Liston and Turkhud (1923).—The Results of an Investigation of Dracontiasis in the Village of Desai. Private communication.

Chitale (1912).—Observations on Three Hundred Cases of Guinea-worm. *Indian Medical Gazette.* Vol. XLVII, pp. 318-320.

Gupta (1917).—*Indian Medical Gazette.* Vol. 52, No. 11, p. 419.

Béclère (1923).—A Method for the Extraction of Filaria. *British Medical Journal.* Aug. 1, p. 345.

Lefebvre (1908).—Extraction d'une filaire de Mediné par cocainisation du parasite. *Presse. Med.* Oct. 24.

THE DIAGNOSIS OF KALA-AZAR BY EXAMINATION OF THICK BLOOD FILMS.

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THE diagnosis of kala-azar can only be properly confirmed by the finding of *Leishmania donovani* in films taken from the blood, spleen juice, liver juice, etc., of the patient. However valuable the serological tests may be, they cannot be said to be absolutely infallibly diagnostic or to be free from possible misinterpretations and fallacies. Even with Napier's aldehyde test, which we regard as the most reliable of all, the serum only commences to give a positive reaction at about the fourth or fifth month of the disease.

To establish the diagnosis of kala-azar by discovery of the specific parasite, we have always held and still hold that the readiest, most reliable and best method is to do spleen puncture and examine films of and N. N. N. cultures from such material. We are accustomed to performing some two or three spleen punctures daily in our laboratory upon out-patients, and have been in the habit of constantly performing this trifling operation now for seven years, without having seen a single accident or harmful symptom resulting.

Yet the ordinary medical practitioner in Bengal rarely carries out spleen or liver puncture, he and his patients alike being under the erroneous impression that these are dangerous procedures. There are still two further ways in which the diagnosis can be clinched by the discovery of the parasite:—

(*a*) Culture of the peripheral blood by Row's method. As shewn by the junior author (Das Gupta, 1922) and others, such a method yields positive findings in some 98 per cent. of untreated cases. Yet it requires full laboratory facilities, which are not available to the ordinary general practitioner.

(*b*) Searching the leucocyte edge of some 6 to 12 thin films of the peripheral blood for parasites. To quote a remark made by a candidate at a recent examination, however, "the method requires the technique of an expert and the patience of Job."

Accordingly, we have from time to time during the past seven years experimented with different thick blood film methods, methods of dehæmoglobinizing blood and of preparing leucocyte films, etc., with previously disappointing results: (e.g., Knowles, 1920, pp. 164-165).

It is curious indeed how diametrically opposite are the views held by different workers as to the value of examination of thick blood films for the presence or absence of the blood-inhabiting protozoa. Thus, as regards malaria, Stitt (1923, p. 300) regards the method as "of the greatest practical assistance in searching for malaria parasites, when in very small numbers in the peripheral circulation." James (1920, p. 172) considers that "the thick-drop method as a whole is crude, and provides opportunities for many diagnostic errors. It should be employed only to supplement the ordinary thin film method, never as a substitute for that method." Until recently our opinion coincided with that of James. Yet some workers appear to have brilliant results with the thick film method and to rely upon it as a routine diagnostic procedure in malaria,—others to consider it of very little value. We are inclined to consider that this difference of opinion may be due largely to differences in the technique employed.

In July 1923, there appeared a paper by Szilard which we read with great interest. In it he described a method of isolating and collecting together living leucocytes in enormous numbers for opsonic and similar work. The technique employed was to hæmolyse the blood by a mixture of acetic and tartaric acids, to exactly neutralize the hæmolysed blood with a solution of potassium hydrate, to collect the leucocytes by centrifuging, and to wash them in Locke's solution. Two very striking illustrations shew the results obtained, microscope fields crammed with myriads of leucocytes which are viable and which retain their staining properties.

It appeared to us that this method would be of great value in the diagnosis of kala-azar. The full technique of Szilard demands laboratory facilities which are not at the disposal of the general practitioner, whilst the time employed would render the full technique unsuitable for routine use in the diagnosis of kala-azar. After trial of two or three different modifications, however, we evolved a technique, founded upon Szilard's paper, which has given us excellent results.

Technique.—In all thick blood film work, the most meticulous attention must be paid to details; nothing spoils a thick film for examination more than the presence of deposit of stain, dirt or bacteria. The glass slides used for the films must be perfectly clean and polished, and should be freed from grease by flaming.

(1) The thick film should be prepared by the method of James (1920, p. 172), with full aseptic technique. Four large drops of blood are placed at the corners of a small square, ½ in. by ½ in., near the centre of the slide. With a round needle or glass rod they are then pooled so that the blood covers the ½ in. square thickly and evenly: "puddling" must be avoided.

(2) The slides are now laid flat on the table, are covered with a Petri dish and are allowed to dry completely. *This is the most important point of the whole process.* A thick film may appear to be dry in half an hour, but the leucocytes have not yet emigrated to and become adherent to the slide. At least two hours at room temperature, or an hour in the 37° C. incubator, is required; otherwise the film gets washed away during subsequent manipulations.

(3) Lay the perfectly dry thick film,—surface upwards,—flat on a staining rack. Flood the slide very gently with the following mixed solution:—

Glacial acetic acid; 2.5% in distilled water .. .. .. 4 parts.
Tartaric acid crystalline; 2% in distilled water .. .. .. 1 part.

This solution dehæmoglobinizes the film, and the process should be watched. An ordinary thick film will be completely dehæmoglobinized in 5 to 10 minutes, but films with thicker patches may require a little longer.

(4) As soon as dehæmoglobinization is complete, drain off the fluid by tilting the slide. Next, flood the slide with methyl alcohol. Allow to remain on for one minute. The film is now fixed.

(5) Drain off the methyl alcohol and wash the film *very thoroughly* in distilled water. Every trace of acid must be removed from the film, or the subsequent staining will be unsatisfactory.

(6) Stain the film with Giemsa's stain, one drop to one c.c. of distilled water, for 10 minutes. Differentiate in distilled water. Do not blot the film, but allow it to dry in air, placing the slide tilted against any vertical surface, with the film side downwards to protect it from dust.

(7) Examine with the 1|12th-in. oil immersion lens and a fairly high, e.g., No. 6 ocular. The leucocytes are seen to be evenly scattered, field by field, over the half-inch square, and can be rapidly examined for *L. donovani*. As contrasted with control thick films for healthy blood, the leucopænia of kala-azar at once becomes most strikingly apparent.

Results in kala-azar.—Up to the date of writing—(25th June 1924)—we have examined the blood of 70 persons suffering from fever and splenic enlargement by this method. We would have preferred to have collected a much larger series of observations for analysis before publishing this paper, but we have received so many enquiries from students and others as to the details of technique that we have decided to here give the technique in full detail, and to present a preliminary analysis only of the results to date. We hope at a later period to analyse the results obtained upon a larger series of observations.

Of these 70 persons, 55 were found to be suffering from kala-azar as *proved* either by finding *L. donovani* in thick films of the peripheral blood or in spleen puncture films, whilst 35 of them gave either a + + or a + + + aldehyde reaction; the aldehyde reaction in the other cases being either negative or only partially positive. *In no less than* 34 *out of these* 55 *kala-azar cases, the necessity for spleen puncture for diagnosis was done away with as parasites were met with in the thick films of the peripheral blood, and the diagnosis thereby proved.*

As a control to results with the thick film method, we may take our collected results of examination of thin films of the peripheral blood from 140 proved cases of kala-azar in Calcutta in 1922, as published in a previous memoir,—(Knowles, Napier and Das Gupta, 1923, p. 321). The comparative value of the two methods is shewn in the following table:—

	Thin films, 1922.	Thick films, 1924.
Number of proved kala-azar cases examined.	140	55
Number shewing parasites in peripheral blood.	27=19 %	37=67 %
Total number of films examined.	442=3·2 per patient.	82=1·5 per patient.
Number of films positive	53=12 %	37*=45 %
Total number of parasites encountered.	102 in 442 films.	65* in 82 films.

* The smallness of these numbers is due to the fact that further search for parasites was usually discontinued as soon as parasites were found, and the diagnosis thereby proved.

As seen in thick blood films by this method, *L. donovani* presents the same appearances as it does in thin blood films. When free or in the large hyaline mononuclear leucocytes it stains well and shews up clearly: some of our findings include clusters of 2, 4, 4, 6, 7 and 12 leishmania respectively per large mononuclear. When seen phagocytosed in a polymorphonuclear leucocyte, however, it tends to stain badly; the capsular outline may be almost lost, the cytoplasm staining very faintly; but the macronucleus and especially the micronucleus stain deeply. It is because the identification of such forms is often difficult for the beginner, that we insist upon the need for the most scrupulous attention to details in preparing and staining the thick films.

Some special cases may now be commented on:—

(1) In two instances, where examination of thick blood films failed to shew parasites, culture of the peripheral blood shewed leishmania. The method, therefore, is far less reliable than is the cultural one.

(2) In one instance parasites were still found after the second intravenous injection of sodium antimony tartrate.

(3) Apart from the above 55 cases, in 8 partially treated kala-azar cases, where from 8 to 75 intravenous injections of antimony per case had been given, spleen puncture films still shewed parasites in 7 and spleen puncture juice culture parasites in 1, but no parasites were detected in the thick films. In other words, spleen puncture is a far more reliable procedure for diagnosis.

(4) In one instance spleen puncture films shewed leishmania, with no malarial parasites detected; the thick films shewed the gametocytes of *L. malariæ* but

no leishmania. The dual infection present was only detected by the dual examination.

Results in other diseases.—Not only is the method of considerable value in the diagnosis of kala-azar; it is also of special value in the diagnosis of malaria. Here, indeed, we have been forced to revise entirely our original estimate of the value of examination of thick blood films in malaria. For cases with very scanty parasites in the peripheral blood, the method is invaluable. It is also of special value in that all too common type of malarial case encountered in general practice in the tropics,—the patient who has taken sufficient quinine to render the search for parasites in thin blood films hopeless, but insufficient to control the fever.

In malaria the thick film method shews up growing trophozoite, schizont and gametocyte forms far better than it does young ring forms, and in the latter case it may often be difficult to determine the species present. In one case the microscope field appeared to be full of myriads of little dots of chromatin: only careful examination shewed in each instance the attached tag of faintly staining cytoplasm; the case was one of severe malignant tertian malaria with innumerable fine hair-like ring forms present. In such cases diagnosis is better established of course by the examination of thin films.

Our results to date in malaria are insufficient for analysis, but a few interesting cases may be mentioned:—

(1) An Anglo-Indian patient was admitted from the Andamans suffering from very severe chronic malignant tertian malaria, as proved on examination of thin blood films. He was put on to cinchona febrifuge mixture. On the 7th day the patient was still mildly febrile. Examination of thin blood films shewed nothing: but several young trophozoites of *L. malariæ* were encountered in the first thick film examined. Enquiries shewed that the patient had been taking the mixture irregularly.

(2) Prolonged search of a thin film from a case of quartan malaria shewed only two young ring forms. A thick film, taken at the same moment, shewed 10 mature rosettes and some free merozoites.

(3) In three patients, where kala-azar was suspected, the aldehyde test was completely negative. Examination of thin films shewed nothing. Before doing spleen puncture, thick films were examined, and *L. malariæ* encountered.

(4) A sample of a newly introduced remedy for malaria,—"Smalarina,"—having been received for trial, a suitable case of benign tertian malaria was selected and admitted to hospital. The treatment was administered strictly in accordance with the instructions on the pamphlet accompanying the sample. Up to the 17th day of treatment the patient ran a high intermittent fever, at first with rigors on alternate days, later with daily rigors and an irregularly febrile chart. Thin blood films shewed *P. vivax* up to the 16th day of treatment, but not subsequently. Parasites were, however, present in thick blood films on the 17th, 19th, 20th and 21st days of treatment. The patient's condition, however, was now so bad that the "Smalarina" treatment had to be discontinued, and cinchona febrifuge mixture given, with successful results.

A further matter in which we have found such thick films useful is in carrying out the differential lecocyte count in cases of leucopænia, as all types of leucocytes stain well and can be readily identified (with a few exceptions). The differential leucocyte count is often a matter of considerable difficulty in kala-azar, owing to the leucopænia present. In such thick films, however, a differential count upon 500 leucocytes can be relatively easily carried out. Lastly, the method is probably applicable to all cases where the search for very scanty blood-inhabiting protozoa in thin fims is very difficult, in man, birds and animals. In brief, we have here what we have for a long time desired, but never previously obtained in our own experience, a method of dehæmoglobinizing and staining thick blood films, which does not destroy or distort cell fixation images, and which gives good results.

SUMMARY.

A method of taking, dehæmoglobinizing and staining thick blood films is described, which, in the authors' experience (on only some 70 cases) enables a positive diagnosis to be given in kala-azar by the discovery of the parasite in such films in some 67 per cent. of cases, and which has also proved to be of value in the diagnosis of difficult cases of malaria, and in carrying out the differential leucocyte count in cases of leucopænia.

REFERENCES.

Das Gupta, B. M., 1922.—The Diagnosis of Kala-Azar by Peripheral Blood Culture. *Indian Med. Gaz.*, LVII, 6, p. 217 (Abstract).

James, S. P., 1920.—"Malaria at Home and Abroad." London, John Bale, Sons & Danielsson.

Knowles, R., 1920.—A Study of Kala-Azar. *Indian Journ. Med. Res.*, VIII, 1, p. 140.

Knowles, R., Napier, L. E., and Das Gupta, B. M., 1923.—The Kala-Azar Transmission Problem. *Indian Med. Gaz.*, LVIII, 7, p. 321.

Stitt, E. R., 1923.—"Practical Bacteriology, Blood Work and Animal Parasitology." 7th edition, London, H. K. Lewis & Co.

Szilard, P., 1923.—A method for the Isolation of White Blood Cells. *Journ. of Lab. & Clin. Med.*, VIII, 10, p. 661.

OF *ENTAMŒBA HISTOLYTICA* CARRIERS.

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To the biologist, the conception of the ideal relationship between host and parasite is that of perfect symbiosis; both host and parasite living together in mutual agreement, each leading an independent existence, yet each helpful to the other. Such an ideal relationship is reached in the case of the lichens; it may also possibly be