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9. ABSTRACT

This paper describes studies conducted to evaluate an indirect hemagglutination test for malaria. Antigen was prepared from mature schizonts of *Plasmodium knowlesi* (one of the four human malaria parasite species) harvested from rhesus monkey blood. The plasmodia were freed from red blood cells with distilled water and then disrupted in a French pressure cell. A 2% NaCl extract of the disrupted plasmodia was used to sensitize group O tanned erythrocytes from human blood. The IHA test detected antibody titers of 1:16 or greater in 98% of slide-proved cases of malaria, and in less than 1% of serum from persons without a history of malaria. Problems in this technique included harvesting the correct stage of plasmodia for antigen, using the French pressure cell, and storing the labile disrupted preparation. These variables have been controlled in a test that can detect antibody against a spectrum of plasmodia that infect primates, with a minimum expenditure of antigen. Evaluation of the technique for field studies indicates that plasma eluted from filter paper rectangles are suitable for titration. This method requires further study. Seroepidemiological studies with 10,956 sera of military recruits from four countries in the Western Hemisphere suggest that the IHA test may be useful in the study of epidemiology of malaria. Discrepancies between the prevalence of malaria estimated by serological methods and by active and passive surveillance require further study and evaluation.

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EVALUATION OF THE INDIRECT MICROSALMAGGLUTINATION TEST FOR MALARIA<sup>1</sup>

1. Introduction

Stein & Desowitz (1964) described an indirect haemagglutination (IHA) test utilizing formalin and tannic acid treated sheep red cells, sensitized with antigens from Plasmodium cynomolgi and P. coatneyi. This test was subsequently used in a field study of malaria immunity in Australian New Guinea (Desowitz & Saave, 1965). Bray & El-Nahal (1966) reported difficulties with this test system and recommended a technique using fresh sheep red cells treated with tannic acid. Mahoney et al. (1966) fractionated antigens prepared from the plasmodia of P. knowlesi following disruption of the parasites in a French press. They found antigens active in IHA tests in both the supernatant and the residue after centrifugation at 125 000 g for 90 minutes.

Since the IHA test has proven to be useful in sero-epidemiologic studies on toxoplasmosis (Walls et al., 1967; Walls & Kagan, 1967) and other parasitic diseases (Cuadrado & Kagan, 1967), an attempt was made to standardize and evaluate an IHA test for malaria. Such a test might be employed as a supplement to the fluorescent antibody test for diagnostic purposes and may be useful in studying the epidemiology of malaria.

2. Materials and methods

Antigen preparation. Splenectomized rhesus monkeys were infected with the Anopheles hackeri strain of P. knowlesi<sup>2</sup> by blood transfer. The animals were anaesthetized and exsanguinated when 10% or more of their red blood cells contained mature schizonts. Either heparin or sodium citrate was used as an anticoagulant. All further procedures were carried out at 4°C.

The blood cells were washed three times in 10 volumes of 0.85% saline solution by centrifuging at 1100 g for five minutes, and lysed by adding at least 10 volumes of distilled water. After centrifugation at 3000 g for five minutes, the sediment consisted of plasmodia, leukocyte nuclei, and some red cell stroma. Washing the sediment three times with saline solution facilitated removal of most of the fluffy white stroma. The final sediment was stored at -70°C before further processing. Blood from a 3-kg monkey could be processed in four hours to yield 5-20 ml of plasmodial sediment.

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For further preparation, the plasmodia were thawed in tepid water, placed in a cooled French pressure cell,<sup>1</sup> and two volumes of antigen diluent (equal volumes of 4% NaCl and M/15 phosphate buffer at pH 6.1) were added. The cell was operated at 20 000 lbs/in<sup>2</sup> (1400 kg/cm<sup>2</sup>). Antigen diluent used for rinsing the cell brought the final volume to four times the volume of the plasmodial sediment. The disrupted material was centrifuged at 8000 g for 15 minutes. The supernatant was tan to pink in colour and opalescent; the residue was black and appeared to consist of malaria pigment and amorphous debris. The supernatant was considered undiluted antigen and used as such, or diluted with antigen diluent when titration tests showed that dilution was compatible with suitable cell sensitivity. The undiluted antigen was stored at -70°C.

Test procedure. Human group O blood, either fresh or stored for less than 21 days in acid citrate dextrose at 4°C, was washed three times with phosphate buffered saline solution (PBS-equal volumes of 0.85% NaCl and M/15 phosphate buffer at pH 7.2). The buffy coat was discarded. The erythrocytes were suspended in 40 volumes of PBS, and an equal volume of 1:20 000 (w/v) tannic acid in PBS was added. The mixture was incubated for 15 minutes in an ice bath. After they were washed twice with PBS, the cells were ready for sensitizing.

The packed tanned cells were sensitized by adding five volumes of antigen, mixing and incubating for 15 minutes at 37°C. To decrease nonspecific agglutination, an equal volume of PBS with 5% normal rabbit serum (NRS) was added, and this mixture was incubated for an additional five minutes. After one wash in PBS at pH 7.2 containing 1% NRS, the cells were suspended in 50 volumes of this solution and used immediately. Cells with 1:10 000 merthiolate were useful for one week if stored at 4°C. Stored cells were re-suspended in fresh PBS with 1% NRS before use.

Plastic plates<sup>2</sup> with an 8 x 12 array of wells were used. Each well first received 0.05 ml of PBS with 1% NRS. A 0.05 ml diluting loop filled with test serum was placed in the first well in each row, and the serum was serially diluted in a doubling step-wise manner, beginning with a 1:2 dilution.

During agitation, 0.025 ml of the 2% sensitized cell suspension was dropped into each test well. Positive and negative control sera were titrated each day. The completed test was read 45 to 90 minutes later. A smooth mat of agglutinated cells, a 2+ reading according to Stavitsky (1954) was considered a positive reaction. In order to detect nonspecific agglutination reactions, positive sera were tested with a 2% suspension of tanned red blood cells sensitized with 5% NRS.

Sera titrated. To evaluate the test for specificity, sera from 61 residents of St Lawrence Island, Alaska, 11 chronic tuberculosis patients from Atlanta, Georgia, and 43 syphilis patients from throughout the United States of America were assumed to be from malaria-free areas. A parasitology diagnostic battery of 166 sera was also tested. The sera contained 12 subgroups of specimens known to be serologically positive for echinococcosis, filariasis, schistosomiasis, trichinosis or other non-malarious diseases, or else negative for all these diseases.

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<sup>1</sup> American Instrument Company Inc., Silver Spring, Maryland.

<sup>2</sup> Cooke Engineering Company, Alexandria, Virginia.

To evaluate the test for sensitivity, sera assumed to contain antibody to malaria were taken from patients with blood-film-proven infections. Sera from 17 cases of P. falciparum infection primarily represented United States citizens who contracted malaria in a country with known endemic malaria. The sera from 130 persons with P. vivax infection were collected in a hyperendemic focus of malaria in Honduras.

To evaluate the test for sero-epidemiologic studies, 10 956 serum specimens representing collections from military recruits in the United States (Paul et al., 1964), Brazil (Florey et al., 1967), Colombia and Argentina were evaluated. In addition, sera eluted from filter-paper were studied to evaluate this technique for use in field studies.

### 3. Results

Factors influencing the test. Since the sensitivity, specificity and reproducibility of the IHA test for malaria appeared equivocal from an earlier report (Bray & Fl-Nahal, 1966), preliminary studies were carried out to characterize the variables which influenced the test. Conditions having an insignificant effect on the sensitivity of the test were splenectomy of the monkey, contamination of the antigen with host components, pressure of the French press, centrifuge speed, PBS osmolarity, tannic acid concentration, temperature and pH during sensitization of the cells.

Collins et al. (1965) previously reported that rhesus monkeys received in the United States of America for medical research may have had prior experience with malaria. They found that 65% of the rhesus monkeys received from New Delhi had sera reactive in the IFA test when tested with several different simian malaria antigens. We have found that over 50% of our monkeys from the same source had sera reactive by IHA using P. knowlesi antigens. This prior experience and, at times, partial immunity may have been responsible for unpredictable infections in experimentally induced malaria. For antigen production, all monkeys were splenectomized prior to infection. The use of splenectomized animals made the course of infection more predictable.

The plasmodial preparation, prior to disruption in the French press, contained white blood cell nuclei and some red cell stroma as well as unknown quantities of adsorbed host elements. These antigenic materials have not appeared to affect the specificity of the test, but further delineation of their influence awaits the production of a purer preparation.

Antigen extraction from the whole plasmodia was carried out in a cooled French press at 20 000 lbs/in<sup>2</sup> (1400 kg/cm<sup>2</sup>). We found the antigen to be useful in the IHA test when produced at lower pressures, but felt that the best yield was obtained at the maximal pressure. The disrupted material was centrifuged with forces varying from 3000 g to 48 000 g without affecting the activity of the final product.

The PBS used as buffer had an osmolarity of 228 milliosmoles. Another formula of PBS with 308 milliosmoles of solute did not greatly alter the sensitivity of the test. In agreement with Shioiri (1964), we found that the concentration of tannic acid could be widely varied, but that 1:20 000 as used above was the most effective. Our antigen preparation was effective in sensitizing tanned red cells when incubated at 37°, 22° or 4°C within the pH range 5.6 to 7.2.

Several variables must be controlled for standardization of this technique. These included controlling the stage of parasite prepared for antigen, physical characteristics of antigen storage, species of red blood cells, temperature of tanning the red cells, and osmolarity of the antigen diluent.

The activity of antigens from P. knowlesi varied according to the stage at which they were harvested. Antigens which were reactive with sera from animals immune to P. knowlesi could be extracted from several stages of the erythrocytic cycle.

Antigens having the greatest degree of cross reactivity with sera from other primate malaria infections were present in increasing concentrations as the parasite became more mature. These antigens have detected antibodies in our laboratory against the following species of primate malaria: P. gouldi, P. coatneyi, P. cynomolgi, P. fieldi, P. knowlesi, P. brasilianum, P. malartae, P. ovale, P. vivax and P. falciparum.

The best preparations, made from blood containing an equal number of mature schizonts and of young rings, could be diluted 20 times and still obtain maximal sensitization of tanned cells. Preparations of ring stages were not as active. The P. knowlesi infections in the splenectomized animals generally developed synchronously and required harvesting within a critical two-hour period to yield the highest quality of preparation.

The active antigens of P. knowlesi were quite labile. Plasmodia stored at  $-70^{\circ}\text{C}$  showed a gradual loss in antigen activity over a three-month period. Disrupted plasmodia stored in antigen diluent at  $-70^{\circ}\text{C}$  lost activity in two weeks. When stored at  $4^{\circ}\text{C}$ , the disrupted preparation was inactivated in three days. Once adsorbed on to red cells and washed free of other plasmodial constituents, the antigens appeared to remain stable until contamination occurred or the cells spontaneously agglutinated.

The use of human group O or rhesus monkey erythrocytes improved the reliability of sensitization. Sera were tested with red cells from the same species. Rabbit and sheep cells have occasionally been sensitized to a degree that equals human cell sensitization, but have been less reliable. Human group O cells from five different donors have been of equal quality. Those which were drawn while the donor was fasting and were stored in ACD have been least fragile, confirming the report of Indeglia et al. (1967).

A water bath at  $4^{\circ}\text{C}$  was necessary for adequate tanning of cells. A  $37^{\circ}\text{C}$  water bath did not promote red blood cell changes that allowed sufficient adsorption of the malaria antigens.

The antigen diluent found effective for obtaining maximum sensitivity of human O cells, tanned at  $4^{\circ}\text{C}$ , was 2% NaCl with phosphate buffer. Use of this diluent was not essential during disruption of the plasmodia. The sensitization of red cells with a weak antigen was enhanced when salt solution approaching 2% concentration was used (Table 1). Concentrations of 3.8% NaCl or greater led to spontaneous agglutination of the red blood cells. Sensitization of red cells with a 2% NaCl antigen solution was chosen to increase sensitivity without risk of frequent spontaneous agglutination. Concentrated solutions of antigen have adequately sensitized cells using an antigen diluent with 0.5% NaCl.

Test reproducibility. In an evaluation of titre reproducibility, 30 sera were titrated as blind duplicates six times (Table 2). As expected, tests performed on the same day using the same lot of cells had the least variation in results. One determination differed from its duplicate by one dilution or less 90% of the time and by two dilutions or less 95% of the time. Similar reproducibility within two serum dilutions could be obtained when different lots of cells were used on different days if the titres were adjusted by observing the reaction of the positive control sera. Similar results were also obtained with different technicians and different test readers.

Test specificity and sensitivity. To evaluate the diagnostic significance of titre, various groups of sera were tested. Titres of 1:16 and greater were uncommon (0.9%) in sera assumed to be free of antibody against malaria (Table 3). In the parasitology diagnostic battery, sera with a titre of 1:16 or greater (6%) may represent true positives because they were collected from individuals with infections from areas where malaria may be endemic. Testing sera from persons with malaria diagnosed by blood smear examination revealed a sensitivity of 96% when a titre of 1:16 or greater was considered a positive reaction (Table 4). Sera were collected and stored at  $-20^{\circ}\text{C}$  without a change of titre over a 12-month period.

Filter-paper studies. Preliminary results on sera collected by filter-paper absorption of capillary blood and compared with that collected by venipuncture have indicated that a filter-paper technique will yield comparable results and thus aid the collection of specimens. The technique was evaluated with seven different papers from five manufacturers. The most suitable paper was evaluated by testing eluted specimens in parallel with serum specimens.

Desirable characteristics of a filter-paper for this purpose were considered to be: sufficient stiffness of a 1 x 3-in (2.5 x 7.6 cm) rectangle to allow storage in a microscope slide box, absorption of sufficient blood in a 14-mm circle for serologic tests, integrity and inertness in relation to the blood specimen and its elution, availability and low cost.

The filter-paper (KOPACO 1023,028, Rochester Paper Company, Rochester, Michigan)<sup>1</sup> which met most of these requirements was purchased in 20 x 20-in (50.8 x 50.8 cm) sheets, cut to 1 x 3-in (2.5 x 7.6 cm) rectangles, and printed with two circles, each 14 mm in diameter. For field studies, the filter-paper rectangles (112) were returned to the diagnostic laboratory in plastic bags with glassine interleaves and a desiccant.

The blood was collected by placing the FPR on top of the bleeding point with the circle uppermost, allowing the blood to soak through the paper and fill the circle. Approximately 0.14 ml of blood (three drops) was necessary to saturate each circle. Identifying data were written on one end of the FPR. The paper was dried at ambient temperatures and then kept as cold and dry as possible.

In the laboratory, a hand-operated paper punch (13 32-in (11.7 mm) single hole punch, "Pet" No. 59, Wilson Jones Co., Chicago, Illinois) was used to remove a 13 32-in (11.7 mm) disc from within the filled circle. The disc was immersed in 0.2 ml of phosphate buffered saline solution (PBSS) and agitated twice during the 30-minute soaking period. The disc was removed with a rod, rolling the rod over the disc to express some of the eluate. Approximately 0.13 ml of eluate could be obtained in this manner: centrifugation of the disc at 1000 g for 10 minutes on top of a short segment of plastic tubing allowed recovery of 0.17 ml of eluate. It may be used immediately or stored at -20°C.

Seventeen sera were tested in parallel with the eluates from FPR specimens that had been stored at -20°C. The sera were positive three two-fold dilutions higher than the eluates. This indicated that the eluate was equal to a 1:8 dilution of serum. In a microhaemagglutination titration of filter-paper eluates, the first well of the plastic plate would contain a 1:16 dilution. The reproducibility of the IHA test was not altered when eluates were made from FPR that were stored at -20°C.

When FPR and sera were stored at 4°, 24° or 10°C in a dry atmosphere for 15 days, or at 37°C with 100% humidity for five days, both specimens partially lost their antibody activity. The filter-paper eluate of the above specimens varied from a 1:11 to a 1:22 dilution of serum as compared to 1:8 in the frozen specimens.

The use of FPR to absorb capillary blood greatly simplifies the collection of blood specimens in the field. These dried specimens can then be easily shipped to a central laboratory for testing. Each 13 32-in (11.7 mm) disc will yield sufficient eluate for one to three tests beginning at a dilution of 1:32. If necessary, the remaining portion of the 14 mm circle can be cut out and processed to yield additional tests. Without centrifugation, several hundred filter-paper specimens have been tested in one day by one technician.

Sero-epidemiologic studies. Four collections of military recruit sera were tested. They consisted of 2237 sera from the United States of America, 281 sera from Brazil, 201 sera from Colombia and 3977 sera from Argentina. Table 5 lists the titres obtained with the

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<sup>1</sup> Use of trade names is for identification only and does not constitute endorsement by the World Health Organization, the United States Public Health Service or the United States Department of Health, Education and Welfare.

military recruit sera. For epidemiologic purposes, a titre of 1:8 or greater was considered a positive test. This decision was based on the frequency distribution plot of the titres in each collection as shown in Fig. 1. Based on this premise, the following prevalence of positive reactors was obtained: United States of America 20 of 2237 (1%) specimens positive; Brazil 558 of 2281 (21%) specimens positive; Colombia 629 of 2961 (21%) specimens positive; and Argentina 112 of 3077 (1.6%) specimens positive.

#### Distribution of positive reactors within each country

United States of America: Fig. 2 shows the States of the United States of America from which a serum specimen with a titre of 1:8 or greater was obtained. The distribution appears random, and no area had more than one positive specimen.

Brazil: Fig. 3 shows the prevalence of positive malaria reactors in Brazil by States. Each dot represents 2% or fraction of 2% positive of the number of sera tested in each area. States with no markings were not adequately sampled (less than 24 samples). Fig. 4 presents the prevalence distribution of malaria in Brazil as determined by active surveillance in 1963-1964. Each dot represents 1% positive slides. Although the dots in Figs 3 and 4 represent different parameters, there is a correlation between States of high and low malaria prevalence.

Colombia: Fig. 5 represents the distribution of positive malaria reactors in Colombia. Only States with 21 or more sera are represented. Fig. 6 represents the geographic distribution of malaria as determined by surveillance in 1966. The distribution within the country is similar by both methods.

Argentina: Fig. 7 represents the prevalence of positive reactors in Argentina as determined by the IHA test. Fig. 8 represents the prevalence of malaria in Argentina as determined by both active and passive surveillance.

Note the discrepancies in results obtained by the two methods. In Tucuman we found 28% of a sample of 50 sera to be positive (1:9 or greater). The surveillance mechanism did not detect any infected individuals. In Chaco we obtained 6% positive reactors; surveillance data shows 3.3% positive slides. This indicates that our sample of 38 sera may not be representative of the State.

Serological testing revealed a high percentage of positive reactors in the area south of the endemic area (Cordoba, 9.3%; Santa Fe, 4.5%; Entre Rios, 3.8%; Mendoza, 11%; Santa Luis, 5%). This discrepancy should be investigated further. Although the serologic prevalence of malaria in Argentina is only 1.6%, the distribution of these reactors indicates a much broader distribution than is acknowledged by the eradication programme, especially in the State of Mendoza, an area considered to be malaria-free.

#### 4. Discussion

Previously described IHA tests have demonstrated a moderate degree of cross-reactivity between human and simian plasmodia. Stein & Desowitz (1961) found that antigens from P. cynocephali gave a high titre with P. vivax sera, but a low titre with P. falciparum sera. Antigens from P. cootzei gave high titres with P. falciparum but low titres with P. vivax sera. Mahoney et al. (1966) used an extract of L. knowlesi in an IHA test which gave high-titred reactions with all P. vivax sera. Titres of 1:16 or greater were found in only 19% of the P. falciparum sera. In the IHA test performed in our laboratory, very broad cross-reactivity between human and simian malariae was observed and the sensitivity with sera from individuals infected with P. falciparum was high.

The thermostability of malaria antigens has not often been commented upon in the literature. Most preparations have apparently been quite stable. An antigen prepared by Tolorovic et al. (1968) from acute infections of P. gallinaceum in chickens is stable for

several months at 4°C. Fluorescent antibody test (FEA) antigens are generally stored as dry smears at -70°C and remain usable for one year or more. Our crude extract of P. knowlesi plasmodia has been very labile. Storage at -70°C has been essential. This lability suggests that reactions continue in the undiluted preparation and result in alteration of the effective antigens. Effective preservations need to be developed.

The IHA test offers certain advantages in the serology of malaria. It requires little time per test: a technician working with sensitized cells can titrate over 400 sera per day. This may be further improved by the use of automated techniques. The antigen requirement of IHA is certainly much higher than that of the IFA test, but it utilizes simian plasmodia. Each monkey can provide sufficient antigen for 2500 complete titrations of more than 10,000 screening tests. These characteristics make possible the use of the IHA test in studies of the sero-epidemiology of malaria.

The use of filter-paper for the collection of capillary blood also extends the usefulness of the IHA test for field studies. Sera collected on filter-paper in tropical areas have been mailed to the United States and have been successfully titrated for antibody.

The aim of the IHA programme has been to provide a sensitive serologic tool that can be used effectively on a broad scale, both efficiently, inexpensively, and with a high degree of sensitivity and specificity. Such a serologic test may be a useful ancillary method in the evaluation and surveillance of malaria eradication programmes. The test as described in this manuscript is both sensitive and specific, and it meets the criteria for the study of the prevalence of malaria antibody in a population. The field evaluation of this technique must be carried out under careful control situations. Its ultimate usefulness in malaria eradication merits support and collaboration in such evaluation studies.

## 5. Summary

An indirect haemagglutination test for malaria has been evaluated. Antigen was prepared from mature schizonts of Plasmodium knowlesi harvested from the blood of the rhesus monkey. The plasmodia were freed from red blood cells with distilled water and then disrupted in a French pressure cell. A 2% NaCl extract of the disrupted plasmodia was used to sensitize tanned human group O erythrocytes.

This IHA test has detected antibody titres of 1:16 or greater in 98% of slide-proven cases of malaria and in less than 1% of sera from individuals without a history of malaria. The major problems in this technique included harvesting the correct stage of plasmodia for antigen, using the French pressure cell and storing the labile disrupted preparation. Control of these variables has made possible a test that can detect antibody against a spectrum of plasmodia infecting primates with a minimal expenditure of antigen.

Evaluation of the technique for field studies indicates that plasma eluted from filter paper rectangles are suitable for titration. This method requires further study.

Sero-epidemiological studies with 10,956 sera of military recruits from four countries in the Western Hemisphere suggest that the IHA test may be useful in the study of the epidemiology of malaria. Discrepancies between the prevalence of malaria estimated by serological methods and by active and passive surveillance require further study and evaluation.

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TABLE 1. EFFECT OF SALT CONCENTRATION ON THE SENSITIZING OF HUMAN RED BLOOD CELLS

	Titres			
	Normal human	<i>P. malariae</i> human	<i>P. vivax</i> human	<i>P. knowlesi</i> monkey
Human O cells, tanned at 4				
Dilute antigen in 0.7% NaCl	Negative	1:40	1:40	1:10
Dilute antigen in 0.8% NaCl	Negative	1:320	1:160	1:1280
Dilute antigen in 1.0% NaCl	Negative	1:640	1:640	1:1280
Dilute antigen in 1.3% NaCl	Negative	1:5120	1:5120	1:5120

TABLE 2. AGREEMENT OF DUPLICATE TITRATIONS IN AN EVALUATION OF 30 SERA

Test conditions	Number of duplicate determinations	Per cent. of duplicate determinations differing by	
		1 dilution or less	2 dilutions or less
Tests made on the same day using the same lot of cells	330	96%	97%
Tests made on different days using the same lot of cells	180	80%	95%
Tests made on different days using different lots of cells	360	69%	86%
Tests made on different days using different lots of cells but adjusted according to control sera results	360	69%	93%

TABLE 3. TITRE OF THE SERA OF INDIVIDUALS PROBABLY WITHOUT A HISTORY OF MALARIA

Source	Titre						Total
	0	2	4	8	16	32	
Normal Alaskans	51	3	1				61
Syphilis, primary and secondary	28	2	8	1	1		43
Chronic tuberculosis	5	1	2				11
Parasitology battery	105	13	19	19	6*	4*	166

\* Includes three cases of schistosomiasis and four cases of filariasis.

TABLE 4. TESTS OF THE SERA OF INDIVIDUALS WITH SLIDE-PROVEN MALARIA

Species	Titre						Total
	0	2	4	8	16	32	
<i>P. vivax</i> , Honduras	1	0	0	0	2	127	130
<i>P. falciparum</i> , United States	0	1	0	0	1	15	17
Mixed species, United States	2	0	0	3	3	77	85
Mixed species, United States Vietnam	5	0	0	1	2	127	135

TABLE 5. TITRES OBTAINED WITH MILITARY RECRUIT SERA FOR MALARIA ANTIBODY

Country	Titres																	
	0	2	3	4	8	9	16	27	32	64	81	128	213	256	512	1 024	2 048	4 096
United States of America	2 293	7		7	13		7											
Brazil	1 979	76		68	153		134		129	59		31		27	12	8	2	
Colombia	2 162	62		108	134		166		146	93		46		31	8	2	1	2
Argentina	2 877		58			100		30			10		2					

Fig. 1  
FREQUENCY DISTRIBUTION OF IHA-MALARIA TITRES OBTAINED BY TESTING SERA OF MILITARY RECRUITS

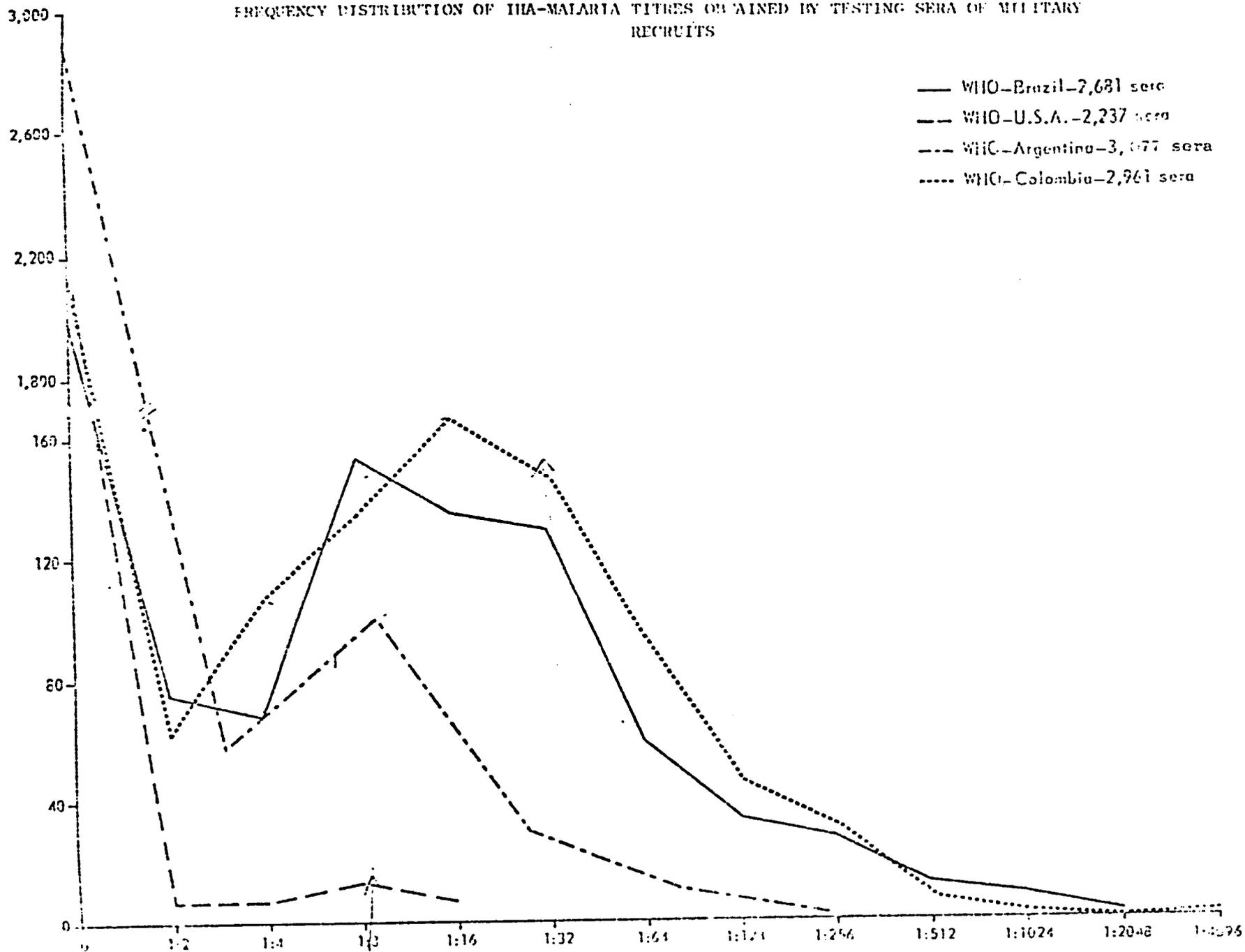
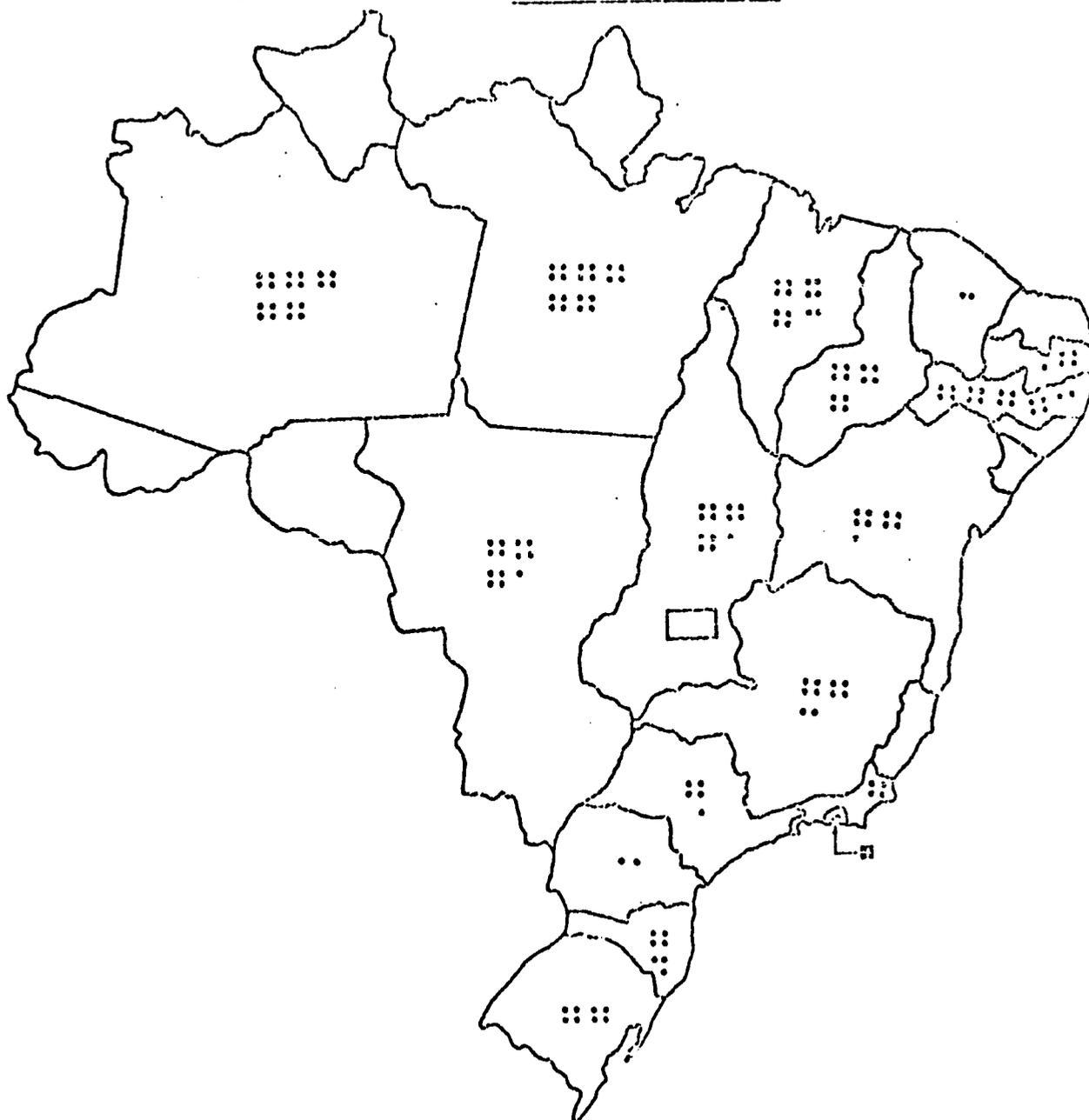




Fig. 3

THE GEOGRAPHIC DISTRIBUTION OF POSITIVE SEROLOGIC REACTORS IN BRAZILIAN MILITARY RECRUITS (1961) FOR MALARIA ANTIBODY AS DETERMINED BY PASSIVE MICRO-HAEMAGGLUTINATION WITH AN ANTIGEN PREPARED FROM PLASMODIUM KNOWLESII



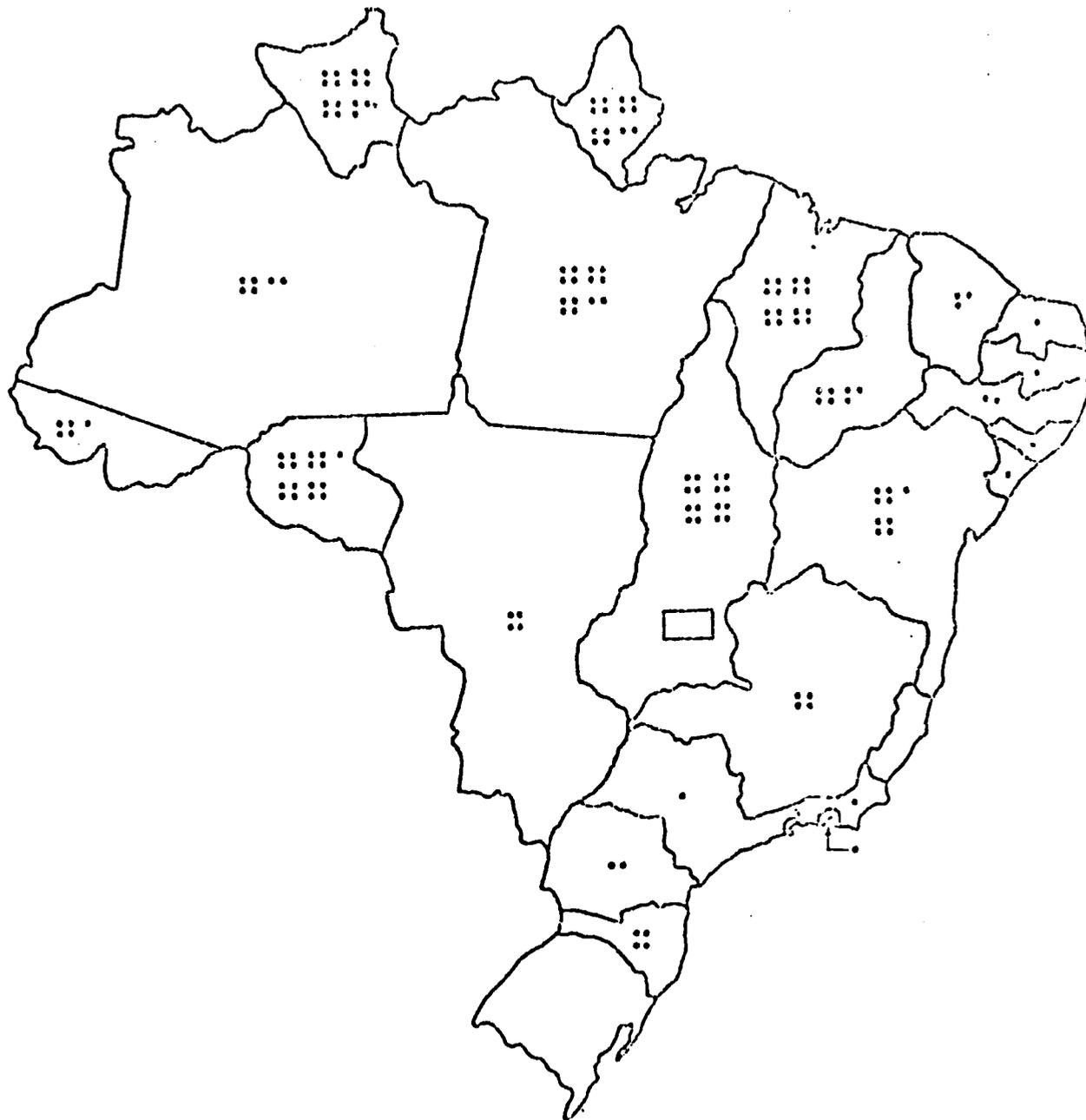
• Each dot represents 2% (or fraction of 2%) of sera reactive in the IHA test at a titre of 1:8 or greater.

Only states with 24 or more sera are represented.

■ 0% positive.

Fig. 4

THE GEOGRAPHIC DISTRIBUTION OF MALARIA IN BRAZIL (1963-1961) AS DETERMINED BY ACTIVE SURVEILLANCE



• Each dot represents 1% (or fraction of 1%) of positive slides.

Fig. 5

THE GEOGRAPHIC DISTRIBUTION OF POSITIVE SEROLOGIC REACTORS IN COLOMBIA MILITARY RECRUITS (1966) FOR MALARIA ANTIBODY AS DETERMINED BY THE PASSIVE MICRO-HEMAGGLUTINATION TEST WITH AN ANTIGEN PREPARED FROM PLASMODIUM KNOWLTONI



• Each dot represents 5% (or fraction of 5%) of sera reactive in the HIA test at a titre of 1:8 or greater.

FIG. 6

THE GEOGRAPHIC DISTRIBUTION OF MALARIA IN COLOMBIA (1966) AS DETERMINED BY ACTIVE AND PASSIVE SURVEILLANCE

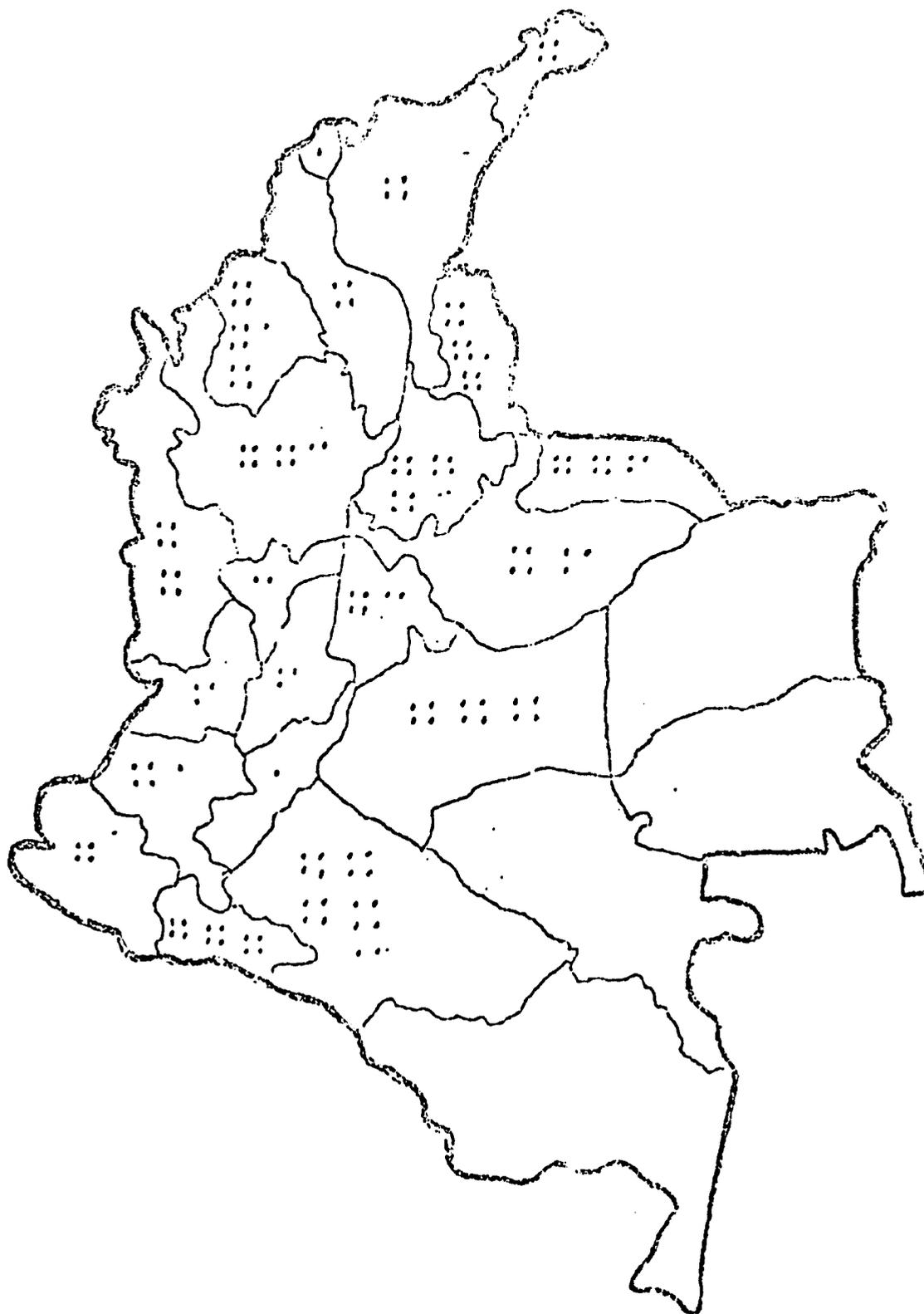
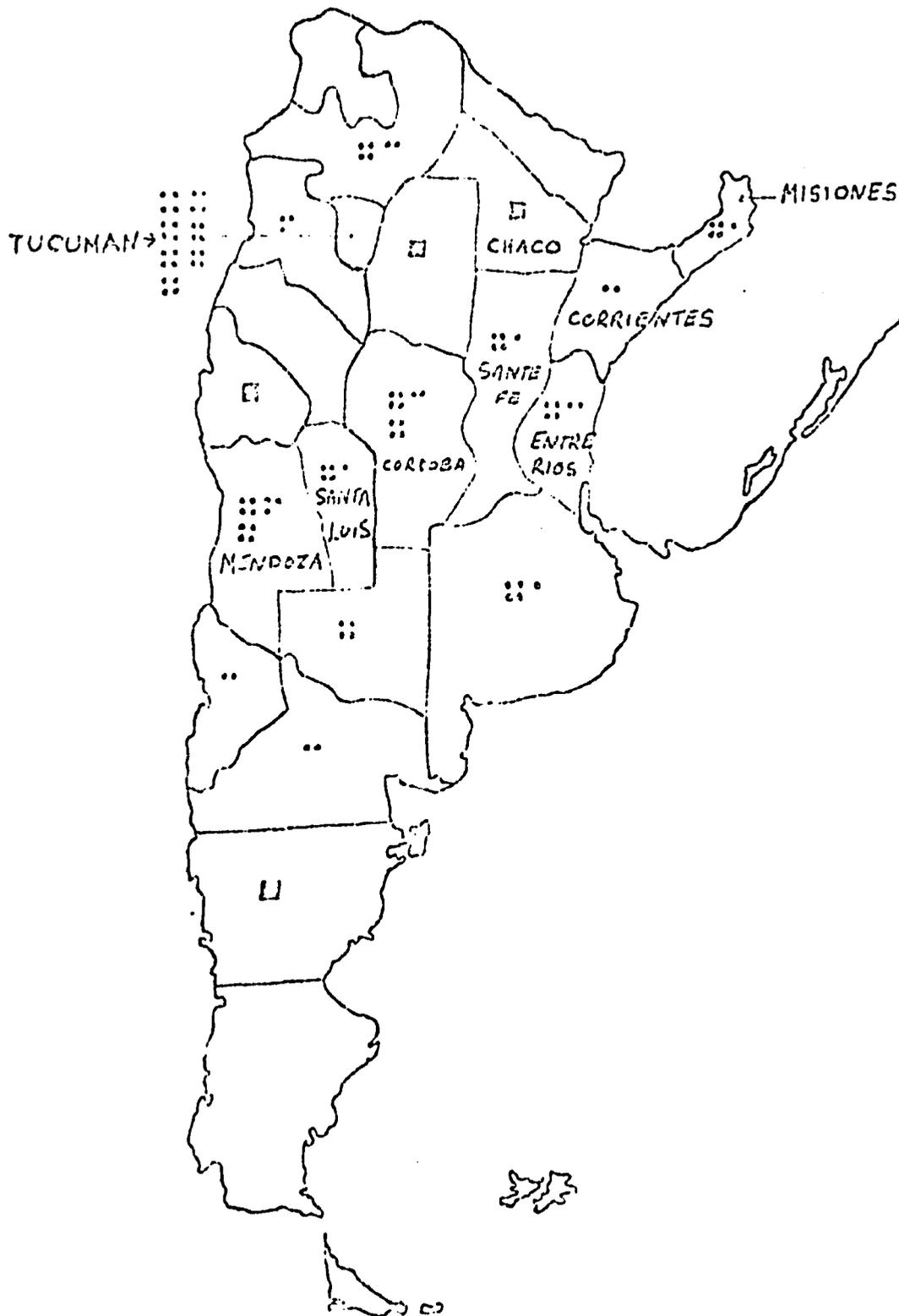


Fig. 7

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THE GEOGRAPHIC DISTRIBUTION OF POSITIVE SEROLOGIC FACTORS IN ARGENTINEAN MILITARY RECRUITS (1961-1965) FOR MALARIA ANTIBODY AS DETERMINED BY THE PASSIVE MICRO-PARTICULATE AGGLUTINATION TEST WITH AN ANTIGEN PREPARED FROM PLASMODIUM KNOWLESII



• Even for the presence of a concentration of 15% of seropositives in the IFA test a score of 1.2 or greater.  
Only states with a score of 1.2 or greater are represented.  
■ 0% positive

Fig. 8

THE GEOGRAPHIC DISTRIBUTION OF MALARIA IN ARGENTINA (1964-1965) AS DETERMINED BY ACTIVE AND PASSIVE SURVEILLANCE



• Each dot represents 0.1% (or fraction of 0.1%) of positive slides.