. A mixture of o-phenylene diamine and H_2O_2 was used as the substrate, and the reactions were stopped with 8 M sulphuric acid. Optical density was measured at 492 nm with a Hitachi-Corona Microplate Reader. All sera were placed into antigen-filled wells and antigen-free wells in duplicate and the mean ELISA values (after subtracting background values of sera) were analyzed. A judgement of positive was determined on the basis of an upper critical value of 99% rejection (Matsuda *et al.*, 1981).

Statistical analysis

Chi-squared Test or Fisher's Direct Method was used.

Results

When ELISA was repeated using different lot numbers of ELISA plates, the ELISA values obtained with negative control sera were found to be greatly different, as the mean values and 99% upper critical values were 0.063, 0.096 and 0.122, 0.313. However, no difference was seen in judgement of positive for several positive control sera. In the present study, data obtained from the first experiment were analyzed and values more than 0.1 were regarded as positive.

Table 1 shows the summarized serological results. When specificity of the antigen was examined using the 46 sera of Japanese para-

Table 1 Results of serological tests

		No. of sera tested	Positive No. (%)		
			ELISA	COPT	CIE
S. haematobium egg	Positive group	52	48 (92.3)	49 (94.2)	48 (92.3)
	Negative group	52	36 (69.2)*	44 (84.6)	37 (71.2)*
S. mansoni egg	Positive group	67	57 (85.1)	61 (91.0)	61 (91.0)
	Negative group	42	37 (88.1)	38 (90.5)	35 (83.3)
Japanese control sera		46	1 (2.2)	0 (0)	4 (8.7)

*P < 0.05 compared to an egg-positive group.

site-free individuals, positive reactions in the ELISA and COPT were very rare, but in the CIE, 4 sera (8.7%) showed non-specific reactions. In cross-reactivity excepting for *S. mansoni* sera, 2 (ELISA and CIE) and 1 (COPT) out of the 3 sera of the paragonimiasis patients reacted. The other infected sera did not show any reaction in the ELISA and COPT, while in the CIE, reactions with 1 clonorchiasis and 2 anisakisiasis patient's sera appeared.

Sera collected from the *S. haematobium*-endemic area, and from the *S. mansoni*-endemic area showed high rates of positive reactions (Table 1). Sensitivities observed with homologous *S. haematobium*-infected sera were 92.3%, 94.2% and 92.3% in the ELISA, COPT and CIE, respectively. With sera from the *S. mansoni*-endemic area, the sensitivities were 85.1% in the ELISA, and 91.0% in both the COPT and CIE, slightly less than the above homologous reactions. In egg negative from the *S. haematobium*-endemic area, the positive rates were significantly lower then those in egg positives, especially in the ELISA and CIE, while no difference was seen in any of the tests between the two groups in *S. mansoni*-endemic area.

The correlation between serological results and egg number excreted in *S. haematobium* infection is shown in Table 2. The positive rates gradually rose as the number of eggs increased. Contrastingly, in *S. mansoni* infection (Table 3), no difference could be seen among the groupos classified. Furthermore, no difference in the serological results by sex or age was observed in subjects from the both endemic areas (data not shown).

Table 4 shows the results of a comparative

Table 2 Relationship between serological results and excreted egg number in subjects from *Schistosoma haematobium* endemic area, Kwale District, Kenya

S. haematobium egg No.	No. of subjects	Positive No. (%)		
		ELISA	COPT	CIE
0	52	36 (69.2)*	44 (84.6)	37 (71.2)†
1–10	21	19 (90.5)	19 (90.5)	18 (85.7)
11–100	12	11 (91.7)	11 (91.7)	12 (100)
101–3000	19	18 (94.7)	19 (100)	18 (94.7)

Egg No. in 10 ml sample of urine.

*$P < 0.05$ compared to a group excreted 101 eggs or more.

†$P < 0.05$ compared to groups excreted 11 eggs or more.

Table 3 Relationship between serological results and excreted egg number in subjects from *Schistosoma mansoni* endemic area, Kirinyaga District, Kenya

S. mansoni egg No.	No. of subjects	Positive No. (%)		
		ELISA	COPT	CIE
0	42	37 (88.1)	38 (90.5)	35 (83.3)
1	23	19 (82.6)	21 (91.3)	21 (91.3)
2–3	20	18 (90.0)	18 (90.0)	18 (90.0)
4–61	24	20 (83.3)	22 (91.7)	22 (91.7)

Egg No. in a 20 mg sample of stool.

Table 4 Comparison of COP reactivity between *S. haematobium* eggs collected from human urine and from hamster liver

Endemic area	Source of eggs	No. of sera examined	Positive No. (%)	Incidence of COP grade (%)		
				I	II	III
S. haematobium	Human	32	28 (87.5)	10 (31.3)	9 (28.1)	9 (28.1)
	Hamster	32	28 (87.5)	5 (15.6)	13 (40.6)	10 (31.3)
S. mansoni	Human	60	52 (86.7)	16 (26.7)	14 (23.3)	22 (36.7)*
	Hamster	60	57 (95.0)	8 (13.3)	7 (11.7)	42 (70.0)

*$P < 0.01$ compared with eggs collected from hamster liver.

study on COP reactivity between eggs collected from human urine and those from hamster liver. Randomly sampled sera from both the endemic areas were used in testing. When sera from the *S. haematobium*-endemic area were incubated with eggs from human urine, grade I (regarded as a weak reaction) occurred at a higher rate (31.3%) than grade II (28.1%) or grade III (28.1%) (regarded as strong reactions). In contrast to this, the incubation with eggs from hamster liver produced a higher rate of grade II (40.6%) and grade III (31.3%) than that of grade I (15.6%). Similar results were obtained with sera from the *S. mansoni*-endemic area, though with a remarkable incidence of grade III (70.0%) (the strongest reaction) by eggs from hamster liver, being almost two times that (36.7%) by eggs from human urine. Furthermore, it was noted that sera from the *S. mansoni*-endemic area showed lower rates of grade I and higher rates of grade III in comparison with sera from the *S. haematobium*-endemic sera – indicating higher reactivity of *S. mansoni*-infected sera.

Discussion

Reports which evaluated *S. haematobium* eggs collected from human urine as immunodiagnostic antigen for ELISA are few and they are conflicting. Ogumba *et al.* (1982) attemted to use the eggs for the diagnosis of schistosomiasis haematobia, but sensitivity obtained by the extract from the eggs was found to be extremely low. In contrast, Humberger *et al.* (1982) fractionated a glycoprotein by ion exchange chromatography from the extract of the eggs and found the substance was highly reactive with a serum pool from schistosomiasis haematobia patients, though a large scale of study was not done. In the present study, crude extract from the eggs treated with

pepsin-HCl solution was found to give satisfactory sensitivities (92.3%–94.2%), in comparison with reported sensitivities (83.0%–100%) by ELISA using *S. haematobium* or *S. mansoni* adult worm antigen (Farag *et al.*, 1978; Salih *et al.*, 1978; Ismail *et al.*, 1979; Janitschke *et al.*, 1981; Ogunba *et al.*, 1982).

On parasitological examination, inhabitants living in the surveyed endemic areas were found to have various parasitic infections (hook worm, *Ascaris lumbricoides, Trichura trichuris, Entamoeba histrytica* and other species). There has been concern that infections of these species are involved in high percentages of sero-reactions in egg negatives (Table 1). However, they appear to be much less involved and evidenced by the cross-reactivity of schistosome species antigens in the ELISA (Hillyer and Risos, 1979; Ogumba *et al.*, 1982; Ishii and Owhashi, 1982). In this study, apart from *S. mansoni* sera the apparent cross-reactivity was noted in only sera of paragonimiasis patients. With regard to the high prevalence (97%) of *S. haematobium* fround in the school children in this area, the high positive rates in egg negatives may therefore be ascribable to the low sensitivity of urine or stool testing: Yogore *et al.* (1983) have reported that a single stool examination underestimated sero-positives by 50% and two stool examination reduced the value to 29% in school children with *S. japonicum* infection. In subjects from the *S. haematobium*-endemic area, positive rates in egg negatives were significantly lower than those in egg positives. This may be a reflection of the higher sensitivity of the urine testing compared to that of the stool testing.

Farag *et al.* (1978) compared *S. haematobium* and *S. mansoni* adult worm antigens in the diagnosis of schistosomiasis haematobia using ELISA and found that the homologous antigen was higher in sensitivity. In the present study, similar tendency was found with egg antigen in all the tests (Table 1) though the extent of difference was not remarkable. With regard to the degree of COP reaction, however, the antigen was less reactive with *S. haematobium* sera than with *S. mansoni* sera (Table 4). It has been reported that *S. mansoni* sera are generally more reactive than *S. haematobium* sera (Huldt, *et al.*, 1975; Schinski *et al.*, 1976; Michael *et al.*, 1979; Janitscke *et al.*, 1981).

With regard to the COP reactivity of eggs from humans, there are a number of different observations. Newsome (1958) has claimed that the use of this kind of egg appeared inadvisable due to the frequent formation of non-segmented precipitates which were not true reactions. In contrast, Hillyer and his coworkers (1980; 1981) observed much stronger reactions (blebs plus septates), in comparison with those by *S. mansoni* eggs from mouse livers, prompting them to recommend eggs from human urine. In the present study, satisfactory sensitivity and specificity were observed using eggs from human urine, in contrast to the report of Nesome. However the reactivity was not as high as that with eggs from hamster livers. The different observation appears to be attributable to the egg recovery process.

Our aim of the present study is to utilize serological methods to assess the *S. haematobium* control project which is currently underway in the Kwale District, in Kenya. Although a comparative study on the reactivity of *S. haematobium* eggs is still required to be undertaken, it can meanwhile be concluded that the antigen of eggs collected from human urine is practical and useful for the immunodiagnosis of schistosomiasis haematobia.

Summary

S. haematobium eggs collected from human urine were evaluated as an immunodiagnostic antigen for schistosomiasis. The eggs were used after pepsin-HCl treatment to remove the enveloping immune precipitates. Using ELISA, COPT and CIE, high sensitivities (92.3% to 94.2%) were found on reaction with homologous *S. haematobium*-infected sera. Sufficient specificity was observed with ELISA and COPT, but not with CIE. Sera from an *S.*

mansoni-endemic area were used for comparison. Although they were highly reactive, the sensitivities were slightly less in comparison with the homologous reactions and no correlation with excreted egg number was demonstrated. When the difference in the source of the eggs was studied using COPT, eggs from hamster livers gave stronger reactions than those from human urine. In conclusion, the present study reveals that eggs from human urine are practically useful as antigen for the immunodiagnosis of schistosomiasis haematobia, though the degree of COP reactivity is much less than that with the eggs collected from hamster liver.

Acknowledgments

We would like to thank Dr. P. A. Peters for his kind supply of tools for the "quick" Kato smear technique. We are also indebted to Mr. J. H. Ouma and Mr. J. B. Bebora of the Division of Vector-borne Diseases, Kenya, for their cooperation in the collection of samples. We gratefully acknowledge the financial assistance extended by the Japan International Cooperation Agency.

References

1) Farag, H. F., Barakat, R. M. R., Awadalla, H. N. and El-Gohary, Y. (1978): Diagnosis of bilharziasis (*S. haematoboium* and *S. mansoni*) by the ELISA using the homologous antigen. Tropenmed. Parasit., 29, 413–416.
2) Hillyer, G. V. and Rios, I. G. (1979): The enzyme-linked immunosorbent assay (ELISA) for the immunodiagnosis of schistosomiasis. Am. J. Trop. Med. Hyg., 28, 237–241.
3) Hillyer, G. V., Ramzy, R. M. R., El Alamy, M. A. and Cline, B. L. (1980): Immunodiagnosis of infection with *Schistosoma haematobium* and *S. mansoni* in man. Am. J. Trop. Med. Hyg., 29, 1254–1257.
4) Hillyer, G. V., Ramzy, R. M. R., El Alamy, M. A. and Cline, B. L. (1981): The circumoval precipitin test for the serodiagnosis of human schistosomiasis mansoni and haematobia. Am. J. Trop. Med. Hyg., 30, 121–126.
5) Hirata, M., Takamori, K. and Tsutsumi, H. (1977): Circulating antigen in animals infected with *Schistosoma japonicum*. 3. Detection of circulating antigen by counter immunoelectrophoresis. Kurume Med. J., 24, 139–146.
6) Huldt, G., Lagerquist, B., Phillips, T., Draper, C. C. and Voller, A. (1975): Detection of antibodies in schistosomiasis by enzyme-linked immunosorbent assay (ELISA). Ann. Trop. Med. Parasitol., 69, 483–488.
7) Humburger, J., Lustigman, S., Siongok, T. K. A., Ouma, J. H. and Mahmoud, A. A. F. (1982): Analysis and preliminary purification of glycoproteins isolated from eggs in the urine of patients with *Schistosoma haematobium* infection. J. Immunol., 129, 1711–1714.
8) Ishii, A. and Owhashi, M. (1982): Enzyme-linked immunosorbent assay with egg antigens of *Schistosoma japonicum*. Z. Parasitenkd., 67, 279–287.
9) Ismail, M., Draper, C., Ouchterlony, Ö., Nilsson, L. Å. and Terry, R. (1979): A comparison between a new serological method, thin layer immunoassay (TIA), and the enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies in schistosomiasis. Parasite Immunol., 1, 251–258.
10) Janitschke, K., El-Kalouby, A. H., Braun-Munzinger, R. A., El-Baz, H. and Mahmoud, M. (1981): Evaluation of the ELISA test as an epidemiological tool in schistosomiasis. J. Trop. Med. Hyg., 84, 147–154.
11) Koech, D. K., Hirata, M., Shimada, M. and Wambayi, E. (1984): Precipitates found around *Schistosoma haematobium* eggs from human urine prior to circumoval precipitin test. Ann. Trop. Med. Parasitol., 78, 627–632.
12) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.
13) Matsuda, H., Nakao, M., Tanaka, H., Nagata, T., Noseñas, J. S., Blas, B. L., Portillo, G. P. and Santos, A. T. Jr. (1981): A study of ELISA for schistosomiasis japonica using 5-amino-salicylic acid, a substraste of peroxidase-labelled antibody. Jpn. J. Parasitol., 30, 363–372.
14) Michael, A. I., Farag, H. F., Barakat, R. M. R. and Youssef, M. (1979): Indirect immunoperoxidase tests in the diagnosis of schistosomiasis. Tropenmed. Parasit., 30, 423–425.
15) Nakao, M., Matsuda, H., Tanaka, H. and Nagata, T. (1981): Comparison of three kinds of substrates for peroxidase-conjugated antibody in micro-ELISA for schistosomiasis japonica. Jpn. J. Parasitol., 30, 197–204.
16) Newsome, J. (1958): Species-specific serological tests for bilharzia. Ann. Trop. Med. Parasitol., 52, 82–86.
17) Ogunba, E. O., Maddison, S. E., Tsang, V. C. W. and Slemenda, S. B. (1982): Reactivity of fractionated antigens of *Schistosoma mansoni* with

sera of patients infected with *S. haematobium*. Afr. J. Med. Sci., 11, 67–73.
18) Peters, P. A., El Alamy, M., Warren, K. S. and Mahmoud, A. A. F. (1980): Quick Kato smear for field quantification of *Schistosoma mansoni* eggs. Am. J. Trop. Med. Hyg., 29, 217–219.
19) Salih, S. Y., Bartlett, A. and Voller, A. (1978): Detection of antibodies by enzyme-immunoassay in human *Schistosoma mansoni* infections: A clinical and chemothrapeutic study. Tropenmed. Parasit., 29, 409–412.
20) Schinski, V. D., Clutter, W. C. and Murrell, K. D. (1976): Enzyme- and ^{125}I-labeled anti-immunoglobulin assays in the immunodiagnosis of schistosomiasis. Am. J. Trop. Med. Hyg., 25, 824–831.
21) Tsuji, M. (1974): On the immunoelectrophoresis for helminthological researches. Jpn. J. Parasitol., 23, 335–345.
22) World Health Organization (1976): The enzyme-linked immunosorbent assay (ELISA). Bull. W.H.O., 54, 129–139.
23) Yogore, M. G. Jr., Lewert, R. M. and Blas, B. L. (1983): Seroepidemiology of schistosomiasis japonica by ELISA in the Philippines. 1. Underestimation by stool examination of the prevalence of infection in school children. Am. J. Trop. Med. Hyg., 32, 1322–1334.
24) Yokogawa, M. and Sano, M. (1966): Immunosero-diagnosis of schistosomiasis japonica. II. Isolation techniques of the *Schistosoma* eggs from the tissues for circumoval precipitation test. Jpn. J. Parasitol., 15, 394–398.
25) Yokogawa, M., Sano, M. and Araki, K. (1967): Immunosero-diagnosis of schistosomiasis japonica. III. Circumoval precipitation test. Jpn. J. Parasitol., 16, 77–84.

ヒト尿由来虫卵を用いたビルハルツ住血吸虫症の免疫学的診断

平田瑞城[1] 稗田友之[1] 塘　普[1] 嶋田雅暁[2] 佐藤克之[2] DAVY K. KOECH[3] EDITH WAMBAYI[3]
([1] 久留米大学医学部寄生虫学教室, [2] 長崎大学熱帯医学研究所寄生虫部,
[3] *Kenya Medical Research Institute, Nairobi, Kenya*)

住血吸虫症の血清学的診断の為，ヒト尿由来ビルハルツ住血吸虫虫卵の有用性について検討した．虫卵はペプシン塩酸処理し虫卵周囲沈降物を除去後用いた．ELISA, COPT, CIE でビルハルツ住血吸虫感染血清との間に高い感度を認めた（92.3%－94.2%）．特異性では ELISA, COPT に比べ CIE で劣ることを認めた．マンソン住血吸虫感染血清の交叉反応性について検討した時，感度は上記の同種反応に比べて若干低く（85.1%－91.0%）．また抗体陽性率と虫卵排出数との間には一定の傾向を認めなかった．COPTを用いて上記抗原をハムスター肝臓由来の虫卵抗原と比較した時，後者でより強い反応性を示した．結論として，ヒト由来虫卵抗原は COP 反応強度ではハムスター肝臓由来虫卵に劣るが，ビルハルツ住血吸虫症の免疫診断用抗原として有用であると判断した．