

THE APPLICATION OF THE ENZYME LINKED  
IMMUNOSORBENT ASSAY (ELISA) IN THE  
SERODIAGNOSIS OF SCHISTOSOMIASIS

A Thesis

Presented to

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In Partial Fulfillment

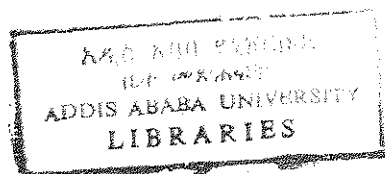
of the Requirements for the Degree

Master of Science in Biology

by

Seyoum Tatischeff

June 1980



DEDICATED TO MY BROTHER

F. V. TATICHEFF

AND THE VERY RARE LIKE HIM

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## ABSTRACT

A description of the schistosome parasite and its life cycle, its immunology and the fact that the parasite causes a disease of world-wide significance is made. The existing distribution of schistosomiasis in Ethiopia and its recorded vectors and the possibility that the disease may rapidly be coming a public health threat is mentioned.

As possible tools for the sero-diagnosis of schistosomiasis, short accounts of the skin test, the indirect immuno-fluorescence test, the complement fixation test and the radio-immunoassay are given. The enzyme linked immunosorbent assay (ELISA) as a promising diagnostic method has been elaborated.

The primary objective of this study was the assesement of ELISA as a sero-epidemiologic tool for schistosomiasis. In this study, samples from S.mansoni and S.haematobium endemic areas and samples from non-endemic area were used. Microscopic examination of stool and/or urine ELISA on blood samples and skin tests for schistosomiasis were done. ELISA with a sensitivity of 86.86 to 96% and a specificity of 80 to 81% was preferred as a sero-diagnostic method for schistosomiasis from: the conventional microscopy where sensitivity was low and from the intradermal test which showed poor specificity (40% positivity was observed in a non-endemic area).

## INTRODUCTION

### A. The Parasite and its Life Cycle.

The digenetic trematodes, the schistosomes, belong to the phylum Platyhelminthes, class Trematoda, superfamily Schistosomatoidea, family Schistosomidae and genus Schistosoma.

Unlike the other trematodes of man, schistosomes are dioecious. Their length range between 6.5 and 26 mm. (1). The males are shorter and more fleshy than the narrowly elongate females and, when mature, the females are carried by the males in a ventral groove of the body known as the gynaecophoric canal. Another unusual character of the schistosomes is their adaptation of the blood system of the definitive host as their adult habitat and hence they are referred to as blood flukes.

The schistosome life cycle is well established. Eggs produced by the female worms have to pass through the wall of the fine blood vessels in which they are laid and then through the wall of the bladder or intestine (according to the species of the schistosome concerned) until they fall into the lumen of the organ and eventually are voided with either the faeces or urine.

In the presence of fresh water, hatching takes place. Hatching is stimulated by warmth and light. Once free, the miracidia which are ciliated larvae, swim actively and enter the snail intermediate host. In the intermediate host, the miracidia change to mother and

daughter sporocysts and eventually into several hundreds of fork-tailed cercariae which are the infective stage to the definitive host. The actively motile cercaria gains entrance to the definitive host usually through the skin and after penetration becomes a schistosomula which migrates and develops into adult worm in specific sites of the body depending on the species of the parasite.

Following copulation, the female worm produces eggs thus completing the cycle.

From the public health point of view the most important species are: Schistosoma mansoni, S.haematobium, S.intercalatum, S.japonicum and S.mekongi (2). Only two species, S.mansoni and S.haematobium cause human schistosomiasis in Ethiopia.

The life cycle of the above schistosomes follows more or less the same pattern, the basic difference is the type of snail intermediate host and the habitats of the adult worms. Adult S.mansoni and S.japonicum commonly reside in the small mesenteric vein whereas S.haematobium adults are usually present in the pelvic venules.

In the Ethiopian situation, Biomphalaria pfeifferi is the principal intermediate host of S.mansoni and occurs widely in the Ethiopian high lands (3). Kloos et al. (4) incriminate B.sudanica in the transmission of the Ethiopian strain of S.mansoni in the human population around Lake Ziway. The only confirmed intermediate host of S.haematobium in Ethiopia is Bulinus (physopsis) abyssinicus (5).

B. The Disease and its Immunology.

Typical clinical manifestations in acute phases, are bloody dysentery in intestinal schistosomiasis and hematuria in the case of S. haematobium. In both instances eggs are observed in microscopic examination. In general the disease is a chronic one, mostly asymptomatic, but in few cases resulting in severe pathological changes (6).

The pathology of all human schistosomiasis is essentially the same differing only in the location and egg-laying capacity of the adult worm. A condition of great importance in the pathology of the disease is the fact that not all eggs laid by the female worm are successful in achieving passage through either the feces or urine. Some are carried back in the venous blood and may become lodged in the liver or other organs, while others may be held in the wall of the bladder or intestine and remain there until they die. Pathological changes are therefore attributed to chronic inflammatory lesions produced in and around blood vessels by eggs or their products, and in some instances by dead adult worms. These lesions are referred to as granulomas.

Whereas in principle damage to tissues of all organs through which the parasite has migrated may be expected the liver, spleen and the renal systems are the ones most commonly affected. In the liver, chronic portal inflammation is conspicuous and frequent occurrence. In the spleen, changes in the lymphoreticular tissues

occur and thus enlarge the organ, In such splenomegaly, cellular proliferation and passive congestion are two important factors. With regards to changes in the renal systems, nephrotic syndromes may be observed. Glomerulonephritis is a common feature in hepatosplenic schistosomiasis due to S.haematobium.

In view of the fact that the parasite, either as an adult or as an egg, may reside in the organs responsible for immune responses, stimulation for the synthesis of immunoglobulins is inevitable. Unfortunately these immune responses, so far as present knowledge goes, are of value only for the serodiagnosis of the disease. Their ability for protection is not yet clearly defined. Nevertheless, there is evidence of concomitant immunity which protects against reinfection in the presence of an already established infection (7). The adult worm residing in the host blood vessels confer this protection by providing a continuous antibody - mediated cell cytotoxicity which may involve immune IgG antibody and a killer cell such as the eosinophil. The action of this lethal antibody is directed only against fresh invading cercaria. Such a concomitant immunity exists not only between same species of parasites but also in parasites of different species (8) and different strains (9). In general, because antibody or cell-mediated activities have not so far been correlated with protection, resistance to reinfection can only be measured by following the fate of a challenge infection.

C. Schistosomiasis as a global problem

The mention of a few facts would suffice to support the statement that schistosomiasis is becoming a public health menace.

To begin with, one needs only look at the global distribution and prevalence of schistosome infections of man to sense their ever existing potential to cause an alarming situation. An estimated number of 200 million people in the world is under the dark shadow of the disease and another 400 million is at risk (1). Another point worth mentioning in recognition to the fact that this trematode infection represents a considerable clinical and public health problem in Asia, Africa and South and Middle America, is that a world wide program for the fight against this parasitic disease is actively being pursued through the encouragement and coordination of the World Health Organization (WHO) (10). Considering the enormity of the task, the 1979-81 budget for this special global program in schistosomiasis allotted by WHO is U.S \$11,592,000. (10).

The mere existence of the infection in a person is of little significance. One should be interested in whether and to what extent the infection causes important injury at individual and community level. Such speculations will no doubt give a deeper insight to the magnitude of the problem caused by the schistosome parasites. It is an undeniable fact that Schistosoma infection can result in severe illness, disability and death, In terms of economic impact, such consequences can not be easily overlooked. For instance, in the

early sixties, the Egyptian government incurred an annual expense of 80 million pounds for the treatment and control of schistosomiasis and 50 million pounds in manpower loss due to incapacitation by the disease.(11). Such a monumental expense (over 900 million E.B. at the then existing rate) to fight against a single disease for a relatively developed country cannot be considered lightly.

D. Epidemiology of Schistosomiasis in Ethiopia.

The situation of schistosomiasis in Ethiopia has a simillar outlook as in any other country where the disease is endemic.

Since the great majority of the Ethiopian community uses streams and other natural water resources for their daily needs, such a condition should in no doubt be undermined as a factor for the continued existence and propagation of the disease.

Recent increases in infection, and the discovery of new endemic foci, may be attributed to mass population mobility and the development of large agricultural schemes. In general, the stepping up incidence of schistosomiasis may be thought of primarily being a repercussion of poor agricultural engeneering. In deed, schistosomiasis may now be called "The agricultural engineers disease". The relationship of irrigation (agricultural engineering) and schistosomiasis has been demonstrated by Sturrock (12) in Tanzania, where a pre- and post-irrigation survey was conducted and showed a marked increase in incidence of schistosomiasis after irrigation

had been largely instituted. A similar circumstance was predicted in Ethiopia by Duncan et al. (13). In both cases, irrigation made a definite and marked contribution of the propagation of the disease. Irrespective of agricultural development and population mobility, however, schistosomiasis has long been endemic in most parts of the country (see map on page 15). Ayad (14), in the first most reliable document on the occurrence of schistosomiasis in the country reviewed earlier reports, made clinical inquiries and microscopic examination of stool and urine, identified suspected snail hosts and finally established the fact that S.mansoni was known to occur in Harrar, Jima and Adwa. He also made a mention of two cases of S.haematobium been reported in a localized focus near Gewane. Buck et al. (15) latter reconfirmed S.mansoni endemicity in the town of Adwa. S.mansoni prevalence in 22.8% of School Children was reported in the northern shore of lake Tana by Chang (16). And according to A. Lemma (17). H.B..Russel is known to have found 48% of 189 Afars suffering from S.haematobium. Sole and Lemma (18) indicated the prevalence of S.haematobium in the flood plain of the Ogaden region. Pollderman (19) generalized that human infection with S.mansoni is common in the Ethiopian highlands. On the other hand, S.mansoni endemicity in altitudes between 500-1000m and 2,000 - 2,200m., in different parts of the country, was indicated by Kloos et al.(4). Kubasta (20) and Lo et al. (21) have shown that schistosomiasis is endemic in Harrar, Alemaya and the Damotta Valley.

In 1966 S.mansoni was reported from only three children in the Wonji Sugar Estate(22). The 1979 records of the Estate hospital laboratory indicates several hundred cases. If the irrigable areas of the endemic regions should continue to develop at the present rate, with no consideration for control measures, then an increase in schistosomiasis prevalence would seem likely to follow soon.

Although it has never been attempted to determine the actual site of contracting the infection, schistosomiasis has been reported throughout the 14 Administrative regions of Ethiopia. The escalating yearly incidence is a cause of apprehension. Reports from all 14 Administrative regions show an increase from 1024 cases in 1972 to 21,801 in 1976 (23). It is clear that the trend is towards an increased incidence which may partly be due to health services reaching the masses and as a result existing cases becoming more apparent. In addition, the increase may be of man's own making because by the construction of dams, irrigation systems and other water construction work in an attempt to introduce development, the very conditions that favour the spread of the disease are being created.

#### E. Diagnostic Methods for Schistosomiasis

Microscopic examination for the recovery of the characteristic ova are the most specific means of diagnosis. Various concentration techniques have been devised and all have shown satisfactory results. However, the fact that: microscopic works are tedious, particularly in large population surveys, the undesirability of examining urine and/or stool samples for aesthetic reasons, some cultural ethics being prohibitive to the provision of stool and/or urine samples, discourage microscopic work.

the diagnosis of individual cases. A high sensitivity has almost always been noted. A sensitivity of 93-96% was observed in patients passing S.haematobium eggs (27,28). Its high sensitivity and relatively low specificity limits its application to epidemiologic investigations (29,30). Sadun (31) attributes these variations in sensitivity and specificity to differences in the character of the antigen employed, age or sex of patients, duration of active infection or site of injection. The fact that the skin test is sex and age dependant: reaction is more intense in adults than in children and is more pronounced in males than in females has been noted in a study conducted in the town of Adwa (15). Such a finding therefore, indicates that skin test is unsuitable for sex and age stratified study. Cross reactions are observed in areas endemic for bovine schistosomiasis (30) and therefore, in such areas where non-human mammalian schistosomiasis or other trematode infections are prevalent, a large portion of false positive reactions might be anticipated when this test is used.

Pioneers in the list of serologic tests for schistosomiasis are the indirect immunofluorescence (IFA) test and the complement fixation (C.F) test. Thomas (32) and Wilson (33) have proved that IFA is more sensitive and specific than the C.F test and according to Draper (34) the counter-current immunoelectrophoresis (C C I E) was the least sensitive and specific as compared to I F A and C.F

Recent study by Hillyer et al. (35) has added the Radioimmunoassay (RIA) and the Circumoval precipitin (COP) test to the already

expanding alternatives for the diagnosis of schistosomiasis. They consider that the RIA and COP have a diagnostic sensitivity of 95% and detected 100% of individuals excreting ten or more eggs per gram of stool.

The use of RIA appears however to be discouraged because of the short shelf-life of the conjugates, its being impractical in field situations and because of the **obvious** health hazards (35).

A significant addition to existing serological tests is the enzyme-linked immunosorbent assay ELISA (36,37).

The following, outlines the principles of ELISA. The principles is also illustrated in diagram 1. on page 16

1. The specific antigen is first made to coat to a solid phase
2. Excess antigen (not coated to the plate) is removed by washing.
3. Serum suspected of containing specific antibody is added to the sensitized carrier. Specific antigen antibody coupling will take place.
4. Excess serum components are washed away.
5. Enzyme-labelled antiglobulin (conjugate) is added. This will specifically react with the antibody. A final antigen, antibody and enzyme-labelled antiglobulin complex is formed.
6. Excess reactants are washed out.
7. Substrate, specific to the enzyme on the antiglobulin, is added. Hydrolysis occurs. The extent of hydrolysis is proportional to the amount of antibody present.

The fact that most serologic methods often fail to detect antibodies in patients with schistosomiasis, even in some cases with active infection, has been documented by Kagan et al (25). Such a difficulty in some patients is a limiting factor in sero-epidemiological studies of the disease. It emphasises the fact that there is a need for very sensitive serologic methods capable of detecting antigens and antibodies. It is possible that ELISA, originally described by Engvall and Perlmann (38) might help in this context.

Huldt et al (39) are of the opinion that ELISA has a potential for being a useful tool for epidemiological purposes but as yet very little is known about its specificity for schistosomiasis. These same co-workers reported that ELISA shows a higher reading in S.mansoni than in S.haematobium infection but nevertheless they pointed out that it is possible to detect schistosome infection in many patients using ELISA, even in cases serologically negative by conventional methods, such as the immunofluorescence technique.

Schinski et al (40) in their comparative studies of various immunodiagnostic methods for schistosomiasis concluded that ELISA would appear to be the most adaptable of the labelled anti-globulin assays, particularly in field situations. The sensitivity of ELISA is attributed to the ability of a single enzyme molecule to react with several molecules of substrate, thereby expanding the sensitivity of detection of the enzyme (40). As is true of most serologic test for schistosomiasis false-negative and false positive results may also be obtained in ELISA (41)

For the reason that the antigen used in ELISA for schistosomiasis could be made from either the egg, as soluble egg antigen (SEA) or the cercarial antigen (CA) or even the adult worm antigen (AWA) the possibility of improving the sensitivity and specificity of the test, and also the potential for using it to differentiate active from chronic infections, is promising. Using the SEA, ELISA had a sensitivity of only 75% and much cross-reactivity was obtained in serum samples from patients with helminth infections with Echinococcus, trichinosis, cysticercosis and fascioliasis (42). Thus ELISA done with SEA lacks immunologic specificity and further purification of the antigen is recommended.

Lunde et al (43) demonstrated that by the use of both the C.A. and the AWA, ELISA can be of help in differentiating acute from chronic cases of human schistosomiasis. ELISA could thus be considered a tool for seroepidemiologic studies as well as a guide to monitor clinical conditions.

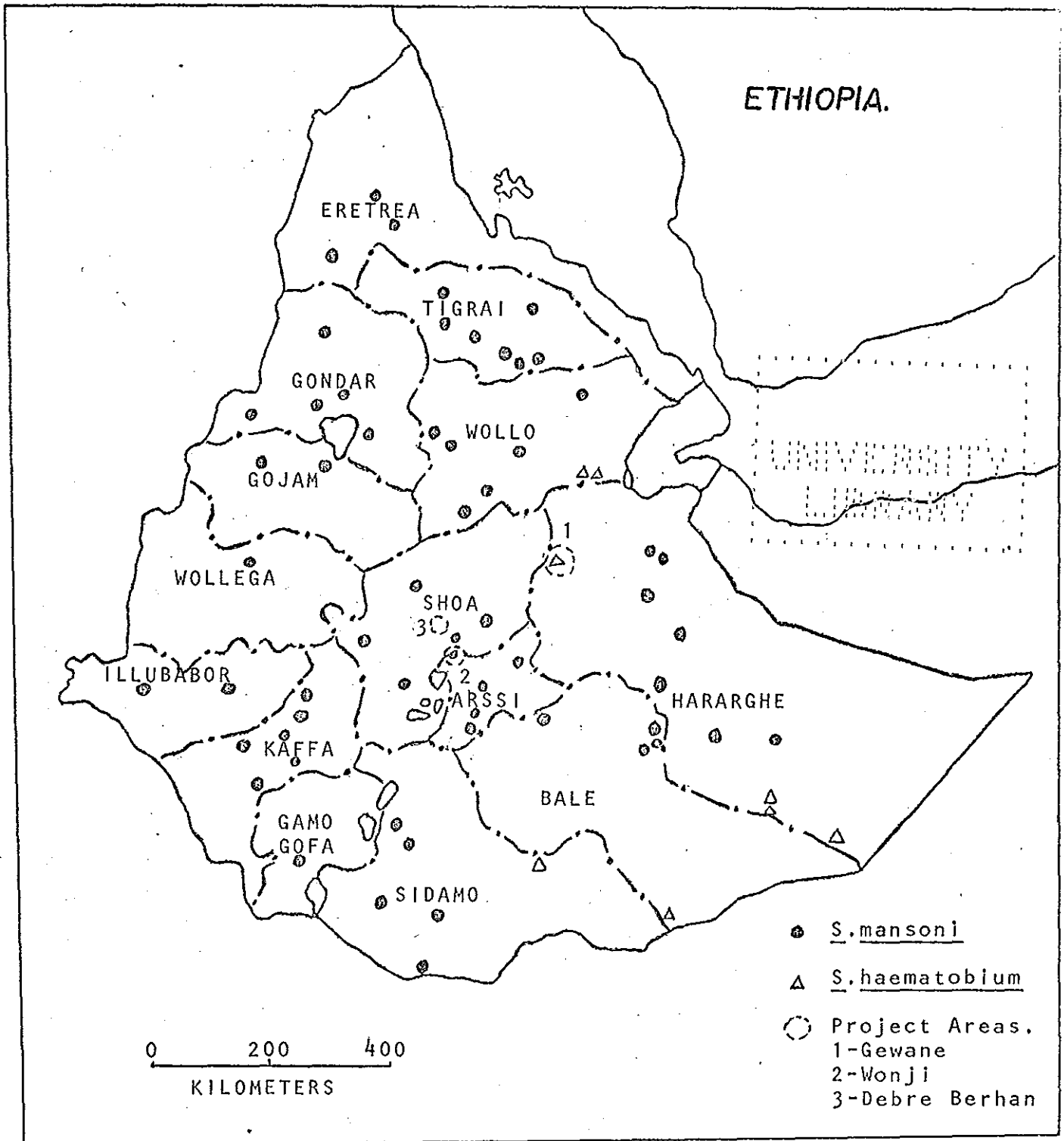
#### F. Aim of the Present Study

The primary objective of this study is to appraise the use of ELISA in sero-epidemiologic studies of schistosomiasis in the Ethiopian situation.

Attempts in the last three years using ELISA have shown encouraging results, however, its application in epidemiologic investigations, its comparable results with existing diagnostic methods and the feasibility of its wide scale use in the Ethiopian situation need to be assessed.

It is one thing (and definitely important) to know the existence and extent of prevalence of schistosomiasis in Ethiopia but quite a different issue to speculate on what should be done about it. An attempt to control and eventually erradicate the disease is the most and only logical solution to the ever-prevailing problem. Such an attempt must, at least partly, be based on accurate information regarding the prevalence of infection and therefore reliable methods of diagnosis must accordingly be instituted. It is with this view in mind that an attempt to develop ELISA for the sero-diagnosis of schistosomiasis is made.

Map 1.

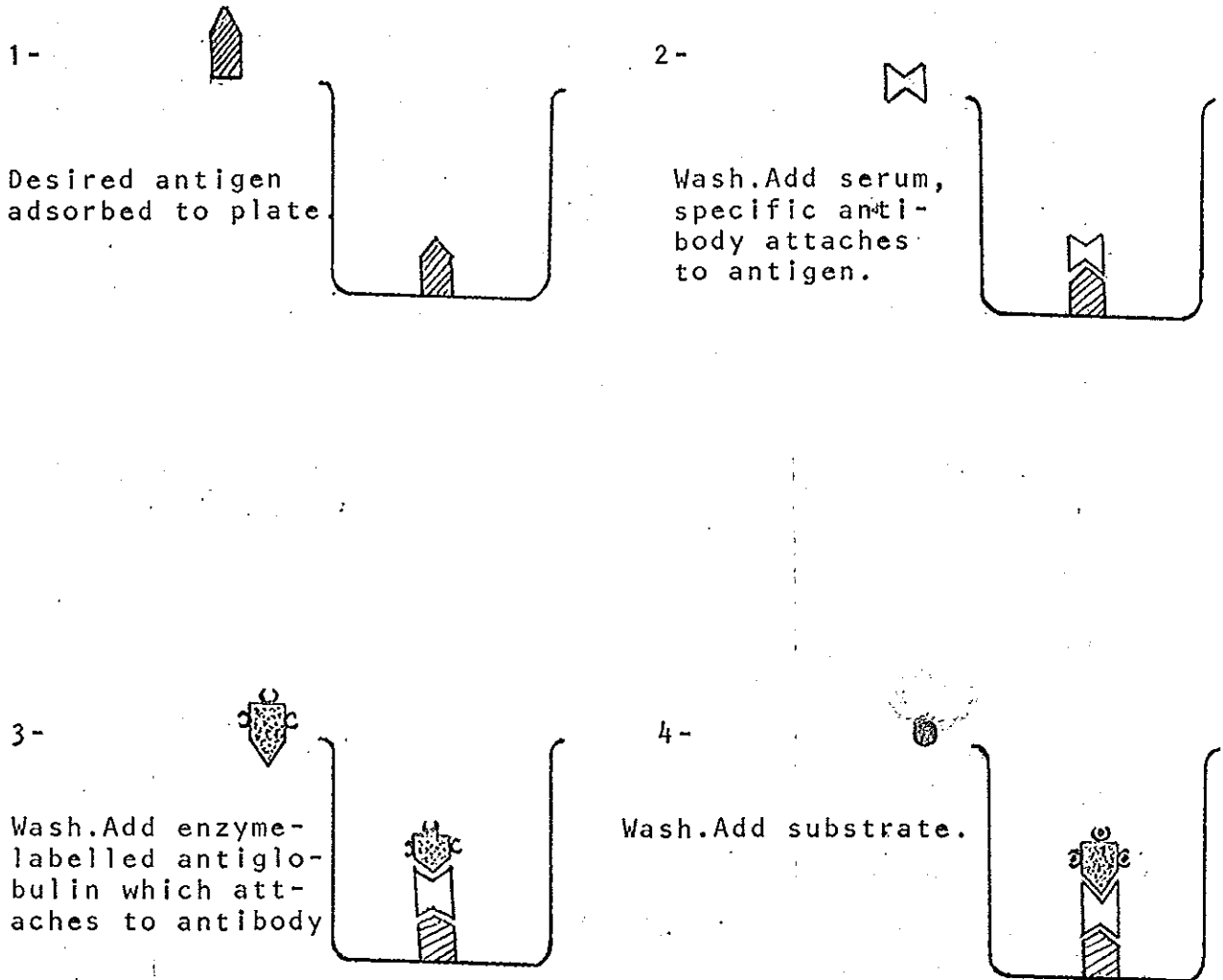


DISTRIBUTION OF SCHISTOSOMIASIS IN ETHIOPIA.

Compilation of the Pathobiology  
Institute, Addis Ababa University  
(Aklilu Lemma). 1970-'73.

Fig.1

PRINCIPLES OF ELISA FOR MEASURING ANTIBODIES.



Result. Amount hydrolyzed as noted by intensity of color developed=Amount of antibody present.

## MATERIALS AND METHODS

Blood samples from individuals of S.mansoni endemic and S.haematobium endemic areas and also of non endemic areas for schistosome are assayed by ELISA. These same groups have microscopic examination of stool and/or urine for parasites and intradermal test for schistosomiasis. The results of the microscopic findings, ELISA and skin test are used for the final analysis of the study.

### A. Study Areas (see map on page 15)

Three different localities were selected for the study. The Gewane area was used for the S.haematobium population, the Wonji area was used for the S.mansoni study and the town of Debre-Berhan was used as a negative control.

#### 1. The Gewane Area

Gewane is a warm area 350 km. Northeast of Addis Ababa and has an average altitude of 700m. The population, essentially made up of a nomadic Afar Tribe, is estimated to be around 25,000 people. Dwellings are scattered along the Awash river bank. During the wet season, the area becomes a huge marsh which eventually completely dries up except for two or three permanent water collections. Both the Awash river and the swamp water are the exclusive source of water for a day-to-day use.

The area is within the Awash valley development scheme and therefore the risk for the dissemination of the S.haematobium infection is very high.

## 2. Wonji

This is a town 118 km South East of Addis Ababa. It has an elevation of 1650m. The town has one of the largest and oldest sugar producing estate of the country. Irrigation is used for the plantation and S.mansoni infection has been in existence ever since the sugar industry was developed some twenty years ago.

## 3. Debre-Berhan

Debre-Berhan is 135 km North north east of Addis Ababa. It is a road-side town of about 30,000 people. Its average elevation is 2,700m.

This place was selected for negative control because a 3 years retrospective analysis of the laboratory finding at the Haile-Mariam Mamo Hospital shows that out of over 7,200 patients who had had stool examination for parasite, there were only three S. mansoni cases (probably imported) and for this reason Debre-Berhan was considered as a non endemic area for schistosomiasis.

## B. Sample Collection and Examinations

In all the three areas used for the study, attempts to obtain random samples failed because either there was an over whelming demand for participation among those not selected in the sample, or the number of participants was so little that it was mandatory to use the same age and/or sex group in order to have a statistically significant number of the population in the study. Nevertheless, a

minimum of five years stay in the particular area, with very much limited mobility was the primary criteria for accepting individuals for the study.

#### 1. Stool Samples

A total of 596 samples were collected in screw cap bottles with 5% formalin as preservative. Of these 152 were from Wonji, 257 were from Guewane and 187 were from Debre-Berhan.

The stool samples were examined for parasites at the Central Laboratory and Research Institute (CLRI) using the Ritchie (44) concentration method. A minimum of two slides were carefully examined under the microscope and the results recorded against coded identification number. Results were recorded as either being negative for parasites or positive for specific parasites.

#### 2. Urine Samples

Urine samples were collected only from Guewane. A total of 257 urine samples were collected in labelled tubes. The tubes were centrifuged on the spot in a clinical centrifuge at 2000 RPM for 10 minutes. The supernatants were decanted and a minimum of two smears, made from a sediment, were examined under the microscope. The presence of S.haematobium egg (s) was recorded as positive. Absence of S.haematobium egg was recorded as negative.

### 3. Blood Samples

A total of 596 blood samples were collected by finger pricks using capillary tubes. 257 were from Gewane, 152 from Wonji and 187 from Debre-Berhan. Blood samples were appropriately labelled and kept in cold ( $5-10^{\circ}\text{C}$ ) during transit. Collected blood samples were either centrifuged in the field (when longer stays in the field were anticipated) or taken to the CLRI and centrifuged there.

Each collected sample was assayed in duplicate - by ELISA. All tests were carried out using soluble egg antigen (SEA) from S.mansoni species. The antigen was provided, lyophilized, through the courtesy of the London School of Hygiene and Tropical Medicine.

The assay was performed using the following procedure adapted from McLaren et al. (45)

#### ELISA Procedure:

##### DAY 1

##### A. Dilutions of blood samples:

In appropriately labelled tubes prepare a 1/300 dilution of serum samples by adding 5ml of serum to 1.5 ml. incubation buffer. Cover diluted samples with aluminium foil and keep at  $+4^{\circ}\text{C}$  until the next day.

##### B. Preparation of antigen coated plates:

The stock soluble egg antigen (SEA) with an initial concentration of 500 ug/ml protein is first diluted with coating buffer so that the final protein concentration is 2.5 ug/ml.

i.e a 1/200 dilution. 200 ul. of the diluted antigen is dispensed to each well of the ELISA microtitre plate.

Each pair of the well on the plate is labelled with numbers corresponding to those of the diluted serum sample.

The plate is covered and left at room temperature overnight in a sealed container.

## DAY 2

### A. Preparation of control sera.

Make a 1/300 dilutions of positive and negative control sera in incubation buffer. The positive control sera are used for end point determinations and to assess reproducibility of test measurements and the negative control sera are used to check for a nonspecific background reaction and determine the cut off points for negative results.

### B. Test run:

1. Rinse antigen coated plate with incubation buffer. Leave the washing solution on for three minutes and repeat this procedure twice more.
2. Shake off plate to remove all traces of solution
3. Add 200 ul control sera to the correspondingly labelled wells on the plate.
4. Add 200 ul of the diluted serum samples in the appropriate well.
5. Cover plate and incubate for 2 hrs. at room temperature.

6. Record sample numbers on the assay test sheets
7. Rinse plate as in 1 above.
8. Dilute desired enzyme conjugate in incubation buffer during the washing cycle: make a 1:1000 dilution if peroxidase is used and a 1:1500 if alkaline phosphatase is used.
9. Shake off plate to remove all traces of solution.
10. Add 200 ul. of conjugate to each well. Cover plate and incubate for 2 hrs. at room temperature.
11. Repeat washing procedure as in 7.
12. During washing cycle prepare appropriate substrate solution for peroxidase enzyme conjugate, the substrate to be used is orthophenylene diamine (O.P.D). A stock solution of O.P.D is prepared by thoroughly mixing 100 mg. O.P.D with 10 ml. absolute methanol. This solution is stable for 1-2 weeks in the dark at 4°C. Working O.P.D solution is prepared, fresh, by mixing 99 ml. distilled water, 1.0 ml. stock O.P.D and 0.05 ml. 6% H<sub>2</sub>O<sub>2</sub>.

For alkaline phosphatase enzyme conjugate, the substrate to be used is P-nitrophenyl phosphate solution. This is prepared by dissolving 5 mg P-nitrophenyl phosphate tablet (sigma) in 5.0 ml. 10% diethanolamine buffer.

13. Add 200 ul. of substrate to each well
14. Switch on the photometer to allow it to warm up.  
Select the appropriate filter for enzyme system used:  
450 nm. for phosphatase and 492 nm. for peroxidase.
15. Stop the reaction by appropriate inhibitor in 2-3 wells with no serum and use this to set the photometer absorbance scale to zero. 3M NaOH and 8N H<sub>2</sub>SO<sub>4</sub> are, respectively, used for phosphatase and peroxidase inhibitors.
16. Monitor optical density levels of positive and negative control sera progressively until a pre-determined desired O.D. is reached, Then stop reaction in the rest of the plate.
17. Take photometric readings of all sample.

Reagents:

1. Coating buffer

Na<sub>2</sub>CO<sub>3</sub> 1.59 gm, NaHCO<sub>3</sub> 2.93 gm, NaN<sub>3</sub> 0.2 gm, Distilled water 1000 ml. stable for two weeks if stored at + 4°C.

2. Incubation buffer (Pbs-tween) PH = 7.4

NaCl	8.0 gm
KH <sub>2</sub> PO <sub>4</sub> · 12H <sub>2</sub> O	2.9 gm
KCl	0.2 gm
Tween 20	0.5 ml
NaN <sub>3</sub>	0.2 gm
Distilled water	1000 ml.

3. 10% diethanolamine buffer PH = 9.8

Diethanolamine	97 ml
Distilled water	800 ml
NaN <sub>3</sub>	0.2 gm
Distilled water-to make up 1000 ml.	

Note:-

- a) Sodium azide (NaN<sub>3</sub>) is never used in any of the reagents if peroxidase is used
- b) Coating buffer is used only in diluting the antigen for coating the plates.
- c) Incubation buffer (Pbs-tween) is used only in serum and conjugate dilutions and for washings.

With each daily run positive controls (Pre ELISA tested sera from acute Schistosoma patients with high egg counts) and negative controls (pre ELISA tested sera from people who had as far as is known, had no schistosoma infection and were negative for parasites by stool examination) were used. The cut off point for positive ELISA results depended on the daily readings of the negative results with an upper extinction ranging from 0.3 to 0.5.

4. Skin Tests:

The skin tests were done according to the technique described by Kagan et al. (46). 596 skin tests were done. Of these, 257 were in Gewane, 152 in Wonji and 187 in Debre-Berhan. Skin test kits prepared from adult worms of S.mansoni were obtained from Hoechst Pharmaceuticals. Exactly 0.05 ml of antigen were administered intradermally in the flexor surface of the forearm

and with another syringe a similar quantity of control diluent was injected into the other arm. Results were read after 15 minutes. Areas of wheals were measured directly using a transparent plastic grid specially designed for the purpose. A test was called positive if the area of the test wheal was at least  $10\text{mm}^2$  and at least twice the area of the control wheal.

## RESULTS

The comparison of stool microscopy, ELISA and skin test in S.mansoni and S.haematobium endemic areas and in a non-endemic area for schistosomiasis are, respectively, illustrated in tables 1, 2 and 3.

In table 4, the results of the ELISA and skin tests on samples parasitologically positive for Schistosoma eggs only and Schistosoma eggs and other parasites are given.

Table 5 depicts results obtained by ELISA and skin tests on samples from schistosome endemic area microscopically negative for schistosomes but variously positive and negative for other parasites.

The results of ELISA and skin tests on samples collected from the non-endemic area for schistosomiasis and microscopically positive and negative for other parasites are indicated on table 6.

Table 7 and 8, represent results of stool microscopy, ELISA and skin tests in adults and children in, respectively, S.mansoni and S.haematobium endemic areas.

Findings by sex are given in tables 9 and 10 for, respectively, S.mansoni and S.haematobium endemic localities.

The sensitivity of ELISA and skin tests for microscopically proved S.mansoni only and S.haematobium and other parasites and also for S.haematobium only and S.haematobium and other parasites is given in table 11.

The specificity of both ELISA and skin tests were determined using values obtained from Debre-Berhan. This is summarized in table 12.

The correlation of ELISA and skin tests are analyzed by the product moment correlation coefficient for schistosome endemic and non-endemic zones and also based on microscopic findings of schistosome and other parasites. This is given in table 13.

The distribution of ELISA optical density (O.D) readings in schistosome endemic and non-endemic areas is given in figure 2. Since the discriminating levels for positive and negative results varied from day-to-day, the histogram is made using ELISA O.D. from a one day determination only.

TABLE 1  
ELISA AND SKIN TESTS  
IN S.mansoni ENDEMIC AREA

Microscopic Findings	Number	ELISA Pos.	Skin Pos.
No parasite	18 (12%)	8 (42%)	13 (68%)
<u>S.mansoni</u>	46 (30%)	43 (93%)	43 (93%)
<u>S.mansoni</u> & Hook Worm	23	23	20
<u>S.mansoni</u> & <u>Ascaris</u> l.	20	17	18
<u>S.mansoni</u> , Hook Worm & <u>Ascaris</u> l.	7	6	7
<u>S.mansoni</u> & <u>Trichuris</u> t.	10	9	9
<u>S.mansoni</u> , Hook Worm & <u>Strongyloides</u> s.	6	5	5
<u>S.mansoni</u> & <u>Ascaris</u> l. and <u>Trichuris</u> t.	4	4	4
<u>S.mansoni</u> & Hook worm and <u>Ascaris</u> & <u>Strongy-</u> <u>loides</u> s.	3	3	2
<u>Ascaris</u> l.	5	3	4
Hook worm	3	1	2
<u>E.histolytica</u> cysts	2	1	0
<u>Hymenolepis</u> sp.	2	1	0
<u>Taenia</u> sp.	2	0	1
<u>Giardia</u> l. cysts	1	0	0
Total	152	123(81%)	128(84%)

73 (48%)

66 (90%)

65 (92%)

15 (10%)

6 (40%)

7 (46%)

TABLE 2

ELISA AND SKIN TESTS

IN S.haematobium ENDEMIC AREA.

Microscopic Findings	Number	ELISA Pos.	Skin Pos.
No parasites	100(39%)	55(55%)	66(66%)
<u>S.haematobium</u>	99(39%)	70(71%)	88(89%)
<u>S.haematobium</u> & <u>Trichuris t.</u>	10	4	5
<u>S.haematobium</u> & <u>Ascaris l.</u>	8	7	5
<u>S.haematobium</u> <u>E.histolytica</u> cysts	6	6	6
<u>S.haematobium</u> & Hook Worm	1	1	0
<u>E.histolytica</u> cysts	3	3	4
<u>Ascaris l.</u>	7	3	3
Hook Worm	7	1	1
<u>Taenia sp.</u>	6	0	4
<u>Giardia l.</u> cysts	5	2	1
Total	257	152(59%)	183(71%)

25 (10%)

18 (72%)

16 (64%)

33 (13%)

9 (27%)

13 (39%)

TABLE 3

ELISA AND SKIN TESTS

IN NON-ENDEMIC AREA FOR SCHISTOSOMIASIS

Microscopic Findings	Number	ELISA Pos.	Skin Pos.
No parasite	61(33%)	10(16%)	15(24%)
<u>Ascaris l.</u>	53	8	14
<u>Ascaris l.</u> & <u>Trichuris t.</u>	10	2	4
<u>Ascaris l.</u> & Hookworm	10	2	0
<u>Ascaris l.</u> & <u>Hymenolepis sp.</u>	15	1	3
<u>Ascaris l.</u> & <u>E.histolytica</u> cysts	3	1	0
<u>Ascaris l.</u> & <u>E.vermicularis</u>	4 126 (67%)	0 18 (14%)	0 26 (21%)
<u>E.vermicularis</u> & <u>Trichuris t.</u>	2	0	0
<u>Hymenolpis sp.</u>	9	1	0
Hook worm	2	0	0
<u>E.histolytica</u> cysts	5	1	2
<u>E.vermicularis</u>	4	0	2
<u>Trichuris t.</u>	9	2	1
<b>Total</b>	187	28(15%)	41(22%)

TABLE 4  
 ELISA AND SKIN TESTS  
 ON PARASITOLOGICALLY PROVED SCHISTOSOME CASES

ELISA and Skin Test Results	Microscopic Results:			
	<u>S.mansoni</u> only (Tot. =46cases)	<u>S.mansoni</u> & other para- sites (Total = 70 cases)	<u>S.haematobium</u> only (Tot.=99cases)	<u>S.haematobium</u> and other parasites (Total = 25 cases)
ELISA Pos. Skin Pos	87%	97%	85%	75%
ELISA Pos Skin Neg.	2%	0%	2%	0%
ELISA neg. Skin Pos.	9%	3%	13%	25%
ELISA Neg. Skin Neg.	2%	0%	0%	0%

TABLE 5

ELISA AND SKIN TESTS IN ENDEMIC AREA

ON PARASITOLOGICALLY PROVED NEGATIVE SHISTOSOME CASES:

	Microscopic Results			
	<u>S.mansoni</u>	endemic area	<u>S.haematobium</u>	endemic area
ELISA and Skin Test Results	Pos. for other parasites (Total=15 cases)	Neg. for other parasites (Total=18 cases)	Pos. for other parasites (Total=33 cases)	Neg. for other parasites (Total=100 cases)
ELISA Pos. Skin Pos.	36%	32%	35%	52%
ELISA Pos. Skin Neg.	36%	7%	12%	3%
ELISA Neg. Skin Pos.	7%	39%	21%	31%
ELISA Neg. Skin Neg.	21%	22%	32%	14%

TABLE 6  
STOOL MICROSCOPY,  
ELISA AND SKIN TESTS FINDINGS  
IN ADULTS AND CHILDREN IN S.mansoni ENDEMIC AREA

Number Examined	No. Positive for <u>S.mansoni</u> eggs	No. Positive	
		ELISA	SKIN
Adults 98 (>19 years old)	78 (79.6%)	81 (82.6%)	85 (86.2%)
Children 54 (<19 years old)	41 (75.9%)	42 (77.7%)	43 (79.4%)
Total 152	119 (78.2%)	123 (80.9%)	128 (84.2%)

TABLE 7  
STOOL MICROSCOPY,  
ELISA AND SKIN TEST FINDINGS  
IN ADULTS AND CHILDREN IN S.haematobium ENDEMIC AREA.

Number Examined	No. Positive for <u>S.haematobium</u> eggs	No. Positive	
		ELISA	SKIN
Adults 138 (>19 years old)	94 (68.1%)	98 (71%)	123 (89.1%)
Children 119 (< 19 years old)	30 (25.2%)	54 (39.1%)	60 (50.4%)
Total 257	124 (48.2%)	152 (59%)	183 (71.2%)

TABLE 8  
STOOL MICROSCOPY, ELISA AND SKIN TESTS  
FINDINGS BY SEX

S.mansoni endemic area

Number Examined	No. Positive for S.mansoni ova	No. Positive	
		ELISA	SKIN
Male 84	77 (91.7%)	79 (94.0%)	80 (95.2%)
Female 68	42 (61.8%)	44 (64.7%)	48 (70.6%)
Total 152	119 (78.2%)	123 (80.9%)	128 (84.2%)

TABLE 9  
STOOL MICROSCOPY, ELISA AND SKIN TESTS  
FINDINGS BY SEX

S.haematobium endemic area

Number Examined	No. Positive For <u>S.haematobium</u>	No. Positive	
		ELISA	SKIN
Male 97	28 (28.8%)	56 (57.7%)	73 (75.2%)
Female 160	96 (60%)	96 (60%)	110 (68.7%)
Total 257	124 (48.2%)	152 (59.1%)	183 (71.2%)

TABLE 10

\*SENSITIVITY OF ELISA AND SKIN TESTS  
IN SCHISTOSOMA ENDEMIC AREAS

Microscopic Findings	Sensitivity	
	ELISA	SKIN
<u>S.haematobium</u> only	86.86%	97.97%
<u>S.haematobium</u> and other parasites	91.66%	100%
<u>S.mansoni</u> only	89.14%	95%
<u>S.mansoni</u> and other parasites	96%	100%

\* Formula used for calculation:

$$\text{Sensitivity} = \frac{a}{a + c} \times 100$$

Where a = No. of microscopically proved schistosome cases and positive ELISA or skin test.

c = No. of microscopically proved schistosome cases but negative by ELISA or skin tests.

Note: ELISA results are used for computing ELISA sensitivity and skin test results are used for computing skin test sensitivity.

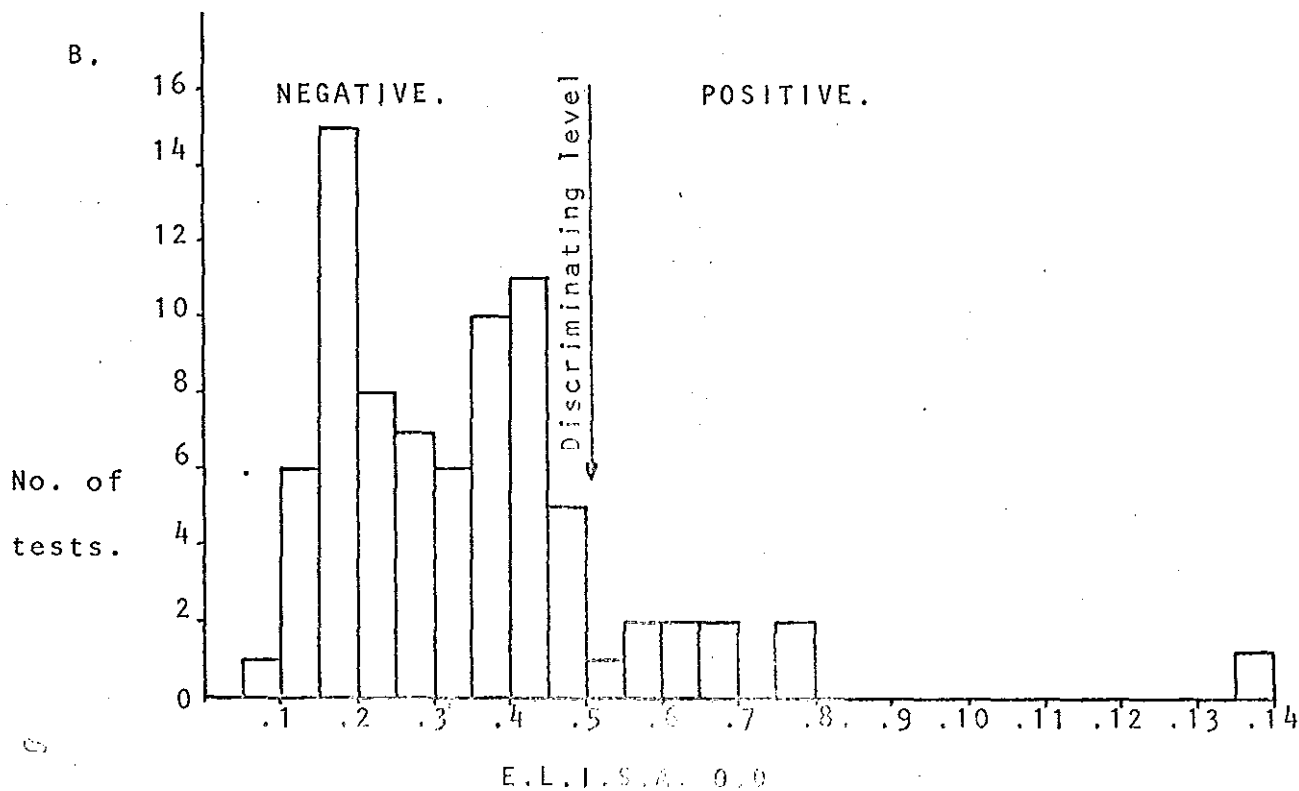
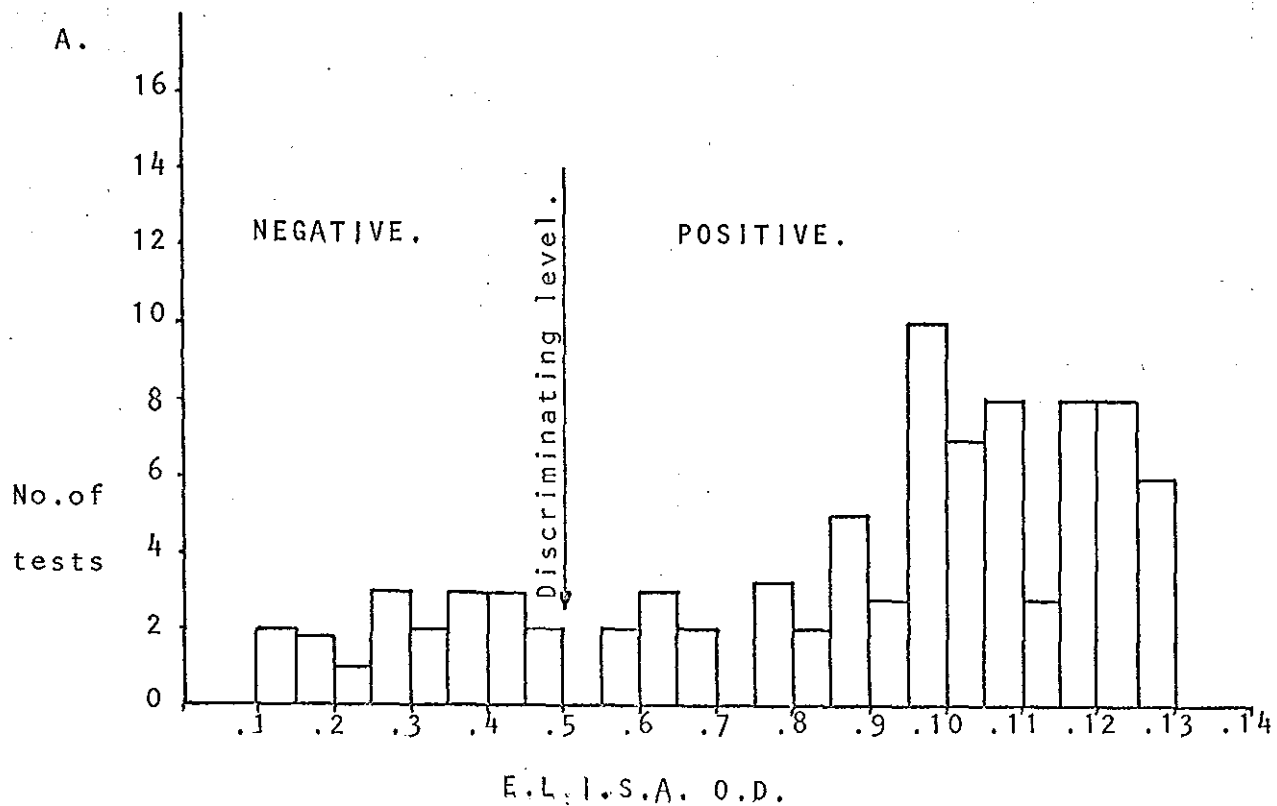
TABLE 12

ELISA AND SKIN TESTS CORRELATION

Microscopic Findings	Correlation(=r)	P
A <u>S.haematobium</u> endemic area		
<u>S.haematobium</u> only	0.47	< 0.001
<u>S.haematobium</u> & other parasites	0.35	> 0.2
Other Parasites only	0.40	< 0.02
No Parasite	0.28	< 0.005
B <u>S.mansoni</u> endemic area		
<u>S.mansoni</u> only	0.62	< 0.001
<u>S.mansoni</u> & other Parasites	0.35	< 0.01
Other Parasites only	0.20	> 0.4
No Parasite	0.30	> 0.1
C Non-endemic area for <u>Schistosoma</u>		
Positive for Parsites	0.30	< 0.001
No Parasite	0.20	< 0.1

Fig.2

DISTRIBUTION OF E.L.I.S.A. O.D. READINGS  
IN SCHISTOSOME ENDEMIC(A) & NON-ENDEMIC(B) AREAS.



## DISCUSSION

### 1. Epidemiology by Microscopic Examination and Serology

The endemicity of schistosomiasis as measured by microscopic examination in the three areas studied were 78% in the S.mansoni endemic area, 48% in the S.haematobium endemic area and nil in the control area. Endemicity by ELISA for the same localities were 81%, 59% and 15%, respectively, for Wonji, Gewane and Debre-Berhan and by skin tests it was 84%, 71% and 22%. The above findings indicate the value of using ELISA as a sero-epidemiologic tool for Schistosoma infection.

The higher endemicity by serological test as compared to microscopy may be due to:

- a. Schistosoma eggs may have been missed by microscopy.
- b. An old infection where egg excretion is very low or has ceased.
- c. Past infection with adult worms expelled out but antibodies are still present.
- d. False positivity or cross reaction with other helminth.

This means that item (d) above may account for some of the discrepancy between the level of endemicity as measured by egg counts compared with serological tests. However, the higher level of positivity found in microscopically negative cases in endemic areas (tables 1 & 2) as compared to the non-endemic area (table 3) indicates that the facts (a) to (d) above must also play a part in explaining the results.

The immuno assay is evidently more sensitive than the microscopy, i.e. the presence of antibodies could be detected even in the absence of the parasite in microscopic examination. Therefore, despite the above uncertainty, ELISA could still be considered of value in determining endemicity for schistosomiasis.

False positive reactions are known to occur in ELISA (41,47). Although the rate of false reactions may be negligible for a mass study of this nature, as has been stipulated following similar studies by other authors (36,39), further purification of the ELISA antigen and the standardization of its procedure is required.

Antibodies cross reacting with S.mansoni and/or S.haematobium antigens could be produced:

- a. Following exposure to cercariae of animal schistosomes
- b. Possibly following exposure to non-schistosome trematodes
- c. Possibly following exposure to non-trematode parasites.

ELISA is sufficiently specific enough to exclude the majority of non-schistosomal infections from those caused by the schistosomes as there was a positivity of only 15% in samples with non-schistosomal parasites from non-endemic area (table 3). Even when a positive ELISA was obtained in a Schistosoma non-endemic area, the O.D. values were distributed at the low end of the positive range (Fig. 2)

In the present study the sensitivity of ELISA ranges from 86.8 to 96%. The presence of other parasites appears to have the result in the S.mansoni study with  $P < 0.05$  at the 5% level. In the S.haematobium study the presence of other intestinal parasites was not significant at the 5% level ( $P > 0.2$ ). Such a discrepancy may have been attributed to several factors among which may be the fact that there may have been more than one species of phylogenetically related species of parasites to the schistosomes. In the S.mansoni study, 48% of all cases of S.mansoni and other parasites were multiple other parasites finding whereas the finding in S.haematobium was only 10%. Such an influence on the ELISA result (i.e. cross-reaction) may have to be tolerated particularly, as in this case, when a crude SEA is used

Even though a crude antigen was used in this study, the specificity of ELISA (80-81%) was satisfactory and better than the skin test (74%). It should be anticipated that superior specificity may be obtained with a highly purified antigen. This was proved by McLaren (41, 43) where a specificity of 99-100% was established.

In the computation of the specificity of ELISA and the skin tests, values from non-endemic area only were used. Values from schistosome endemic areas were not used because of the possibility that subjects used in these localities may have had previous infection and also because acute infections may have been missed by microscopic examinations. Such a possibility is sustained in the

findings of the present study where, in the presence of no parasites at all, the rate of positivity of ELISA was 42% and 52% and that of the skin test was 68% and 66% in respectively, S.mansoni and S.haematobium endemic areas.

2. Results by age and by sex:

By arbitrarily considering subjects under 19 years old as children and those above 19 years old as adult, there appears to be no great difference in the S.mansoni infection. This may be attributed to the fact that both adults and children have similar possibility for being exposed to infected waters. Children use the irrigation canals as "swimming pools" and adults work in the fields. The children: adults distribution of S.haematobium infection is, however, different 25%, 39% and 50% of the children were, respectively, positive by microscopy, ELISA and skin tests whereas 68%, 71% and 89% were, by the same tests, positive in the adult groups. Variations of prevalence of infection in adults and children could be ascribed to the degree of exposure to the infective agent. In general, children, are restricted to the dwelling quarters where a relatively lower risk of exposure exists and adults are mostly in the fields, including marshy areas, herding their cattle and farming.

On the basis of the findings of the present study, the incidence of schistosomiasis by sex appears to follow the established norm of labour division. A higher rate of S.mansoni infection was observed in males with 92%, 94% and 95% positivity in, respectively microscopy

ELISA and skin test. In the females, the values were 62%, 65% and 71%. In the Gewane area, although no great difference by serology was observed, 60% of the females and only 29% of the males had S.haematobium eggs in their urine. The higher occurrence of S.mansoni infection in males could be due to the fact that the male population is the one mostly involved in the plantation work. Increased S.haematobium infections in the female may be accounted for the traditional way of life in the Afar tribes where females have a greater role than males in farming and the over-all procurement of food for the family.

## CONCLUSION

In evaluating the relative values of the microscopic examinations, ELISA and the skin tests for measuring the level of Schistosoma endemicity in an unknown area, the following observations and conclusion could be made:

1. Microscopic examinations are tedious, monotonous and time consuming as a tool for mass survey. On the average about 10 minutes are required for the processing and the actual microscopic work of a single sample. They also give a low prevalence of infection. In the S.mansoni endemic area 42% and in the S.haematobium endemic area 55% of the samples with negative microscopy were positive by ELISA. Findings by other workers (41, 43) also indicate similar opinions. Therefore, on the basis of the present study, microscopic examinations are not recommended for the measurement of Schistosoma endemicity level.
2. The skin test is easy to perform but requires strict adherence to the procedure. The injection has to strictly be intradermal, the volume of the antigen has to be exact and the reading time should be precise. These restrictions make the skin test unpopular particularly when a study is conducted (as in this one) by one person. Of greater significance in the discrimination of the skin test however, is its very high sensitivity and low specificity. In the present study, the sensitivity of the skin test was 95-100% and the specificity was only 75%.

3. ELISA is easy to perform. It requires small amount of serum and reagents. Serum samples could be collected by finger pricks and actual assays could, if need arise, be done in a small local laboratory. The sensitivity of ELISA, in this study, was 88-96% and its specificity was 80-81%.

As a first attempt in the use of ELISA for sero-epidemiologic studies of Schistosomiasis in the Ethiopian situation, the findings of the present study appear promising. It must be pointed out however, that more meaningful results might have emanated had the study been conducted using purified strain of S.mansoni and S.haematobium species of Ethiopian origin.

In the absence of other supporting documents, the speculation that the Schistosoma species in Ethiopia may be of unique strain, is based on a report made by the Pathobiology Institute (50) which indicates that the S.haematobium occurring in the Ethiopian lowlands seems to be a distinct strain which uses B.abyslnicus as its sole intermediate host.

Similar studies will have to be conducted before coming to a final conclusion, however, on the basis of the present study, it is recommended that ELISA could be of use as a sero-epidemiological tool for schistosomiasis.

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