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STUDY OF MALARIA PARASITE DENSITIES  
FOUND IN VILLAGE SURVEYS IN THAILAND (1960-1961)

by

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During the period 1960-1961 a number of malarimetric surveys were carried out in the North-Eastern and Southern regions of Thailand by the WHO Malaria Advisory Team. The chief purpose of these surveys was to obtain a general picture of the malaria situation, and to relate malaria incidence to such factors as types of terrain, patterns of rural life, and previous spraying of houses with residual insecticides.

The results of these surveys were of immediate practical importance, but a series of data obtained from the examination of blood for malaria parasites are of particular interest from the methodological point of view and might be of value to other workers.

The object of this paper is to present the parasite density findings in infected bloods and to relate them to the age-group and fever history of investigated subjects. The importance of good thick-film technique is indicated by the results of this study.

No regular house visits or drug treatment had been instituted in either region at the time of these surveys.

The principal malaria vector in the country is Anopheles minimus. A. balabacensis is found in forest areas, and other species known to be vectors in other countries are found in the South.

Villages surveyed and sampling procedure

South Thailand. Thirty-four villages in Trang and Songkhla provinces were surveyed between March and June 1960. Most of the villages were in rubber plantations, where houses are widely scattered. Spraying with residual insecticides in previous years had been intermittent and incomplete. Villages were chosen for survey to cover different categories of spraying history, but without other consideration or other

knowledge about them. Surveys were also made in a few coastal villages where malaria had been found by national staff, and in a few forest villages. One group of labourers at a tin mine was also examined. Parasite rates in villages surveyed averaged 8.6% (adults) and 19% (children). Rainfall in the Southern peninsula is intermittent throughout the year. There is little seasonal variation in temperature.

North-Eastern Thailand. One hundred and twenty-six villages were surveyed in the provinces of Saraburi, Korat, Khon-kaen, Roi-et and Ubon, between November 1960 and May 1961. This region includes several distinctive types of terrain, ranging from densely forested hills, where parasite rates averaged 35% in adults and 55% in children to flat, open rice-fields with average parasite rates of 0.5% (adults) and 1% (children). Most of the highly malarious villages had been sprayed intermittently and incompletely. Most of the villages with little or no transmission had never been sprayed. Villages were chosen for survey to cover different categories of terrain in adequate proportion, but without other consideration.

Weather in the North-East has considerable seasonal variation. Average lowest monthly temperature ranges from 5°-8°C in January to 21°C in May-September. The monsoon is from June to September. There is often extreme drought in the dry season, especially in the unforested plains. Most of the infected adults found in these areas said they had visited a forested area before getting fever. It is usual for some men to leave their villages during the dry season and seek work elsewhere.

The headman of a village was usually informed the day before a survey, and requested to gather as many people as possible at a convenient centre. Children were often examined at school, where the teacher would line them up for inspection by class, and by village where one school served several villages. All available children were examined for spleen enlargement.

In the South, blood films were usually taken from all available adults, children and infants. In the North-East, blood films were taken from the first 25 children who presented themselves, or, in small villages, from all available children; in some areas blood films were taken from the first 10 or 25 adults and from all available infants.

Where the presence of transmission was in doubt, in the unforested areas of the North-East, blood films were taken additional to the random samples, from people who gave evidence of possible malaria.

The following figures give the percentage of available children of two to nine years of age whose blood was taken:

<u>Region</u>	<u>Number of spleen examinations</u>	<u>Number of bloods in "random" sample</u>	<u>Blood examination as % of available subjects</u>
South	1821	1798	99%
North-East	4653	2836	61%

To see if there were any tendency for those who presented themselves first to have more or fewer infections, the number of positives in the first halves of the samples are here presented for comparison with the number of positives in the second halves:

	<u>Number of positives in first halves of samples</u>	<u>Number of positives in second halves of samples</u>
<u>Children:</u>		
South	180	161
North-East (forested areas)	185	183
<u>Adults:</u>		
South	66	61
North-East (forested areas)	37	33

This table indicates that although samples taken were not "random" in the statistical sense, it seems unlikely that sampling errors present would significantly affect the general pattern of parasite findings described below.

Method of investigation

Each person from whom blood was taken (or the subject's mother, in the case of a young child), was asked its name, age, house number (omitted in schoolchildren, who could be identified by class), how long ago he had last had fever, and whether he had visited another district. In the South, adults were asked if they were employed in a rubber plantation. In some villages they were asked if their house had been sprayed the previous year, whether they had a crop-hut, and some other questions relating to epidemiology.

Inquiries were made about fever using the equivalent Thai word and also using local names for malaria. Coughs, sore throats and well-recognized illnesses such as mumps, measles and chickenpox were not included as "history of fever".

All interrogation of subjects and recording of answers was done very carefully by the same Thai interpreter, with an assistant, under the team leader's supervision. The peasants were apparently well disposed towards the team and answered questions readily.

Additional blood films were taken in the North-East from people who gave evidence of possible malaria infection - i.e. from children with enlarged spleens and from children and adults who said they had had fever within the past year. These cases did not of course affect the parasite rate, but some of them contribute to the "fever within two weeks" group shown in Table 4.

One thick blood film was made from each subject; all were made by the same WHO technician (the author of this report). As far as could be managed by judgement alone, the blood films made were the same thickness. Long experience (about 15 000 blood films dealt with in the previous three years) ensured considerable uniformity, but neither direct volume measurement nor leukocyte counts could be undertaken in the field. To prevent the drop of blood running to one side, the slides with fresh thick films were laid horizontally on specially made wooden trays (Avery Jones & Lowy, 1959), blood side underneath to ensure cleanliness.

In order to assess roughly the volume of blood in 100 microscopic fields, blood films of similar thickness to those made in the surveys were made in the laboratory, where leukocyte counts could be carried out on blood from the same subject. It was found in these thick films that blood containing about 8000 leukocytes per  $\text{mm}^3$  gave about 20 leukocytes per microscopic field of thick film, when examined with the microscopes provided (binoculars with x 1.5 body, x 6 oculars, x 95 objective). Examination of 100 fields containing 2000 leukocytes of the thick film was the routine procedure followed which would therefore give an approximate volume of  $\frac{20 \times 100}{8000} \text{mm}^3$ , =  $0.25 \text{mm}^3$  of examined blood.

The area of each blood film was considered relatively unimportant providing that the thickness of it was the same. The thick films were usually about 6 mm by 9 mm, but were made smaller if less blood were available. The area actually seen when 100 fields were examined with the optical equipment described was estimated to be no more than 2.0 mm<sup>2</sup>.

Two alternative staining methods were used, either Giemsa or JSB (Jaswant Singh & Misra, 1956). Giemsa stain, diluted with 4% in water at pH 7.0, was used either alone or following treatment with methylene blue, as indicated by Walker. Giemsa was only used in the highly malarious areas of the North-East, and was preferred when thin films were also being stained. Slides, each bearing a thick and a thin film made from the same subject, were placed film downwards on trays similar to those used for carrying freshly made films flat in the field, but painted to make them watertight. After the fixation of the thin film, diluted stain solution was poured under the slides and left for about 40 minutes, or for 20 minutes if the films had been pre-treated with methylene blue. Only a few slides bearing two thick films from two different subjects were stained in this way; in this case special care was taken in rinsing and draining them, to prevent transfer of parasites from one film to another.

Generally thin films were dispensed with altogether and two thick films, from two different people, put on each slide. Most of these films were stained with JSB using a modified method (Avery Jones & Lowy, 1959) to ensure better haemolysis and quick penetration of the stain.

The procedure was as follows:

- (a) films were allowed to dry for at least one day, and were preferably kept for two or three days;
- (b) slides were immersed in 1:7500 aqueous solution of eosin for five to eight minutes and well drained;
- (c) films were stained in JSB solution I for about one minute;
- (d) films were rinsed in water of pH 5.5-6.5.

Most of the blood proteins were washed out in the eosin solution, which was thrown away after use and replaced by a fresh portion quickly prepared by diluting a 1% stock solution of this stain.

It was found that the staining of the cytoplasm of malaria parasites improves when the eosin solution is prepared with distilled water in place of tap or well water. A further modification consisted of haemolysing the films for five minutes in a dilute aqueous solution of brilliant cresyl blue, then staining for five minutes in the dilute eosin. Using this pre-staining somewhat less time was then needed for JSB solution I. The cresyl blue solution was discarded after use.

For staining by the modified JSB method, the slides were held between wooden slats threaded on a six-inch nail and bound together with elastic bands, as described by Avery Jones & Lowy (1959). Films do not face each other in the clips. Stain solutions were filtered into troughs made to hold a batch of 20 slides. To prevent the possibility of parasites being transferred from the upper film to the lower one on the same slide, the troughs were only half filled with stain so that only the films at the distal ends of the slides were covered. When these had been stained and dried, the slides were reversed in the clips and the films the other end were stained. There was no indication of transference of parasites from one slide to another in the same or succeeding batches.

#### Examination of blood films

All slides were examined by the WHO technician or malariologist. Good binocular microscopes, electric light, good staining and clean films assured a high degree of reliability.

Each film was examined by traversing from edge to edge along parallel paths.<sup>1</sup> A minimum of 100 fields was examined in each film, the fields being counted on hand-operated counters. Fields at the thin edges of a film, containing less than the usual number of leukocytes, were not included in the counted 100, but if scanty parasites were present and one were seen in a field near the edge, then that parasite would be included in the parasite count and two such thin fields would be taken as equivalent to one field of average thickness.

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<sup>1</sup> The term "boustrophedon" describes the procedure: it refers to "the path taken by an ox with a plough".

If the parasites were few then the number of parasites of each species was counted in 100 fields. If the parasitaemia was higher than two or three parasites per field, then they were counted in 10 or 20 fields; if the number of parasites exceeded 10 or 20 per field, then parasites in only two or four fields of average thickness were counted. One hundred fields were examined, in any case, for the presence of other species.

The number of parasites found in 100 fields of standard thickness (equivalent to 0.25 mm<sup>3</sup>) was multiplied by four and recorded as count per mm<sup>3</sup>.

Tabulation of results

Parasites were enumerated in four categories:

1. Plasmodium falciparum asexual forms.
2. P. falciparum gametocytes.
3. P. vivax (all forms).
4. P. malariae (all forms).

Total parasite count was obtained, in the case of mixed infections, by adding together the counts of separate species. For example, a blood containing P. falciparum asexual forms 1600 per mm<sup>3</sup>, P. falciparum gametocytes 20 per mm<sup>3</sup>, and P. malariae 100 per mm<sup>3</sup>, has a total parasite density of 1720 per mm<sup>3</sup>, and the appropriate counts would be recorded under each of the species headings as well as their sum in the "total count" column. An infection with only one species of malaria parasite would be recorded under the relevant species heading, and in addition the same number would be entered as "total parasite count".

Parasite counts were classified according to a doubling geometrical progression multiplying each following class by two. The classification was as follows:

<u>Class</u>	<u>Parasites per mm<sup>3</sup></u>
1	1 to 6
2	7 to 12
3	13 to 25
4	26 to 50
5	51 to 100
. . . . .	
15	51 201 to 102 400
16	over 102 400

The classification is similar to that recommended by Bruce-Chwatt (1958), but with greater breakdown in the low-density range. It is obvious that the parasite densities analysed in this paper are approximations only; but since parasitaemias range from less than one to more than 100 000 per  $\text{mm}^3$ , ". . . the order of infections becomes of greater significance than the accuracy of the results" (Covell, Russell & Swellengrebel, 1953).

As each class covers the range of count double that of the preceding class, an error sufficient to give an estimated density of double or half the true parasite concentration puts the infection only one class too high or too low. For convenience, all the infections within a given class have been treated as if they had the count of the upper limit of that class.

Parasite densities are also expressed here in terms of "leukocytes per parasite", arbitrarily assuming a leukocyte count of 8000 per  $\text{mm}^3$ . Thus a parasite count of 60 parasites per  $\text{mm}^3$  would be recorded in class 5 (51-100 per  $\text{mm}^3$ ) and the leukocyte/parasite ratio for the class upper limit would be  $\frac{8000}{100} = 80$  leukocytes per parasite.

In the graphs (Figs. I-III) a vertical corresponding to a particular parasite density meets a curve at the point corresponding to the percentage of infections found which equal or exceed that density. By finding the appropriate point on a curve, therefore, one may gauge the approximate percentage of positives which would have been found by examining a volume of blood smaller than  $0.25 \text{ mm}^3$ , volume being indicated by the approximate number of leukocytes which must be seen in order to find one parasite. For example, in Fig. I, 29% of infections in adults had a parasite count of 800 or more per  $\text{mm}^3$ . Assuming a leukocyte count of 8000, then 29% of infections would have been found by examining blood of sufficient volume to contain 10 leukocytes.

The simplification of treating all infections in a class as if they had the highest density in that class leads to some unavoidable discrepancy. The lowest parasite density recorded was 4 per  $\text{mm}^3$ , but all such infections are in the class "up to 6 per  $\text{mm}^3$ ", and 6 parasites per  $\text{mm}^3$  is equivalent to about 1300 leukocytes per parasite. It therefore appears on the graphs that 100% of infections would be found by examining that volume of blood which contains 1300 leukocytes; whereas the volume examined was actually that which contains 2000 leukocytes. The term "100%" refers only to the total of infections found, and is not of course an absolute.

Table 4 uses the same density classes, but the cumulative percentage frequencies are in reverse order, starting with low densities. It shows what percentage of infections found had counts below and up to each parasite density given.

Insufficient P. malariae infections were found to warrant separate presentation.

Parasite density findings related to age and fever history of subjects and to parasite species

Parasite densities tended to be highest in infants (median density 1600 per  $\text{mm}^3$ , shown in Fig. I); and slightly higher in children, with a median density of 280, than in adults, whose median parasite density was about 115 per  $\text{mm}^3$ .

In every age-group, those with a history of fever tended to have higher parasite counts than those giving no history (Fig. III).

P. falciparum gametocytes tended to be present in very low numbers (Fig. II). Of the 245 P. falciparum infections in children with a history of fever, 36 showed gametocytes only; in 125 only asexual forms were found, and in 84 both forms were present. Of the 71 P. falciparum infections in adults with a history of fever, gametocytes alone were found in 8, asexual forms alone in 37, and both forms in 26 cases. P. falciparum asexual forms were present in highest concentration, and P. vivax counts were intermediate between those of P. falciparum asexual and P. falciparum gametocytes.

Tables 1-3 were compiled from Figs. I-III and indicate possible ways of interpreting the graphs.

TABLE 1. APPROXIMATE PERCENTAGES OF POSITIVES WHICH WOULD HAVE BEEN MISSED IF VOLUMES OF BLOOD LESS THAN  $0.25 \text{ mm}^3$  HAD BEEN EXAMINED

Data from Fig. I - Random samples

(Volumes expressed as number of leukocytes seen in course of examining film, assuming a leukocyte count of 8000 per  $\text{mm}^3$ .)

Approximate number of leukocytes seen in examining each film	Infants	Children	Adults
660	2%	9%	13%
320	9%	17%	22%
160	15%	27%	36%
80	21%	36%	47%
40	28%	44%	56%

TABLE 2. APPROXIMATE PERCENTAGE OF POSITIVES WHICH WOULD HAVE BEEN MISSED IF VOLUMES OF BLOOD LESS THAN 0.25 mm<sup>3</sup> HAD BEEN EXAMINED

Date from Fig. II - Children and adults with fever history

Approximate number of leukocytes seen in examining each film	Children			Adults		
	Pfa	Pfg	Pv	Pfa	Pfg	Pv
660	2%	32%	10%	0	30%	7%
320	6%	51%	20%	5%	46%	18%
160	11%	64%	31%	13%	61%	36%
80	19%	73%	43%	19%	76%	52%
40	25%	80%	51%	27%	85%	59%

TABLE 3. MEDIAN DENSITIES BY AGE-GROUP, WITH AND WITHOUT HISTORY OF FEVER

Data from Fig. III - Total parasite densities

	With history of fever	Without history of fever
Infants	3 800	230 parasites per mm <sup>3</sup>
Children	400	100 " " "
Adults	270	45 " " "

In Table 4, those who gave a history of having had fever within the previous two weeks can be seen to have higher parasite counts than the first two groups - those without fever history, and those with a history of fever at some unspecified date.





## Discussion

### 1. Fever history

When a subject stated that he had had "fever", the claim was probably based on such symptoms as headache, shivering, and aching joints. It seemed reasonable, therefore, to exclude from the "history of fever" groups those who described other symptoms, clearly attributable to a non-malarial illness. This would give rise to some error due to subjects who may have had malaria concurrently with another illness; but the alternative procedure of recording all sicknesses as "history of fever" would probably have resulted in almost everyone being put into that one category.

Although it is recommended that surveillance workers treat and take blood from all self-designated "fever" cases, this is not, in fact, necessarily the practice. For one thing, there may not always be enough staff to deal with the number of slides which would be taken were no discrimination made between "possibly malarial" and "probably non-malarial" illnesses. One large national malaria eradication organization employed two microscopists per million population; the number of blood films they could examine would be equivalent to about one from each member of the population once in forty years. No proper surveillance activity can be carried out in such conditions.

Thirty-three to forty-four per cent. of infected persons in the surveys described here gave no history of fever according to the questioning method used. There is no means of knowing how many of them had been truly asymptomatic; how many had been febrile but without subjective symptoms or visible signs; how many had had only slight symptoms which did not seem serious enough to mention; and how many had forgotten their illness.

Establishment of a regular surveillance service, making frequent contact with the population, should reduce the number of forgotten symptoms; and reduction of transmission levels may lead to lowered immunity and tolerance to infection, so that symptoms may tend to be more striking in fresh infections.

### 2. Measurement of blood volume

Since all blood films in these surveys were made by one person, some considerable uniformity of thickness of the blood layer could be expected. The estimation of volume by counting fields is very much quicker than estimation by counting leukocytes,

and requires no pipettes such as are used to make films of standard area from measured quantities of blood. Research projects may use various techniques to make more or less accurate measurements of parasite concentration. But when thick films have been made in the ordinary way by several people, the only practicable method of estimating the volume examined and of calculating parasite density is by counting leukocytes. Variation in leukocyte counts may be considered unimportant in comparison to the variation found in parasite counts.

The simplest way in which a supervisor can check on the thickness of films received in his laboratory is to count the approximate number of leukocytes which will be seen in the course of routine examination.

Leukocytes have been used as a measure of volume in this paper because they may make the results more meaningful to workers unaccustomed to measuring blood films in cubic millimetres.

### 3. Low density infections

The lowest density which could be recorded when  $0.25 \text{ mm}^3$  of blood was examined was 4 parasites per  $\text{mm}^3$ , equivalent to 1 per 100 fields; and infections of this density would not be found with any certainty. In fact, the examination of any practicable volume of blood cannot disclose all the parasitaemias present. Some were certainly missed in the series reported here, so that, in Tables 1 and 2, "percentages of positives which would have been missed" are minimum figures.

It seems likely, from their usual low density, that very many P. falciparum gametocytes were missed, and a considerable number of P. vivax also. Judging from their generally high density, few P. falciparum asexual parasites were missed.

Sooner or later, a low-density parasite carrier can be expected either to recover completely or to relapse with fever and increased parasitaemia; but in the meantime he may be capable of infecting mosquitos. It may therefore be of some importance during surveillance operations, when spraying with residual insecticide has been discontinued, to find as many positives as possible, and prevent further transmission.

Infections analysed in this paper were found in areas where conditions were, in many respects, very different from those which pertain in areas under surveillance. Parasite rates were high, and levels of immunity and tolerance presumably higher than

they would be in a population which had been protected from malaria for some years. However, it may be of some interest to observe the following:

(a) The group with highest parasite densities found in this series was that of infants with a history of fever. Only 9% (of an unfortunately small total of 33 cases) had parasite densities of 50 or fewer per  $\text{mm}^3$ .

(b) Twenty-three and twenty-six per cent., respectively, of infected children and adults with a history of fever within the previous two weeks had infections of 50 or fewer parasites per  $\text{mm}^3$ . The proportion of low densities in these groups would have been no lower had history-taking been less selective.

In films containing 20 leukocytes per field, a parasitaemia of 50 per  $\text{mm}^3$  would give 1 parasite per 8 fields - a density reasonably easy to detect. But a film of a thickness of, say, 4 leukocytes per field, would show 1 parasite per 40 fields.

Whereas heavy infections, with parasites in every field, are unlikely to be missed under most conditions, detecting scanty parasitaemias depends very much on technique at all stages. Microscopists must be skilful; but even expert laboratory staff cannot give reliable results if they have to deal with films which are too thin, or dirty, or badly stained; or if they are provided with faulty or inadequate equipment.

#### Summary

1. Parasite densities were estimated in more than 1000 positive slides found in the course of carrying out surveys in Thailand during 1960-1961 and a rapid, approximate method of assessing the malaria parasite count in thick blood films is described.

2. An analysis of obtained data showed that parasite densities were higher in cases with a history of fever than in those giving no history, and highest in those with recent fever. Parasite densities were also highest in infants, and slightly higher in children than in adults. P. falciparum asexual forms were usually found in higher density than P. vivax. P. falciparum gametocytes were usually found in very low concentration.

3. Cumulative frequency graphs and tables indicate what proportion of infections found would probably have been missed by examining volumes less than the  $0.25 \text{ mm}^3$  searched in each film. Thus examination of a quarter of the volume would have reduced the number of positives by 9% in infants, 17% in children and 22% in adults of the investigated group.
4. It is of interest that between 33% and 44% of subjects found infected gave no history of fever on questioning.

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FIG. 1

CUMULATIVE PERCENTAGE FREQUENCY OF TOTAL PARASITE DENSITIES (ALL SPECIES)  
 FOUND IN INFANTS, CHILDREN AND ADULTS  
 IN UNSELECTED SAMPLES

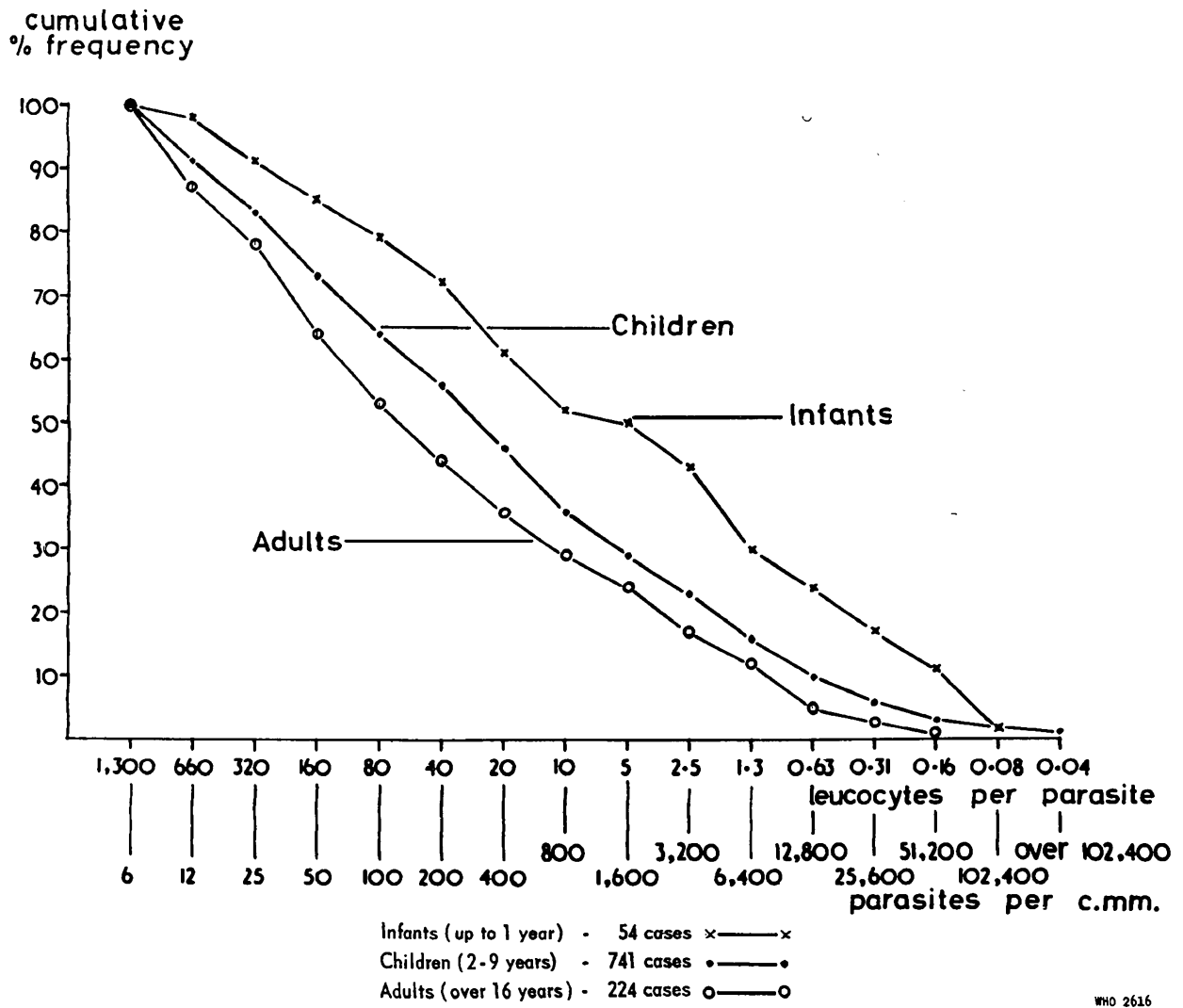
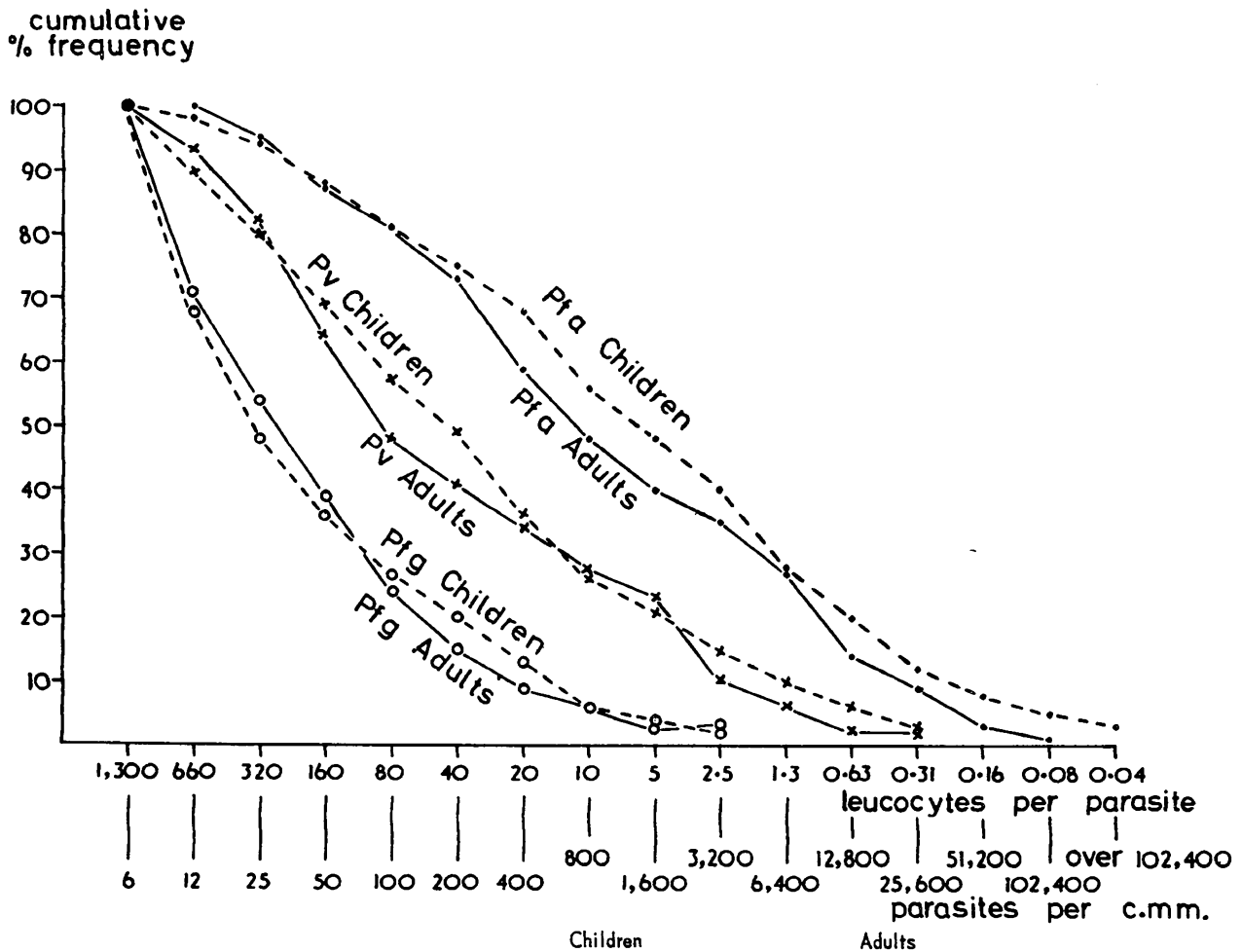


FIG. II

CUMULATIVE PERCENTAGE FREQUENCY OF PARASITE DENSITIES BY SPECIES INFECTIONS  
IN ADULTS AND CHILDREN IN UNSELECTED SAMPLES WITH HISTORY OF FEVER

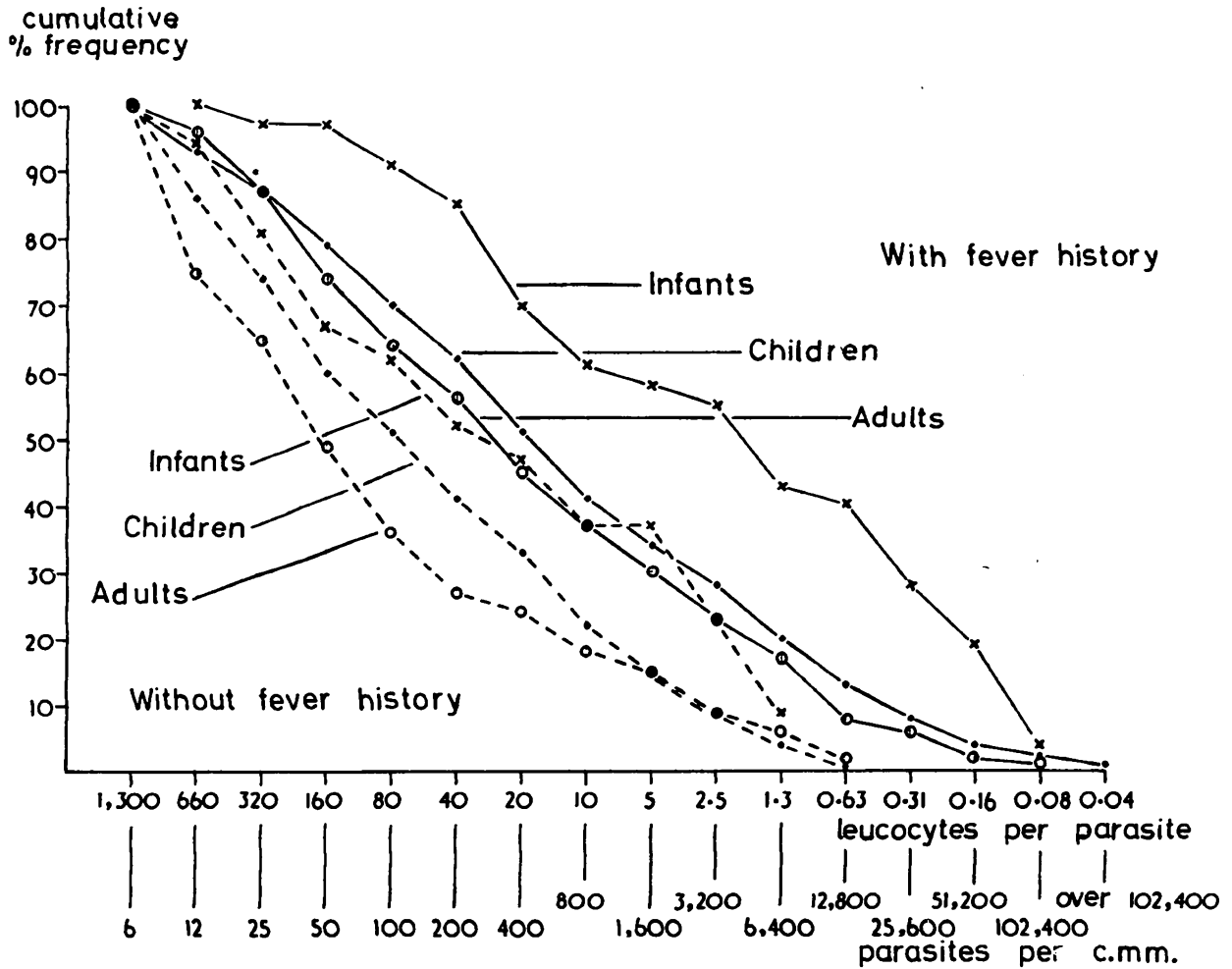


<i>P. falciparum</i> asexual	: (209 cases)	•-----•	(63 cases)	•-----•
<i>P. falciparum</i> gametocytes	: (120 cases)	o-----o	(34 cases)	o-----o
<i>P. vivax</i>	: (286 cases)	x-----x	(55 cases)	x-----x

(Number of *P. falciparum* infections in above cases: Children - 245; Adults - 71)

FIG. III

CUMULATIVE PERCENTAGE FREQUENCY OF TOTAL PARASITE DENSITIES (ALL SPECIES) FOUND IN UNSELECTED SAMPLES. WITH AND WITHOUT HISTORY OF FEVER



With Fever History		Without Fever History	
Infants (up to 1 year) :	( 33 cases) x ——— x	( 21 cases) x - - - - x	
Children (2-9 years) :	( 496 cases) • ——— •	( 245 cases) • - - - - •	
Adults (over 16 years) :	( 126 cases) o ——— o	( 98 cases) o - - - - o	

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