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Serologic Diagnosis of Malaria

ANNUAL REPORT

FY-1972

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AID PASA Control No. RA (HM) 5-68, Amend. #7

"Serologic Diagnosis of Malaria"

ANNUAL REPORT

FY-1972

Submitted by  
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### Purpose and Scope of Project

The program is designed to develop simple, rapid, sensitive, and specific serologic methods for the diagnosis of malaria which are applicable for epidemiologic and clinical purposes.

In an effort to develop methods that may be useful in the worldwide malaria eradication program for surveillance and other epidemiologic purposes, this laboratory has concentrated its efforts on the evaluation and standardization of the indirect hemagglutination (IHA) technique for the detection of malaria antibody. Studies are in progress to define the extent of cross reaction and sensitivity of speciation obtained with the indirect fluorescent antibody (IFA) test.

## SCIENTIFIC ACTIVITIES

- A. Studies on the Indirect Hemagglutination (IHA) test.
  - 1. Antigen studies
  - 2. Immunoglobulin studies
  - 3. Filter paper methodology
  - 4. Stabilization of the Red Cell Carrier
  - 5. Seroepidemiologic studies
  - 6. Epidemic aid
- B. Evaluation and Development of the Indirect Fluorescent Antibody (IFA) test.
  - 1. Antigen studies
  - 2. Occult malaria studies
  - 3. Comparative serologic studies
  - 4. Use of immunoglobulin specific conjugates
  - 5. Radioimmune assay test
  - 6. Other studies
- C. Publications
- D. Fiscal summary
- E. Tables
- F. Figures

A. Studies on the Indirect Hemagglutination (IHA) Test

More than 13,000 specimens were tested in FY 72.

1. Antigen Studies

- a. Efforts to develop a more stable antigen have continued. Antigens extracted from the plasma of Plasmodium knowlesi infected rhesus monkeys were evaluated. These antigens, prepared by Dr. M. Ristic, were similar to those extracted by Rivanol precipitation in that they showed reduced activity when tested against a battery of control sera. One of Dr. Ristic's fractions showed a relatively high rate of false positive reactions.
- b. A retrospective record analysis of Plasmodium knowlesi-infected monkeys used for antigen purposes suggested that optimal antigen development may be associated with rapid passage of the parasite through several monkeys. Several experiments were attempted to enhance the antigenicity of the P. knowlesi used as a source for antigen in the IHA test, by rapid passage through rhesus monkeys prior to parasite harvest. Parasites maintained in chronically infected monkeys or from parasitized blood which had been frozen in nitrogen vapor were passaged through two intact rhesus monkeys prior to inoculation into a splenectomized animal. This procedure resulted in a parasitemia that produced material for antigens of

high quality. The intact animals through which the parasite is initially passed can be cured, subsequently splenectomized, reinfected and exsanguinated for antigen, resulting in no increase in the number of animals required for antigen production. This method of passage has been instituted as the normal procedure.

## 2. Immunoglobulin Studies

Studies on the longevity of immunoglobulins IgM and IgG on filter paper were initiated. Initial data indicates that both immunoglobulin types are stable on filter paper as determined by the Mancini radial diffusion method. Technical problems encountered with the radial immunodiffusion procedure make it necessary to repeat the experiments using additional controls.

## 3. Filter Paper Methodology

In an effort to obtain a greater volume of eluate from filter papers, studies of the effect of increasing the elution volume from 0.2 to 0.4 ml have been initiated. Preliminary tests suggest that the 0.4 ml may be used effectively and may result in slightly greater efficiency of detecting titers in the 1:8 to 1:32 range.

## 4. Stabilization of the red Cell Carrier

### a. Frozen red cells

In cooperation with the Serum Bank Unit/CDC, we have initiated evaluations of human type "O" erythrocytes preserved by freezing. The freezing process, similar to the one used by the Red Cross for preserving blood for transfusion, offers promise for storage of red cells for use in malaria serology. Experiments with cells from Rh positive and negative "O" donors frozen in this manner indicate that cells frozen for up to 8 weeks can be thawed and successfully sensitized with malaria antigen. Furthermore, thawed cells remain usable for up to 6 weeks when stored at 4° C. This exceeds the storage period for fresh cells by 2 to 3 weeks.

b. Aldehyde fixed cells

Double aldehyde fixed cells, sensitized with P. knowlesi were prepared in small quantities and tested with a few negative and positive control sera. A large batch of stable sensitized cells was prepared for evaluation with 24 sera. These sera were collected during October 1970 in Cuiaba, Mato Grosso, Brazil. Seventeen of the sera were from individuals who had parasites in their blood smears (ten P. falciparum, six P. vivax, and one with the species unidentified). Three serum samples were received from the New York Public Health Laboratory and were used as negative

controls. The sera were tested with the stable sensitized cells 6 times during a 90-day period. The results are shown in Table 1. Additional batches of double aldehyde fixed cells were sensitized and 40 serum samples were then tested. The positive samples were from slide proven cases of malaria which had been submitted to the Parasitology Section, CDC, from military hospitals and State health laboratories. The sera were examined at monthly intervals and confirmed the results obtained with the first 24 sera (Table 2).

A batch of double aldehyde fixed cells was sensitized with P. falciparum antigen obtained from an infected Aotus monkey. Sera from 13 P. vivax and 13 P. falciparum infections as well as 17 normal serum samples were tested with the aldehyde cells sensitized with 1) P. knowlesi antigen; 2) P. falciparum antigen; 3) a 1:1 mixture of the two. The results shown in Table 3 indicate that higher titers were found when the mixture of the cells was used to test sera from persons who had P. falciparum malaria rather than with the P. knowlesi antigen. Subsequently, another 3,700 serum samples were tested in an evaluation of the double aldehyde fixed cells as an antigen carrier in the IHA test. These samples included field-collected material from Haiti, Honduras, Brazil, and New Guinea; sera

from patients who had been inoculated with the various human Plasmodium species, from Americans with no history of travel outside the United States; and specimens diagnosed by the IFA test as having antibody to malaria. Analysis of within batch reproducibility of sensitized cells stored at 4°C indicates that 94% of replicate tests produce endpoints within 1 twofold dilution when tested over a 55-day period.

## 5. Seroepidemiologic Studies

### a. Brazil

Parasitologic and serologic (IHA and IFA) data have been examined of the longitudinal study which was conducted in the Cuiaba Sector of the Mato Grosso, Brazil, in collaboration with the Central American Malaria Research Station (CAMRS) in El Salvador. The first three surveys were conducted in May and October 1970 and in February 1971. The data indicate that the intensity of malaria transmission was greater in the northern part of the study area than in the southern part of the area. Some comparative aspects of the study results require further elucidation. Detailed tabulation of the study results was reported earlier.

### b. Nigeria

The initial serologic survey in the WHO-longitudinal Malaria Research Project near Kano, Nigeria was con-

ducted during September and October, 1971, at the end of the wet season. Dr. Hans O. Lobel spent 3 weeks in the area at the invitation of WHO to participate in the demographic, parasitologic and serologic survey. A total of 2039 filter paper samples from the first serologic survey has been tested. They were obtained in two segments of the study area. In area A1, control measures will be initiated in mid-1972; these measures include spraying with OMS-33 during the wet season, use of larviciding in the dry season, and bi-monthly mass distribution of antimalaria drugs. Area C will serve as a control, and no antimalaria activities will be carried out here. The overall slide positivity rate in Area A1 was 67.3%, while the infant parasite rate was 87.5%. In control Area C, these values were 67.3 and 87.3%, respectively. The seropositivity rate (IHA titer  $\geq 1:16$ ) of the filter paper blood specimens collected in the first survey was 89.5% in Area A1 and 83.3% in Area C. The second serologic survey was carried out during the dry season, in May and June, 1972, when approximately 2,500 filter paper specimens were collected.

c. Philippines

The serologic and parasitologic results of studies in the Philippines are analyzed and related to avail-

able malarionetric data in cooperation with Dr. John Mason (CAMRS), previously Chief Malaria Advisor in the Philippines.

d. Tunisia

Filter paper blood specimens collected in Tunisia in April 1970, for WHO were examined by the IFA test by Dr. Ambroise-Thomas (Grenoble) and by the IHA test at CDC. The relative frequency distributions of the IHA and the IFA titers showed that 16.5% of the specimens had a positive titer of 1:16 or greater, while 18.5% had a positive IFA titer ( $\geq$ 1:20). The overall agreement between the IHA and the IFA tests was 76.8% (Table 4).

Only one of 2,169 slides collected was positive (a P. vivax infection in a 14-year-old boy). The results of both the IFA and the IHA tests indicated the absence of recent malaria transmission in most of the 18 localities in the eight gouvemorats sampled throughout Tunisia.

e. CAMRS

Collaborative efforts with CAMRS in the evaluation of the IHA test in Central America have continued. Baseline data have been obtained for localities in the Guayabo area near a large hydroelectric dam project and a coastal area (San Diego) in El Salvador. An attempt was made to retest samples collected in El Salvador during 1970-71. These filter paper samples

were processed by Dr. Collins, NIH/NIAID Chamblee, Ga. using a technique different from the one employed in this laboratory. These samples were found to be unsuitable for re-testing because the presence of filter paper fibers interfered with the IHA reactions. Due to the small volume of material available, it was not feasible to remove the filters.

f. Rhodesia

A detailed protocol has been developed for a pilot study in Rhodesia to test the survey methodology and the use of serologic techniques (IHA and IFA tests) for malaria surveillance in those parts of that country where malaria control activities are carried out.

g. In July, 1971, filter paper specimens were obtained from Nigerian students in Washington, D.C., as part of a longitudinal serologic study of this population group. All students had positive IHA titers but the titer level declined with increasing length of residence in the U.S.

h. A laboratory and field investigation on babesiosis and malaria was carried out in Haiti. Babesia organisms were identified in blood films of 5 patients who also had P. falciparum malaria. None of the patients with Babesia infection had had a splenectomy.

Only one of the 72 household or neighborhood contacts of the malaria-babesia cases recalled having had symptoms compatible with malaria but 46 of these 71 contacts (65%) had antibodies to malaria. An unusual cluster of malaria-babesia cases was found in a locality of 200 inhabitants.

Animal inoculation studies to isolate the Babesia parasite are in progress. Hemoglobin electrophoresis revealed that 25% of the tested individuals had a hemoglobinopathy; HB S and Hb C were found in equal proportions.

- i. A comparison of age related serologic population profiles in five different areas indicated differences in the malaria experience of these populations as measured by a single survey in each area (figure 1). Little relationship was observed between the age specific seropositivity and parasite rates.
- j. The relative frequency distributions of the antibody titers are markedly different in populations living in areas with different levels of malaria endemicity (figure 2).
- k. Seropositivity rates in patients with parasitemia were high and independent of age, both in Ethiopia and the Philippines (figure 3). In contrast, the mean positive titers in the Philippine patients were relatively low in the younger age groups and rose

with age, while they were high in all age groups in the Ethiopian patients. The antibody levels are more likely to reflect the cumulative effect of multiple antigenic stimuli than an age-related ability to produce antibodies. The most notable difference between the two populations from which the patients came was that the parasite rate in the age group under 5 was 62% in Ethiopia as compared with 7% in the Philippines.

1. Epidemic Aid

As a part of epidemic investigations the Epidemiology Program, CDC 50 sera were tested for malaria antibody. Of those sera, 22 were initial or follow-up sera from drug associated malaria cases and 28 were from an investigation of shipboard malaria. In an effort to identify the source of an indigenous case of malaria in Texas, 44 sera from contacts were tested. It was not possible to serologically identify a source.

- B. Evaluation and Development of the Indirect Fluorescent (IFA) Test for Malaria

1. Antigen Studies

- a. Plasmodium ovale studies

A chimpanzee with negative malarial IFA test reactions was acquired, conditioned and splenectomized. Following recovery from the splenectomy the animal was

inoculated with blood from a patient who had been infected with P. ovale parasites. Within a week after the chimpanzee was inoculated a few malaria parasites were observed concomittantly with Babesia-like organisms. No further malaria parasites have been seen and the chimpanzee has not developed any detectable antibodies to malaria. Because the chimpanzee did not develop a useful infection, a new source of inoculum is being sought.

b. Schizont antigens

The evaluation of the schizont antigens for the IFA test has been completed. Two additional variables were included in the evaluation: the use of micro-titration and the use of a better conjugate as compared to ones previously used. For the evaluation, a total of 320 positive and negative sera was tested. The test with the new antigen and new conjugate is very sensitive and new criteria for determination of positive and negative reactions have to be used. Using a dilution of 1:64 for a positive reaction, the test has a sensitivity of 96.5% and a specificity of 94%.

c. Multispecies antigen

Two lots of antigen containing P. vivax, P. falciparum, and P. brasilianum have been made. Titers obtained with the multi-species antigen are equal to or within

one fourfold dilution of the highest titer obtained with any of the single species antigens. In the future, all sera will be screened with the multi-species antigen; this will reduce by two-thirds the number of slides presently used. All positive sera will be titrated with single species antigens for species differentiation.

2. Occult malaria studies

Filter paper blood samples from Nigerian students residing in the United States have again been obtained and tested. No appreciable decline in antibody levels was noted.

3. Comparative serologic studies.

a. Comparison of the CF and IFA tests

Sera from approximately 400 drug addicts, involved in a malaria epidemic in California, were tested by both IFA and CF. Using the criterion of 30% hemolysis as a positive reaction the CF method detected antibody in 27 of 47 P. vivax cases, whereas the IFA test detected antibody in 47 cases. The CF test had a false positive rate of 22%, whereas IFA had less than 1%. Considering 80% hemolysis as a positive reaction, the false positivity rate was reduced to 8% without affecting the sensitivity.

b. Comparison of IFA tests from two laboratories. Two-hundred-eighty-seven specimens collected in El Salvador have been treated by IFA method at NIH, and at CDC. The results indicate that the CDC-IFA test detected approximately twice as many positive reactions as detected by the NIH procedure. Selected sera from a population in Pare, Tanzania, have been tested with IFA by both Dr. C.C. Draper of the London School of Tropical Medicine and CDC. Comparison of results with P. falciparum antigen showed excellent agreement between the two laboratories. Results at CDC with P. vivax, P. ovale, and P. brasilianum antigens, which Dr. Draper did not use, indicate that after P. falciparum, P. malariae is the most prevalent species in this area; P. vivax is third; and P. ovale is last. Though the number of sera tested was small, the serologic results agree with the prevalence determined by blood slide examination in the area.

4. Use of immunoglobulin specific conjugates

a. Evaluation of a CDC anti-IgM conjugate

Sera were tested from 22 patients who had been experimentally infected with malaria to evaluate a CDC-IgM conjugate. This included 16 acute sera

and six sera from individuals who had been treated 6 months previously. The 16 acute specimens gave positive IgM reactions while the other six specimens were negative.

- b. Testing of filter paper blood specimens from the WHO Malaria Research Project IR-0172, Kano-Nigeria. A total of 258 filter paper blood specimens collected in October 1971 in Nigeria has been tested by IFA; 257 specimens were positive at 1:64 or greater when tested with the multispecies antigen. IgM conjugate was used to test an additional 101 specimens, 50 of which had an IgM reaction at 1:32.

5. Radioimmunoassay test for malaria

With the cooperation of the Developmental Virology Unit, Virology Section, a pilot run using the radio-immunoassay (RIA) technique was performed to determine its feasibility in malaria serology. The test procedure is identical to the IFA test except that the conjugate is tagged with  $I^{125}$  instead of fluoresceine isothiocyanate (FITC), permitting an automated reading of the test results with a gamma scintillation counter. The results were promising; with the RIA test, positive and negative sera were distinguishable from each other at low dilutions.

6. Other Studies

- a. A guest researcher, Dr. Francis Nkruma, from Ghana

began a study on malaria and anemia in Ghana on children while at CDC. Sera from 34 Ghanaian children with P. falciparum infections were tested both for total malarial antibody and specific IgM and IgG antibody. All children had malarial antibody titers with very strong IgG responses. However, the highest IgM titer which was observed with P. falciparum antigen was 1:16. Approximately 50% of the children had no detectable specific IgM activity. There was no correlation between anemia and the serologic values.

b. Splenomegaly and malaria in South Vietnamese patients.

A study with Dr. L.C. Butler, NAMRU-2, of Vietnamese patients with and without splenomegaly to determine the relationship of malaria antibodies and splenomegaly has been completed. In total, 90 sera have been tested. Dr. Butler reports that all the splenomegaly patients except one had high antibody titers, but there was also a fairly high frequency of positive titers in patients without splenomegaly.

C. Publications

Sulzer, A.J., and Wilson, M. 1971. The indirect fluorescent antibody test for the detection of occult malaria in blood donors. Bull. Wld. Hlth. Org. 45: 375-379.

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Ludford, C.G., Sulzer, A.J., Wilson, M. and Hall, W.T.K. Antigenic cross-reactions between Babesia argentina and Plasmodium vivax and Plasmodium falciparum. *Exp. Parasit.* (In press).

Butler, T. Wilson, M., Sulzer, A.J., and Thi Loam, N. Chronic Splenomegaly in Vietnam: I. Evidence for malarial etiology. *Am. J. Trop. Med. Hyg.* (in edition).

Lobel, H.O., Mathews, H.M., Kagan, I.G. The Indirect Hemagglutination Test for Malaria and its Application in Anti-malaria Program (In edition).

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Farshy, D., and Kagan, I.G. 1971. Use of Stable Sensitized Cells in Indirect Microhemagglutination Test for Malaria. *Am. J. Trop. Med. & Hyg.* (in press).

(Papers presented at Scientific Meetings)

Kagan, I.G. Evaluation of the Indirect Hemagglutination Test as an Epidemiologic Technique for Malaria. Inter-American Malaria Research Symposium, November 1971, San Salvador, El Salvador.

Lobel, H.O. and Mathews, H.M. Persistence of Antibodies to Malaria detected with the Indirect Hemagglutination Test and Application of the Test in Malaria Surveillance. Ann. Meeting, Am. Soc. Trop. Med. & Hyg. December 1971, Boston.

JUNE 1972 -- FY-72

18.

CENTER FOR DISEASE CONTROL -- Agreement with AGENCY FOR INTERNATIONAL DEVELOPMENT  
CDC Contract No. 3902-15X

AID PASA Control No. RA (IM)5-68 -- PIO/T No. 931-17-111-485-72-3127702

Period covered by this bill: July 1, 1971 -- June 30, 1972

Fiscal Year 1972 Contract Amount	<u>Laboratory Division(Direct)</u> \$92,500.00	<u>CDC (Indirect)</u> \$18,500.00	<u>Total</u> \$111,000.00
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OBJECT CLASSIFICATION OF EXPENDITURES INCURRED:

Object 11	- Personnel Compensation	71,902.79	
12	- Personnel Benefits	1,948.24	
21	- Travel & Transportation of Persons	1,398.84	
23	- Rent, Communications, & Utilities	400.00	
24	- Printing & Reproduction	.00	
25	- Other Services (Contractual)	626.18	
26	- Supplies and Materials	<u>8,223.95</u>	
	TOTAL Direct Cost	\$ 92,500.00	
	Plus 20% CDC Indirect	<u>18,500.00</u>	
	TOTAL COST	<u>\$111,000.00</u>	

D. FISCAL SUMMARY



TABLE 2

## IHA Titers of sera with stable sensitized cells

No.	CDC Code	Days after preparation					No.	CDC Code	Days after preparation				
		0	30	60	90	120			0	30	60	90	120
1	69	32	32	32	32	32	21	166	1024	1024	1024	1024	2048
2	66	4096	4096	4096	4096	4096	22	174	512	256	256	256	128
3	73	0	4	0	0	0	23	146	512	512	256	128	128
4	74	0	0	0	0	0	24	152	1024	2048	1024	2048	2048
5	75	256	128	256	128	64	25	149	512	512	512	512	512
6	76	128	128	256	128	128	26	162	512	512	256	256	256
7	77	256	512	256	256	128	27	173	0	0	0	0	0
8	91	128	64	128	64	64	28	181	1024	1024	1024	1024	2048
9	93	4096	4096	4096	4096	4096	29	178	8	8	8	0	4
10	94	0	0	0	0	2	30	176	4096	2048	4096	4096	4096
11	34	256	128	128	128	64	31	116	512	256	512	256	256
12	33	32	16	16	16	16	32	145	4096	4096	4096	4096	4096
13	10	4096	4096	4096	4096	4096	33	114	4096	4096	4096	4096	4096
14	29	128	64	128	128	32	34	113	0	0	0	0	0
15	57	512	256	256	256	128	35	99	256	512	512	512	512
16	35	0	0	0	0	0	36	98	4096	4096	4096	4096	4096
17	52	2048	4096	4096	4096	4096	37	135	4096	4096	4096	4096	4096
18	53	128	256	128	128	64	38	137	1024	1024	1024	1024	1024
19	37	0	0	0	0	2	39	97	256	512	256	256	128
20	32	256	256	256	256	256	40	139	4096	4096	4096	4096	4096

Table 3

## IHA Titers of Serum from Patients with Malaria Infection and Controls

CDC Code	Slide Diagnosis	<u>P. knowlesi</u> antigen	<u>P. falciparum</u> antigen	<u>P. knowlesi</u> <u>P. falciparum</u> (1:1)
139	<u>P. vivax</u>	4096	4096	4096
9	"	256	256	512
10	"	4096	512	4096
4	"	512	16	256
36	"	2048	128	1024
59	"	1024	64	256
117	"	512	16	256
52	"	512	128	512
66	"	4096	64	1024
77	"	256	16	128
99	"	1024	16	256
S-7	"	2048	64	1024
97	"	256	64	128
6	<u>P. falciparum</u>	256	256	256
8	"	128	4096	2048
15	"	32	1024	256
29	"	16	512	64
64	"	128	2048	256
118	"	128	4096	1024
132	"	64	1024	1024
167	"	256	1024	512
57	"	32	1024	128
137	"	64	512	256
S-A	"	256	4096	1024
S-Be	"	256	4096	1024
S-8	"	256	4096	1024
Seventeen Control Sera (Negative)		Nine 0 Five 2 One 4 Two 8	Ten 0 Six 2 One 8	Thirteen 0 Three 2 One 8

TABLE 4

Agreement between IHA and IFA tests on sera from Tunisia

IHA positive -  $\geq 1:16$

IFA positive -  $\geq 1:20$

		.....IHA.....		
		Neg.	Pos.	Total
IFA	Neg.	1482	232	1714
	Pos.	251	111	362
Total		1733	343	2076

Agreement between IHA and IFA tests

IHA	+	-	+	-		
IFA	+	-	-	+	Total	Agreement
	111	1482	232	251	2076	
Percent	5.4	71.4	11.2	12.1		76.8%

Agreement between IHA and IFA tests for positive IFA and IHA reactors

Reactors only:

111	232	251	594
18.7	39.1	42.3	18.7%

FIGURE 1

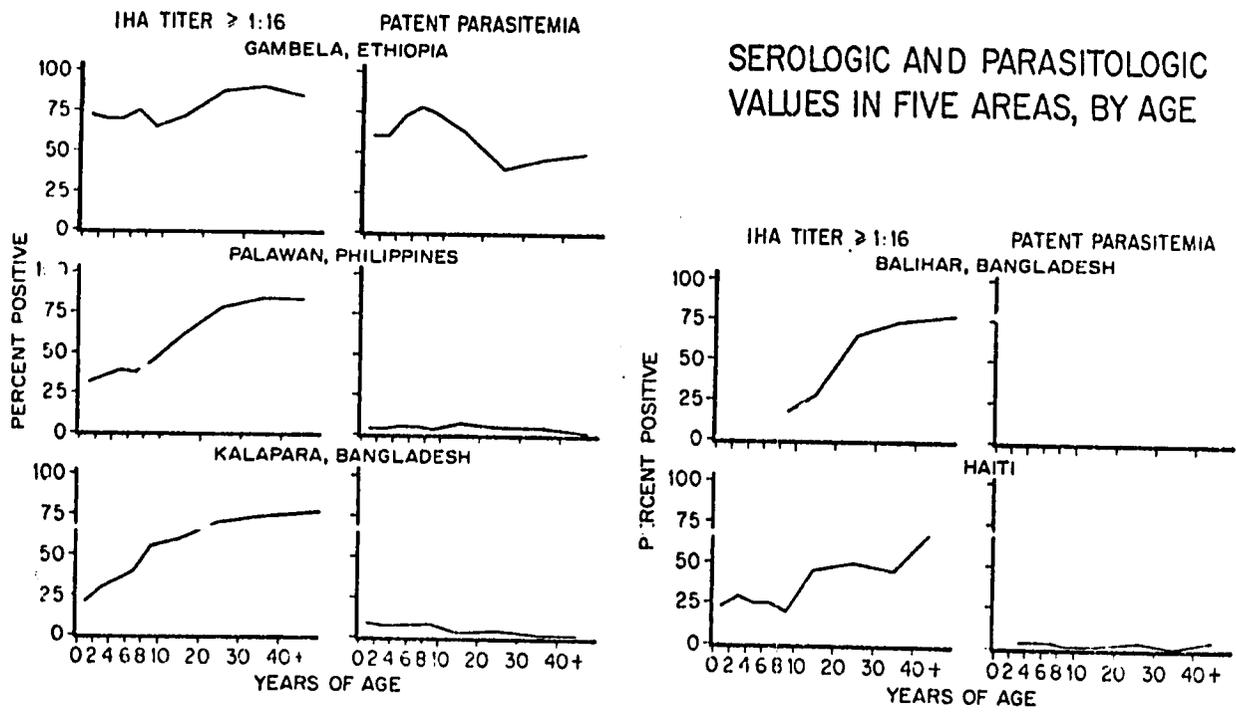


FIGURE 2

RELATIVE FREQUENCY DISTRIBUTION OF IHA TITERS

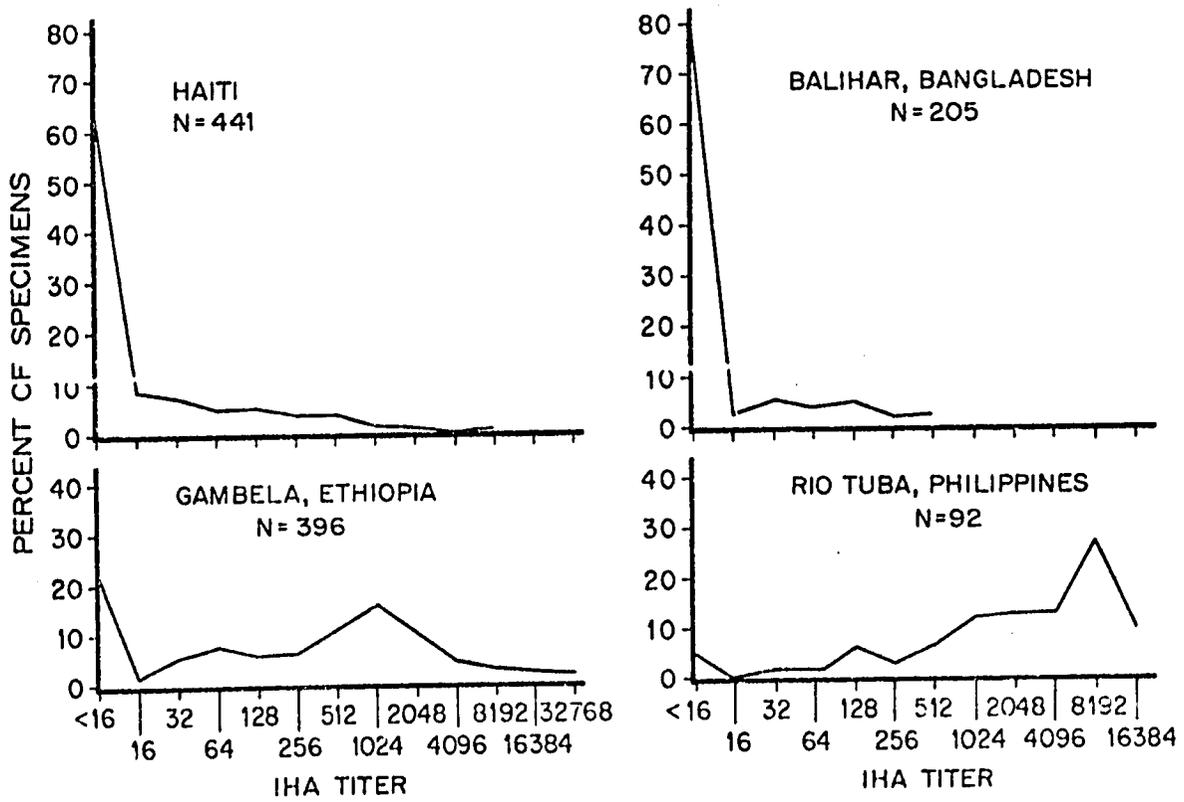


FIGURE 3

