

## The Serological Diagnosis of Amoebiasis in Ceylon

### Part II. The Indirect Fluorescent Antibody Test (IFAT)

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**SUMMARY** Sera from persons with clinical evidence of amoebiasis were tested by the Indirect Fluorescent Antibody Test (IFAT). Sixty one per cent of those with intestinal involvement only showed a positive reaction. In those with hepatic involvement 70% or more showed a positive reaction.

A ++ or +++ fluorescence persisting at a serum dilution of 1:160 or more was taken as being indicative of amoebic infection.

A refinement of technique is advocated before absolute reliance is placed on the test.

In 1954 Goldman used fluorescein tagged antibody to identify cultures of *Entamoeba*. Goldman, Carver and Gleason (1960) used the Fluorescent Antibody Test (FAT) for the antigenic analysis of various strains of amoebae. Goldman (1963, 1966) and Jeannes (1962) reported its use as a diagnostic aid in hepatic and other amoebic infections. In our laboratory this technique has been used in the diagnosis of filarial and other parasitic infections. The present study to determine the value of the IFAT in the diagnosis of amoebiasis was begun at the end of 1967 and continued to early 1970.

#### MATERIALS AND METHODS

Test sera were collected from the same (3) clinical groups as for the Indirect Haemagglutination Test (IHAT) and from a control group. (see Part I).

Antigen: *E. histolytica* was obtained from the stools of patients with acute symptoms of amoebic infection. These were cultured in a modified Boeck and Drbohlav (1925) medium, and sub-cultured every 4th day. The different strains of amoebae were tested with a known serum and conjugated anti-human globulin (CAHG) for evidence of fluorescence. Cultures from which amoebae showed a good fluorescence were sub-cultured as before at intervals of 4 days. A pooled suspension of amoebae was obtained from the tested cultures by picking the surface of the solid phase of several culture tubes. The amoebae were washed down with phosphate buffered saline (PbS) pH 7.2. The washings were centrifuged at a slow speed and the deposit of amoebae collected. These were preserved in 5% formal saline, stored at 4°C and used within 5 days of collection. Prior to use, the pooled suspension was washed three times in PbS, standing between washings for 5 min. in PbS. A drop of suspension contained about 50 amoebae in each low power field.

Serum was diluted from neat to 1:5120. Tests were done in WR tubes. To one drop of antigen, two drops of test sera of the appropriate dilution were added and left overnight at room temperature 30°C. The suspensions of amoebae was then washed in PbS for five minutes (allowing to stand in PbS for five minutes between washings). After this two drops of CAHG (Burroughs Wellcome) in a dilution of 1:10 or 1:20 were added to the suspension and left overnight. Washing was done as before and the final deposit mounted in glycerine buffer for examination under UV light. Each low power field contained about 15 to 20 amoebae.

Examination was with a Reichert's "Binolux" microscope with a bright ground condenser. The filters used were a blue pass exciter filter E 3 BG 12/6 mm and an eye piece filter Sp 3 GG 9/1 + OG 1/1.5 mm. According to the manufacturers of this microscope the development of suitable absorption filters has made it unnecessary to use a dark ground condenser for examination of organisms larger than bacteria (Jayewardene and Wijyaratnam, 1968.)

## RESULTS

A ++ or +++ fluorescence at a dilution of 1:160 is taken as the diagnostic titre.

The results obtained are seen in Table I.

TABLE I  
Results of IFAT with sera from persons with intestinal amoebiasis, amoebic hepatitis, liver abscess and a control group

	Serum Dilutions											
	Neat	1:5	1:10	1:20	1:40	1:80	1:160*	1:320	1:640	1:1280	1:2580	1:5120
<i>Intestinal Amoebiasis</i>												
Group 1												
No. ++	26	24	24	23	22	21	13	9	5	0	0	0
Total done	27	27	27	27	27	27	21	21	4	14	14	12
% +ve	96	88	88	85	81	77	61	41	35	7	0	0
<i>Amoebic Hepatitis</i>												
Group 2												
No. ++	39	40	41	39	36	34	-21	20	12	9	7	6
Total Done	47	47	47	47	47	47	30	30	13	13	13	13
% +ve	80	85	87	82	76	72	70	66	92	69	53	46
<i>Liver Abscess</i>												
Group 3.												
No. ++	42	42	42	41	41	37	31	28	17	13	6	3
Total Done	46	46	46	46	46	46	41	39	24	21	21	21
% +ve	91	91	91	87	87	80	75	70	70	61	28	14
<i>Controls</i>												
Group 4												
No. ++	18	19	16	11	10	8	4	2	0	0	0	0
Total Done	36	36	36	36	36	36	36	36	36	36	36	36
% +ve	50	53	44	30	27	22	11	5	0	0	0	0

\* 1:160 Diagnostic Titre

In Group 1 (intestinal amoebiasis) 61% were positive at the diagnostic titre, 41% at 1:320, 7% at 1:1280 and none over this.

In Group 2 (amoebic hepatitis), 70% were positive at the diagnostic titre, 66% remained positive at 1:320 and 46% at the highest dilution.

In Group 3 (amoebic liver abscess) 75% were positive at the diagnostic titre. At 1:1280 61% were positive, but only 14% were positive at the highest dilution.

In Group 4 (the control group) 11% were positive at the diagnostic titre, 5% at 1:320 and none over this.

#### DISCUSSION

In this series, a higher percentage of positives were seen in the two groups with liver involvement than in the group with only intestinal symptoms.

In the latter group (Group 1) we had 61% showing a seropositive reaction; whereas with the IHAT we had 78% positive though the total examined was much less (8). We have mentioned (Part I) that Milgram *et al.* (1966) had 63% (21 of 68 being negative) whereas Kessel *et al.* (1966) had 97% positive in their series with the IHAT. We have not seen a similar series using the IFAT for comparison of results.

In Groups 2 and 3 the results obtained with the IFAT at dilutions of 1:160 and 1:320 were similar, being somewhat higher in Group 3. In Group 2 the total examined at dilutions of 1:640 and over, were less than in Group 3, but the results showed a higher degree of sero-positivity over Group 3 at these dilutions. In each group the totals examined at the higher dilutions are insufficient for statistical analysis. The only way of seeing whether there is a significant difference is by doing a larger series. At the diagnostic titre of 1:160, there is no statistically significant difference in the results obtained in the three clinical groups. We repeat that the method employed by us, though laborious, does not in any way detract from the reliability of the results. We recommend the adoption of a more elegant technique as it would be both time and labour saving. It would also be advisable to re-check the false positive rate in a representative control group from an endemic environment. As with any other laboratory test, it is understood that a positive result would be considered together with the clinical condition of the 'patient'.

With the IHAT we had a very clear indication of a higher degree of reactivity in the group with liver abscess over the other groups. Though the percentage of positives is higher at 1:160 in the liver abscess group, the results with the IFAT are not sharply defined in this respect. It does appear, however, that in the two groups with liver involvement, a larger number remain positive at the higher titres than in the group with intestinal involvement only.

For practical purposes it appears sufficient to work with about six dilutions of sera per test run. Crude bacterial cultures were used by us as antigen. It does not seem possible that we could improve on this at present in our country. A good concentration of amoebae is therefore an asset in the reading of results.

An explanation is necessary with regard to the two dilutions of sera used in this series. The batch of conjugate used was the same throughout. We tried the early tests with a 1:10 dilution which gave a very satisfactory staining of amoebae. It was noticed that the fluorescence of the background material, (in the crude cultures used), interfered with the reading of the results. In view of this we used the next dilution, which proved a very satisfactory working dilution. We did not repeat the tests already done with the 1:10 dilution as we did not observe a difference in the results to warrant repetition. Table 2 shows the results obtained with the conjugate at dilutions of 1:10 and 1:20.

TABLE 2

Results with the conjugate at 1:10 and 1:20

Conjugate	1:10		1:20	
Serum Dilution	1:160	1:320	1:160	1:320
<i>Group 3*</i>				
No. +ve	7	6	24	22
No. Done	9	8	32	31
% +ve	77	75	75	71
<i>Controls</i>				
No. +ve	1	0	3	2
No. Done	18	18	18	18
% +ve	5	0	16	11

\*In the final analysis, the difference in dilution only applied to Groups 3 and 4.

In Group 3 (Table 2), in the number examined, the percentage positive was about the same at the dilutions of 1:160 and 1:320. The results are not statistically significant. In the control group the same number was examined at each dilution. These results are also not statistically significant. In view of this in the final analysis, we have combined the results obtained with the two dilutions, assuming that the difference in the dilutions did not make a significant difference to the results obtained, other than facilitate reading of the end point.

With reference to the method itself, we did try a slide method, but did not succeed in retaining a sufficient number of amoebae on the slide, for satisfactory reading of the results. As we were using the tube method for other parasitic material, we continued using this for the fluorescent staining of amoebae as well. We recommend a slide method for future work.

In the present analysis of results, the diagnostic titre has been taken as 1:160. This has been guided by the false positive rate of 11% in the controls, at this dilution. If it is assumed that the diagnostic titre is 1:80 it is seen that the results obtained are more akin to those of the IHAT, namely, 77, 72 and 80% for groups 1 to 3 respectively. As the false positive rate at this dilution in the controls is 22%, the results have been analysed, assuming a diagnostic titre of 1:160. It is very necessary to examine a large number of sera from normal persons in endemic areas to determine the false positive rate which will be realistic for a country like ours where amoebiasis is endemic. We are not aware of a series in which the IFAT has been used for fixing the diagnostic titre\*. Jeannes (1964;1966) states that 6 cases of hepatic amoebiasis had fluorescence titres of 1:64 to 1:16384, but does not fix a titre for diagnosis.

#### CONCLUSIONS

Our examination of sera with the IFAT in the diagnosis of amoebiasis indicates that, in the pathogenic groups, those with hepatic amoebiasis show a higher and a more persistent reaction than those with intestinal involvement only.

A marked difference was not seen between the results obtained with sera from cases of amoebic hepatitis and amoebic liver abscess.

It will be necessary to examine a larger number of sera from clinically normal persons in areas where amoebiasis is endemic before a definite diagnostic titre with the IFAT can be fixed.

The IFAT is a very sensitive test. A simpler technique together with a standard antigen is likely to give greater accuracy of results at a higher end point. If standardised thus, the test would take much less time than the IHAT and be as dependable.

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#### REFERENCES

- BOECK, W. C. & DRBOHLAV, J. (1925). The cultivation of *Entamoeba histolytica*. *American Journal of Hygiene*, **5**, 371-407.
- GOLDMAN, M. (1954). Use of Fluorescein tagged antibody to identify culture of *Entamoeba histolytica* and *Entamoeba coli*. *American Journal of Hygiene*, **59**, 318 - 325.
- GOLDMAN, M. (1963). Fluorescent antibody methods in the diagnosis of amoebiasis. *Proceedings of the International Congresses on Tropical Medicine and Malaria*, **11**, 277 - 280.
- GOLDMAN, M. (1966). Evaluation of FAT for amoebiasis, using two widely different strains as antigen. *American Journal of Hygiene*, **15**, 694.
- GOLDMAN, M., CARVER, R. K. & GLEASON, H. (1960). Antigenic analysis of *Entamoeba histolytica* by means of fluorescent antibody. *11 E. histolytica and E. hartmani*. *Experimental Parasitology*, **10**, 366 - 368.

- HBALY, G. R. (1968). Use of and limitations of the indirect haemagglutination test in the diagnosis of intestinal amoebiasis. *Health Laboratory Science* 5, 174 - 179.
- JAYEWARDENE, L. G. & WIJAYARATNAM, Y. (1968). The fluorescent antibody test in the serological diagnosis of the causative organisms of Tropical Eosinophilia and Filariasis. *Journal of Helminthology*, 42, 57 - 64.
- JEANNES, L. (1962) Antigenic analysis of *E. histolytica* by means of fluorescent antibody. *American Journal of Tropical Medicine* 11, 341 - 346.
- do— (1964) Immuno-fluorescent diagnosis of amoebiasis. *British Medical Journal*, 11, 1531.
- do— (1966) Preliminary communication. Indirect fluorescent antibody test in the diagnosis of hepatic amoebiasis. *British Medical Journal* 1, 1464.
- KAGAN, I. G. (1968) Evaluation of serological tests used in the diagnosis of amoebiasis. *World Health Organisation Expert Committee on Amoebiasis*.
- KESSEL, J. F., LEWIS, W. P., MA, S. & KIM, H. (1961). Preliminary report on the haemagglutination test for *Entamoeba*. *Proceedings, Society of Experimental Medicine and Biology*, 106, 409 - 413.
- KESSEL, J. F., LEWIS, W. P., MOLINA - PASQUAL, C. & TURNER, J. (1965). Indirect haemagglutination tests and complement fixation tests in amoebiasis. *American Journal of Tropical Medicine and Hygiene*, 14, 540 - 550.
- MADDISON, S. E. (1965). Characteristics of *Entamoeba histolytica* antigen antibody reactions by gel diffusion. *Experimental Parasitology* 16, 224.
- MILGRAM, E., HBALY, G., & KAGAN, I. (1966). Studies on the indirect haemagglutination test in the diagnosis of amoebiasis. *Gastroenterology*, 50, 645.

\*After this article was submitted for publication we have read the work of Dr Ambroise-Thomas & Kieng Troung (1972) on this subject. *American Journal of Tropical Medicine and Hygiene*, No. 6, 21, pp 906 - 912. The diagnostic serum dilution is 1/50, with the conjugate dilution at 1/20, with the use of a counterstain.

