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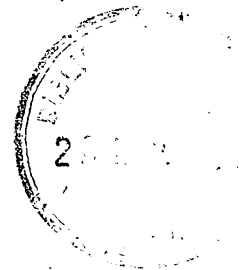
WHO/Mal/280 ✓  
10 November 1960

ORIGINAL: ENGLISH

TRANSFER OF BLOOD PARASITES (PLASMODIA AND TRYPANOSOMA)  
BETWEEN THICK BLOOD FILMS DURING MASS STAINING

by

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Introduction

Malaria parasites were shown to transfer from one blood film to another by Brooke & Donaldson (1948), when films were stained by the mass technique of Barber & Komp. In this technique, slides are packaged together, each slide being separated from its neighbour by the insertion of a small piece of cardboard at one end of the slide. The package is held together by a rubber band; it is then placed in a staining trough and covered with staining solution. In the tests to be described in this paper, slides were not packaged in this way. When stained vertically in a staining jar, they were separated by the inverts of the jar. This ensures a considerably greater gap between blood films than do the pieces of cardboard in the Barber & Komp technique. In further studies, Brooke & Donaldson (1950) and Donaldson & Brooke (1950), showed that the addition of 0.5 per cent. of a 33 per cent. aqueous solution of Triton X-100 (alkylated aryl poly-aether alcohol) in Giemsa staining solutions almost eliminated the transfer of malaria parasites from one blood film to another. These authors suggested that some of the flakes of blood which had become detached from slides during staining, rose to the surface of the staining solution and were held there by surface tension. They might then become attached to other slides when the package was lifted from the stain. It was further postulated that lowering the surface tension of staining solutions by the addition of alkylated aryl poly-aether alcohol, allowed blood flakes to sink to the bottom of the staining trough. Under certain staining conditions, these authors demonstrated a malaria parasite transfer rate of over 40 per cent. The work to be described in this paper was carried out partly to confirm the observations of Brooke & Donaldson. The

principal objectives however, were to obtain information concerning the mechanism of parasite transfer and to find the common factors in staining which are most likely to produce it. It was hoped to perfect a technique suitable for use in field laboratories which would reduce malaria parasite transfer to a minimum

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<sup>1</sup> This investigation of the transfer of malaria parasites from positive thick blood films during mass staining is of considerable practical importance. It is obvious that an erroneous diagnosis of malaria due to the "contamination" of a negative blood film by flocula from a positive blood film undermines the value of blood examination for detection of cases of malaria and upsets the whole system of surveillance activities. The whole problem of the most reliable technique of blood examination needs some re-assessment and this will certainly be stimulated and guided at the regional level by those responsible for the operational side of the terminal phase of malaria eradication programmes.

The results of the present investigation should be confirmed (or otherwise) by other workers and their observations will be greatly appreciated.

The immediate practical recommendations based on Mr Shute's observations are as follows:

1. The technique of taking multiple thick films from several individuals on the same slide should be avoided. The safest practice is "one subject - one slide".
2. The possibility of the parasite transfer from slide to slide exists also with the present method of mass staining using staining jars or slide blocks. This does not necessarily mean that such rapid, convenient and economical mass staining methods should be abandoned without further attempts to improve their reliability. Increased vigilance should be exerted with regard to the proper technique of staining and to the scrupulous attention to every detail when handling blood slides (thick films in particular) for examination for malaria parasites.
3. The most important elements of the correct technique for prevention of parasite transfers are:
  - (a) avoidance of agitation of the staining solution during staining or during further processing of a block or a group of slides;
  - (b) careful flushing off of the upper layer of the staining solution before rinsing, and gentle handling of thick films;
  - (c) addition of 0.5 per cent. solution of 33 per cent. Triton X - 100 to the staining solution before staining.
4. For small numbers of slides the use of the staining methods based on staining slides flat with the blood side turned downwards is preferred. In such cases the addition of detergents is not necessary.
5. For block staining of groups of slides using JSB stain, in addition to points (a), and (b) of paragraph 3 above, it is necessary either to change the JSB solution I for each group of slides or to pass it through a filter paper.  
(Editor's remark)

### Experimental procedure

In Tests 1 to 4, blood films were stained with Giemsa, diluted with a phosphate buffer solution with a pH of 7.2. The strength of the staining solution was 3.5 ml of Giemsa stain per 100 ml of buffer, i.e.  $1\frac{1}{4}$  drops of stain per ml of buffer. Blood films were stained for thirty minutes and were taken 24 to 48 hours prior to staining. This time was chosen because field experience in tropical East Africa has shown that, generally speaking, thick blood films stain better when one to two days old than at any other time.

#### Test 1

A single thick film of parasitized blood was placed on each of a series of slides. Thick films of non-parasitized blood were made on a separate series of slides. The slides were placed in staining jars, each positive film facing a negative one. After staining, the scum on the surface of the stain was removed by running distilled water gently into the jars until they overflowed. Care was taken to disturb the staining solution as little as possible while blood films were immersed in it. The slides were then lifted individually from the stain, washed under a stream of distilled water and stood on end to drain and dry. Two tests were carried out with this technique; the details are shown under sub-headings 1.1 and 1.2 and the results are summarized in Table 1.

1.1 The positive thick blood films of this test contained parasites of Plasmodium vivax at a density of 13 000 per  $\text{mm}^3$  of blood. Eighty-eight of the negative blood films were examined. No parasites were found to have been transferred from the positive to the negative films.

1.2 Blood films containing parasites of Trypanosoma rhodesiense, having a density of about 1 000 000 per  $\text{mm}^3$  of blood were used in this test. The blood was taken from a rat that had been infected in the laboratory by subcutaneous injection, several days previously. The parasite density was calculated from the average number of parasites per microscope field in a thin blood film. Fifty-three of the negative blood films were examined. Trypanosomes were found to have been transferred to 13 of them. The parasites were found in small groups and as individuals.

## Test 2

The staining procedure described in Test 1 was repeated in this series of tests. After staining, the stain solution was poured from the jar; fresh distilled water was run in and the jar rocked to wash the slides. The distilled water was then poured off, the slides were removed from the jar and stood on end to dry. Three tests were carried out in this series. They are described under sub-headings 2.1, 2.2 and 2.3 and their results are summarized in Table 2.

2.1 Thick blood films containing parasites of P. vivax at a density of 13 600 per  $\text{mm}^3$  of blood were used. Examination of 30 of the negative blood films showed that parasites had been transferred to ten of them. The transferred parasites were scattered over the blood films, single parasites usually being found in separate microscopic fields but small groups of parasites were also seen on several occasions.

2.2 In view of the high parasite transfer rate found in Test 2.1 and because this method of washing blood films is a common one, this test was repeated using blood having a parasite density of 600 per  $\text{mm}^3$ . In an examination of 45 of the negative blood films, parasites were found to have been transferred to one of them. This film contained three immature trophozoites.

2.3 Results of examinations of twenty negative blood films which had been stained facing films infected with parasites of T. rhodesiense, showed that trypanosomes had become transferred to 12 of them. The density of the trypanosome infection in the original positive blood films was so high that the appearance of the parasites after transfer to the negative films was frequently as small intertwined groups, with areas free of parasites between them. However, individual trypanosomes were common and the impression was gained that at least some of the parasites that had become dislodged from the infected films had not been transferred while attached to a flake of blood. If this impression were correct, it seemed possible that individual red blood cells might behave in the same way. Transfer of malaria parasites could then occur, not only by the breaking away of a flake of blood but also by the detachment of individual red blood corpuscles. Provided the blood

films were not too fresh, and the slides were free from grease and other contaminants, the breaking away of a flake of blood from a thick film would probably be due to some disturbance of the film. This disturbance might be a slight one, such as flow of water over the film while being washed. On the other hand, if individual red blood cells became detached from the film, it might occur in the apparent absence of disturbance of the solution in which the film was being stained, possibly by the process of dehaemoglobinization. This factor was tested with malaria parasites and trypanosomes and is described under Test 5.

### Test 3

Four thick blood films were made on each of a series of slides, alternate films being made from blood infected with parasites of P. vivax at a density of 3260 per mm<sup>3</sup> of blood. The other films were made from non-parasitized blood. The slides were placed in a staining jar, with the films on one slide facing those on the adjacent slide. After staining, distilled water was run into the jar to remove the surface scum; the slides were then lifted out of the stain individually, washed under a stream of distilled water and stood on end to drain and dry. Thirty of the negative films were examined from this test. Parasites of P. vivax were found in eight of them. The transferred parasites were generally scattered over the surface of the blood films; no single large group of parasites was found and in three films, only one parasite was found in each. In only two of the other five films was more than one parasite found in a single microscopic field. The results of this test are summarized in Table 3.

In all the tests so far described, blood films were stained in rectangular jars in which the slides were stood on edge and the stain poured in until it covered them. The method adopted for Test 1 was the only one in which the transfer of malaria parasites did not occur. However, when this test was repeated using blood with a high density of trypanosomes, parasite transfer did occur. It is suspected, therefore, that the transfer of malaria parasites might also be not infrequent when this method of staining is used. In Test 4, staining plates were used, in which slides are laid across the plate and stained with the blood films facing downwards, instead of being vertical as they are in staining jars.

#### Test 4

Slides were laid, with their edges touching, across a series of staining plates. On each slide a single thick blood film had been made and the slides were laid so that positive and negative blood films alternated along the plate. Each plate contained five slides, three of which were positive. After staining, the slides were lifted off the plates and washed under a gentle stream of distilled water. Two series of tests were carried out. They are described under sub-headings 4.1 and 4.2 and the results are summarized in Table 4.

4.1 One hundred and twenty thick blood films containing no malaria parasites were alternated with positive films made from blood infected with P. vivax at a density of 12 000 per  $\text{mm}^3$  of blood. On examination after staining, no malaria parasites were found to have been transferred from the positive to any of the 120 negative films.

4.2 Thirty negative thick blood films were alternated with thick blood films made from the blood of a rat infected with T. rhodesiense having a density of about 1 000 000 per  $\text{mm}^3$  of blood. After staining, an examination of the negative blood films failed to reveal any trypanosomes transferred to them from the positive films.

The impression was gained while examining the material of Test 2.3, that individual red blood corpuscles and small blood particles might become detached from blood films during the dehaemoglobinizing process. Previous tests had shown that disturbance of staining and washing media would create a comparatively high rate of parasite transfer, but it was still not clear whether blood elements may become detached in the absence of disturbance of the media. Test 5 was carried out to clarify this point.

#### Test 5

A parasitized thick blood film was made at one end of each of a series of slides. They were allowed to dry and were kept for 24 to 48 hours. The slides were then laid on a tray with the blood films uppermost. Two to three drops of phosphate buffer solution (pH 7.2), were then dropped on to each blood film, so that while all, or most, of the film was covered with buffer, some of it extended beyond the

film and on to the clean part of the slide. The buffer was allowed to dehaemoglobinize the blood for three minutes. The tray was then slightly tilted, but not sufficiently to cause the buffer to flow unaided down the length of the slide. The end of a thin piece of glass rod was then brought into contact with the part of the buffer solution that extended beyond the blood film and was drawn down the slide, the solution being encouraged to spread slightly over the surface of the slide. The result of this action was that most of the solution which covered the blood film and which now contained haemoglobin in solution followed the liquid trail down the slide. When the slides were dry, the tray was lowered to a horizontal position. Methyl alcohol was then poured over the whole slide to fix the thick blood film and any elements of it that had spread on to the slide in the buffer solution. The slides were then immersed in a solution of Giemsa stain for half an hour, after which they were washed under a stream of distilled water. The strength of stain used was 8 ml of Giemsa to 100 ml of buffer solution, i.e., three drops of stain per ml of buffer. The extent of spread of the buffer solution was clearly defined after staining by a brown line of haemoglobin which had formed along the periphery of the area that had been covered by the solution. This brown line was of great assistance in limiting the area of the slide that had to be examined under the microscope. The tests carried out with this technique are described under sub-headings 5.1 and 5.2.

5.1 Each thick blood film was infected with parasites of P. vivax at a density of 10 500 per  $\text{mm}^3$  of blood. Examination of the trail of buffer solution and haemoglobin in 30 slides, showed malaria parasites in 26 of them. The parasites were in association with either small blood particles or individual red blood corpuscles that had become detached from the parent blood film, to be deposited in the trail of buffer solution. The parasites were usually found single but groups of two or three were occasionally seen.

5.2 Each thick blood film was infected with parasites of T. rhodesiense at a density of about 1 000 000 per  $\text{mm}^3$  of blood. Microscopical examination of the trail of buffer solution and haemoglobin in 55 slides, showed that trypanosomes had become detached in 30 of them. The trypanosomes had been deposited over the whole area of the slide covered by the trail of haemoglobin and buffer solution. The parasites were not more numerous at the periphery than in the almost clear area in the middle of the trail. Trypanosomes were found single and also in small groups.

### Discussion

The results of Test 5 show that under whatever conditions thick blood films are stained, elements of blood are liable to become detached from the film. This is probably due to some form of disturbance occurring within the film as haemoglobin is dissolved from the red blood corpuscles. It is therefore to be expected that when thick blood films are stained, blood particles, some probably containing malaria parasites, will be floating free in the staining solution. However, it seems evident that disturbance of solutions, whether it be staining or washing solutions, is the major factor which causes deposition of free-floating parasites onto blood films. This is shown by a comparison of the results of Tests 1 and 2. Therefore, solutions should not be disturbed while blood films are immersed in them.

Test 3 shows the danger of taking multiple blood films on a single slide. The staining technique used in this test was the same as that used in Test 1. Although the parasite density in Test 1.1 was considerably above that of Test 3, the rate of parasite transfer was considerably greater in the latter test. It is considered that this was due to dislodged blood elements being carried down the slide from the positive films, to be deposited on the negative films while the slides were stood on end to drain and dry.

It is difficult to explain why parasite transfer could not be demonstrated when staining plates were used instead of staining jars. That it could not be shown in Test 4.2, when a heavy infection of T. rhodesiense was used, shows that the likelihood of parasites being transferred between blood films of malaria, is at least slight, when this staining technique is used. It may well be that the suggestion of Brooke & Donaldson (1950), that detached blood elements are suspended at the surface of staining media by surface tension, or in the scum which forms across the surface of a jar of Giemsa staining solution, has a bearing on the results obtained with staining plates. When these plates are used, scum can only form at the extreme ends of the row of slides laid across them and can have no influence on blood particles which have become detached from the slides. It is possible that these particles fall to the bottom of the solution in staining plates, as they seem

to do when alkylated aryl poly-aether alcohol is used to reduce surface tension of solutions in staining jars. Blood films that have been kept in a warm climate for a number of days will probably not lose blood particles while being stained and parasite transfer would not then occur. This would be due to partial fixation of the blood by the atmosphere, but since this results in a considerable loss of definition of the stained parasites, it is not considered to be a suitable method of preventing parasite transfer.

### 3. Observations on the morphology of transferred parasites

Parasites of P. vivax that had become transferred from one blood film to another, were examined by the author, and others with more experience in the morphology of this species, in an attempt to detect distortions. In some instances distortions were found. This particularly applied to schizonts, gametocytes and mature trophozoites. Many others, however, appeared to be quite normal and distortions could never be detected in young trophozoites. It is considered that the proportion of distorted parasites was too low to be of any value in determining whether an apparent infection was due to parasites that had been transferred from another blood film or to a true infection. When transferred parasites were examined, it was found that many had become very distorted. Small groups of trypanosomes often seemed to be intertwined, an appearance probably caused by the blood particle to which they were attached having become folded when impinging upon the negative blood film. Individual trypanosomes were often seen to be broken, an observation never made with malaria parasites. It may be that the Plasmodia, being intracellular, are protected by the comparatively tough membrane of their host red blood corpuscles; the extracellular Trypanosoma, on the other hand, have no such protection and are therefore liable to be damaged.

#### Summary

Thick blood films, infected with parasites of P. vivax or T. rhodesiense were used to demonstrate parasite transfer between blood films during mass staining. The parasite transfer rate when staining jars were used increased when solutions were disturbed while blood films were immersed in them and when multiple blood films

comprising positive and negative films were taken on the same slide. Further tests showed that individual red blood corpuscles may become detached from a thick blood film during the dehaemoglobinizing process. From this it was concluded that small blood elements are always likely to become detached from thick blood films and float as free entities in staining solutions with present standard staining techniques. Observations of transferred parasites showed too little distortion in P. vivax to be of any diagnostic value but considerable distortion was found in transferred parasites of T. rhodesiense.

When single blood slides were stained in the flat position, transfer of parasites could not be demonstrated.

#### ACKNOWLEDGEMENTS

My sincere thanks are due to Sir Gordon Covell, Mr P. G. Shute and Miss M. Maryon of the Malaria Reference Laboratory, Medical Research Council, England, for providing parasitized blood and for giving me laboratory facilities to carry out much of this work. Their critical advice and observations were also of great value. I should also like to thank Dr W. J. Stoker for his advice in the writing of this paper and the Director, Division of Malaria Eradication, WHO, for permission to publish it.

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TABLE 1. TRANSFER OF PARASITES TO NEGATIVE BLOOD FILMS WHEN SOLUTIONS IN WHICH THE SLIDES WERE IMMersed WERE NOT DISTURBED.

Test number	Parasites tested		Examinations for parasite transfers		
	Species	Density/ mm <sup>3</sup> of blood	No. of test films examined	No. with transferred parasites	Transfer rate
1.1	<u>P. vivax</u>	13 000	88	0	0
1.2	<u>T. rhodesiense</u>	1 000 000	53	13	24

TABLE 2. TRANSFER OF PARASITES TO NEGATIVE BLOOD FILMS WHEN SOLUTIONS IN WHICH THE SLIDES WERE IMMersed WERE DISTURBED.

Test number	Parasites tested		Examinations for parasite transfers		
	Species	Density/ mm <sup>3</sup> of blood	No. of test films examined	No. with transferred parasites	Transfer rate
2.1	<u>P. vivax</u>	13 600	30	10	33
2.2	<u>P. vivax</u>	600	45	1	2
2.3	<u>T. rhodesiense</u>	1 000 000	20	12	60

TABLE 3. TRANSFER OF PARASITES TO NEGATIVE BLOOD FILMS WHEN POSITIVE AND NEGATIVE FILMS WERE ALTERNATED ON THE SAME SLIDE.

Test number	Parasites tested		Examinations for parasite transfers		
	Species	Density/ mm <sup>3</sup> of blood	No. of test films examined	No. with transferred parasites	Transfer rate
3	<u>P. vivax</u>	3 260	30	8	27

TABLE 4. TRANSFER OF PARASITES WHEN THICK BLOOD FILMS WERE STAINED IN STAINING PLATES.

Test number	Parasites tested		Examinations for parasite transfers		
	Species	Density/ mm <sup>3</sup> of blood	No. of test films examined	No. with transferred parasites	Transfer rate
4.1	<u>P. vivax</u>	12 000	120	0	0
4.2	<u>T. rhodesiense</u>	1 000 000	30	0	0