Chapter II

PREVIOUS LITERATURE
Invasion of the tissue by *Entamoeba histolytica* elicits production of specific antibodies by the host's immune system. Generally, the rise in antibody titres corresponds to the increased severity of the disease and the length of exposure to parasite. Thus, as reported by Milgram *et al.* (1966), Ambroise-Thomas and Kien Truong (1972) and Bos *et al.* (1976), patients with frank hepatic abscess show elevated antibody titres, while subjects who are without apparent symptoms of disease and pass cysts, may show a poor antibody response to *E. histolytica*. There are several serologic methods available for the detection of specific anti-*E. histolytica* antibodies. What follows is an account of the various studies reported on the application of these tests for the purpose of serodiagnosis of amoebic patients.

I. DETECTION OF SERUM ANTIBODIES AGAINST *E. histolytica*

1. IMMobilIZATION TEST

Cole and Kent (1953) demonstrated that the specific immune serum could immobilize the trophozoites of *Entamoeba histolytica* in vitro. The poor sensitivity of the test was evident from the fact that immobilization activity could not be demonstrated at a titre beyond single two-fold dilution of the antiserum. Moreover, the test gave positive reaction in only five of the thirteen human antiamoebic sera from patients. Brown and Whitby (1955) further reported the poor sensitivity of this test. They showed that the test was positive in only 24% of proved cases of active amoebiasis and was not superior to complement fixation test in its sensitivity. Valentino (1956) reported that this test was of limited diagnostic value in detecting cases of intestinal amoebiasis.
Biagi and Buentello (1961) studied the immobilization reaction of sera from human patients with amoebic liver abscess, acute intestinal amoebiasis and chronic intestinal amoebiasis. They obtained a positivity rate of 88%, 90% and 78% respectively for these conditions. They, however, noted that because of low antibody titres the test could not be quantitated.

Beltran et al. (1965) compared the immobilization reaction with the immunofluorescent technique and concluded that the latter was superior to immobilization reaction because it was able to detect concentrations of antibody too low to cause immobilization of \textit{E. histolytica}.

2. \textbf{Precipitin Test}

Wagener (1924) was the first to demonstrate experimentally that infection with \textit{E. histolytica} could lead to production of specific precipitating antibody. Using an antigen prepared from the scrapings of colonic ulcers of the infected cats and extracted with Coca's fluid, Wagener (1924) obtained positive precipitin reactions when the cats had been infected for a week or longer. Menendez (1934) demonstrated the presence of precipitins in the sera of rabbits immunized with \textit{E. histolytica} and \textit{E. barreti}. Rabbits were found to be specifically "hypersensitive" to the corresponding antigen used for immunization.

Moan (1957) employed precipitin test for the serological diagnosis of amoebiasis. The test consisted in allowing the interaction of equal volumes (0.5 ml) of the serum and antigen on a ringed slide. Moan (1957) obtained a high degree of specificity with virtually no false positive result and only a very few false negative results. It may be of interest to point out...
that Moan (1957) observed this test becoming negative within a few weeks after successful treatment, indicating potential value of a precipitin test in detection of active invasive phase of amoebiasis. In the same year, Nakamura and Baker (1957) reported the adaptation of Ouchterlony's agar gel diffusion precipitin test for serodiagnosis of amoebiasis.

Gel-diffusion Precipitin (GDP) Test:

Agar gel diffusion precipitin test has been widely used as a tool for serodiagnosis of antibodies against *E. histolytica* in clinical cases by several earlier workers like Nakamura and Baker (1957). Nakamura (1961), Auernheimer and Atchley (1962), Powell et al. (1966) found the test could be useful for diagnosis of cases of ulcerative post-dysenteric colitis following acute amoebic dysentery from other cases of chronic non-specific ulcerative colitis. Maddison et al. (1965a) showed usefulness of this test for detection of cases of present and past invasive amoebiasis.

Gel-diffusion precipitin test has a unique significance as it can demonstrate the antigenic heterogeneity of an organism in as much as each antigen antibody component of the organism can be demonstrated as distinct precipitin band. Thus, Nakamura and Baker (1957), Siddiqui (1961) and Sen et al. (1961) obtained three precipitin bands against *E. histolytica* antigen. Later Atchley et al. (1963) employed a highly concentrated *E. histolytica* antigen (48 million amoebae/ml) and reported up to ten precipitin bands in the sera from cases of symptomatic infections. However, Goldman and Siddiqui (1965) reported only four bands with sera from symptomatic cases although they had used 30-40 million amoebae/ml for preparation of the antigen.
A micro-adaptation of the Ouchterlony agar gel-diffusion described by Auernheimer and Atchley (1962) has been described in which only 5 μl of amoebic antigen and 40 μl of the test sera can be employed for detection of precipitin lines.

Among the recent studies Vinayak et al. (1974a) have used gel-diffusion precipitin for amoebiasis against sera from 42 cases of amoebic liver abscess, 14 cases of amoebic dysentery, 36 cases of amoebic hepatitis and 35 cases of non-dysenteric amoebic colitis. The test was positive in 90%, 93%, 61% and 18% of the cases respectively. The indirect haemagglutination (IHA) test was also performed on these sera. The haemagglutinins of 30 sera which had given titres of 1 to 4096 or higher by the IHA test, were absorbed with sensitized red cells and the tests repeated. After absorption, the IHA tests were negative whereas gel-diffusion precipitin test was unaffected, indicating thereby that the haemagglutinins and precipitins were different antibodies. Similar findings have been reported in another publication by Vinayak et al. (1975). They also found that GDP and IHA tests with 69 sera from amoebic liver abscess cases were positive in 91.3% and 95.6% of the cases respectively. Out of 30 sera from amoebic dysentery cases GDP was positive in 90% and IHA in 96.6%. They also observed a positive GDP with sera which gave IHA titre of 1 in 256 or higher.

Recently, Mohapatra et al. (1978) in India and Atakpa et al. (1978) in Northern Nigeria have also evaluated the gel-diffusion test in clinical diagnosis of amoebic patients and in epidemiological studies, respectively. Mohapatra et al. (1978) obtained a positive precipitin reaction in 74.3% cases of amoebic liver abscess and in 60% cases of amoebic dysentery.
Atakpa et al., (1978) who reported 89% of the proven amoebic liver abscess cases to be positive with gel diffusion precipitin test, emphasized the usefulness of this test in differentiating cases of liver abscess from those of hepatoma.

Immunoelectrophoresis (IEP) test:

In this technique the antigenic fractions are first separated by electrophoresis and reacted with antisera to obtain the precipitin bands.

Krupp and Powell (1971) have carried out studies on the antigenic fractions of *Entamoeba histolytica* against 448 sera of amoebic dysentery or liver abscess cases by micro-immunoelectrophoresis. They found 7 different patterns with all the sera. The maximum number of lines developed in pattern 1 (i.e. 14 arcs) and the minimum number of lines developed in pattern 7 (i.e. only 2 arcs). According to their findings, 96% sera showed pattern 1 (which were collected before or within 2 months of treatment). Pattern 7 (2 arcs) persisted longest after treatment. They also found in this study that pattern 1 correlated with high IHA titres of these sera from amoebic patients. Some of the sera with pattern 7 showed negative IHA tests. Krupp and Powell (1971) have suggested that combination of micro-electrophoresis and IHA titre level in the patient's sera, can provide differentiation between an active infection and antibodies persisting after cure. These authors conclude that if the titre level is high and microelectrophoresis pattern 1, 2 or 3, corresponding to 14, 9, and 7 arcs respectively, it can be predicted almost with certainty that the infection is active.

Counter-immunoelectrophoresis (CIEP) Test

Counter-immunoelectrophoresis (CIEP) which is a modified gel-diffusion precipitin test, takes the advantages of two facts that, firstly, the antigen moves towards anode,
and, secondly, the antibody owing to its slower electrophoretic mobility and the greater effects of endosmotic forces moves toward the cathode. Originally devised for rapid detection of Australia antigen (Gocke and Howe, 1970; Atler et al., 1971), CIEP was adapted for serodiagnosis of amoebiasis by Sepulveda et al. (1971).

Counter-immunoelectrophoresis (CIEP) has been found to be quick and reliable for the diagnosis of invasive amoebiasis. Krupp (1974) reported that the test did not give any false positive reaction unlike the IHA test. Mahajan et al. (1975a) have compared the efficacy of CIEP and IHA tests using 40 sera of amoebic liver abscess and 60 sera of apparently healthy subjects. CIEP was positive in 95% and IHA in 90% of the cases. Among the healthy subjects, only 1.7% false positive with CIEP and 6.7% with IHA test. Precipitating antibodies against *E. histolytica* antigen have been demonstrated by CIEP in 11 out of 15 'pus' samples from patients although the CIEP titres were low (Mahajan et al. 1975b).

**Cellulose acetate - Diffusion Test**

Siddiqui (1961) demonstrated that cellulose acetate-diffusion tests could be used for rapid detection of antibodies against *E. histolytica* in rabbit immune serum. Precipitin lines develop within half an hr. This test has been recently employed for diagnosis of invasive amoebiasis cases by Sodeman and Dowda (1973). They found that the test was highly sensitive and could be used on even a single serum sample.

3. **AGGLUTINATION TESTS**

**Indirect Haemagglutination (IHA) Test:** Indirect haemagglutination test was initially developed for the diagnosis of both amoebiasis and toxoplasmosis by Lewis and Kessel (1961). Kessel et al. (1961, 1962, 1963) employed
tanned human 'O' type RBC coated with amoebic antigen for this test and found that among 455 sera of amoebic patients the test was positive in 100% of the liver abscess cases, 66% of carrier cases and 98% of amoebic dysentery cases. They considered a titre of 1:8 as positive for diagnosis. Among liver abscess cases they found titres of 1:512 to 1:512,000. The test was false positive in 3% of the 101 controls (Kessel et al. 1965). Maddison et al. (1965b) and Milgram et al. (1966) modified the above test by introducing the use of sheep RBC instead of human RBC for IHA test. Milgram et al. (1966) tested 110 sera from patients with unconfirmed liver abscess and 11 cases of confirmed amoebic liver abscess. The test was positive in 107 unconfirmed liver abscess cases and in 10 cases of confirmed abscess. They reported a lowest titre of 1:256 as diagnostic for amoebiasis. They also found that positive test was obtained in 82% intestinal dysentery cases and in only 9% of the asymptomatic cases.

Yap et al. (1970) reported the use of antigen prepared from cysts of E. invadens for sensitization of red cells which were used for this test against 111 sera from the amoebiasis patients and 130 sera from non-amoebic cases. Nearly 100% positive results were recorded on sera from patients with extra-intestinal amoebiasis, and 96% sera from acute amoebic dysentery. Sera of only 9% of the cases with acute amoebic dysentery gave positive gel-diffusion test with E. invadens antigen and 60% positive test with antigen prepared from trophozoites of E. histolytica. These findings suggest that E. invadens has a strong agglutinogen but only a weak precipitinogen common to it and to E. histolytica.
In most of the studies on IHA test, antigen prepared from trophozoites of *Entamoeba histolytica* has been used for sensitizing the red cells. Krupp (1969) has reported a method of storing the red cells after fixation with chilled 1% glutaraldehyde for periods up to 3 months, so that the same batch could be used in tests done with different batches of sera. The red cells fixed with glutaraldehyde are tanned and then sensitized with *E. histolytica* and stored at -70°C without any loss of titre up to 3 months. But after 6 months the titres were 1 or 2 dilutions lower. Most of the workers agree that IHA titre of 1 in 128 or greater should be considered positive for diagnosis of amoebiasis and titres below this should be considered non-specific (Healy et al. 1970; Wang, 1973; Cross et al., 1975; Jayewardene and Wijayaratnam, 1973; Meerovitch and Healy, 1976). Ali Khan (1974) also employed glutaraldehyde fixed red cells for IHA test. Glutaraldehyde fixed and antigen-sensitized sheep red cells refrigerated or lyophilized were found to be stable for more than 6 months in the IHA tests against amoebiasis. These studies on the stability of stored red cells will be very useful for providing standard sensitized red cells for IHA tests.

Farshy and Healy (1974) have further modified the method of preparation of stabilized sensitized red cells. They treated the human "0" cells with pyruvic aldehyde, then they were tanned and treated with 2% glutaraldehyde, and stored in refrigerator. This method is considered to give greater stability of sensitized red cells which can be supplied to different laboratories.

IHA test is valuable for the diagnosis of hepatic amoebiasis and symptomatic intestinal amoebiasis but in the cases of asymptomatic amoebiasis the serum antibodies IHA test gives very low titres which are sometimes similar to the non-amoebic control cases. Krupp (1970) found very
high titres in hepatic amoebiasis cases and in this group the test was positive in 90% of the cases. Similarly the test was positive in 80% of intestinal amoebiasis cases. Krupp also found that IHA test for amoebiasis was not influenced by the presence of other concurrent infections with nematodes, *Entamoeba coli*, and pathogenic bacteria. Most of the asymptomatic cyst passers (over 90%) gave negative results. Wang (1973) also found the positive IHA test in 100% cases of amoebic liver abscess. Jayewardene and Wijayaratnam (1973) found this test positive in 78% amoebic hepatitis cases and 90% cases of proved aspirated amoebic abscess. Vinayak *et al.* (1974) recorded positive test in 57.6% "amoebic hepatitis" cases and in 89.5% of the amoebic liver abscess cases. Yap *et al.* (1975) reported 89.1% positive test in cases of the intestinal and hepatic amoebiasis. False positive IHA was recorded in 4.3% of healthy blood donors.

Juniper *et al.* (1972) reported that IHA was most sensitive of all the tests used, but it remained positive for longer periods ranging from 6-12 months or even 1-3 yrs., and it would not help to differentiate intestinal from the extra-intestinal cases. Neal *et al.* (1968) also reported that haemagglutination antibodies persisted longer than the complement fixing antibodies after treatment. Krupp and Powell (1971) also recorded the persistance of IHA test after successful cure for periods upto 3 yrs., so that this test can not be used to detect active infection. Stamm *et al.* (1976) have reported the persistence of IHA antibodies for periods upto 11 yrs.

Cross *et al.* (1975) carried out seroepidemiological survey in Borneo with the help of IHA test and also carried out detailed parasitological survey of the 2169 stool samples. In this study 12% cases showed
E. histolytica infection by parasitological examination and 34% of the population showed positive IHA test (titre of 1 in 128 or greater). In another study Meerovitch and Healy (1976) have reported that 1% level of seropositivity (IHA titre above 1 in 128) correlated with the presence of active trophozoites of E. histolytica in stool. Even though 24.2% of the cases showed E. histolytica cysts in stool, the IHA test was not positive.

**Latex Agglutination Test**: Stamm et al. (1973) observed that though latex agglutination test (Serameba test) was less sensitive, it was equivalent to IHA test in clinical and epidemiological work. Absorption of haemagglutinins by sensitized red cells, resulted in negative IHA and latex agglutination test, suggesting thereby that the same antibody is responsible for IHA and latex agglutination tests. Out of 100 sera of amoebic liver abscess cases tested by latex agglutination test, 98 showed detectable antibodies (Morris et al. 1970a). Similarly out of 100 sera of amoebic dysentery, 96 gave positive test suggesting that the test is valuable in invasive amoebiasis. Morris et al. (1970b) found a close correlation between latex agglutination and gel-diffusion tests. Out of 250 amoebic sera tested, only 3 sera negative by latex agglutination test were positive by gel-diffusion, and 5 sera positive by latex agglutination were negative by gel-diffusion. Latex agglutination test has been used by several workers (Mahajan et al. 1972a; Ambroise-Thomas, 1974; Rees et al. 1974; Meyer, 1974). Haider et al. (1974) found this test useful for diagnosis of amoebic liver abscess, acute amoebic dysentery and chronic non-dysenteric amoebiasis. Haider and Rasul (1975) also found this test positive in 86.6% of sera from chronic non-dysenteric amoebiasis cases.
Bentonite Flocculation Test: Tupasi and Healy (1970) have reported the use of Bozicevich Trichinella bentonite flocculation test for the diagnosis of amoebiasis. Bentonite particles sensitized with E. histolytica antigen are employed for the test. None of the 24 asymptomatic carriers gave positive test. However, 93% of the extraintestinal amoebic cases and 86% of the symptomatic carriers were positive by this test. Mahajan et al. (1974b) carried out a comparative evaluation of bentonite flocculation, IHA and indirect fluorescent antibody test (IFA) with 51 sera of amoebic liver abscess, 43 sera of amoebic dysentery, 32 sera of amoebic hepatitis, and 50 sera of normal healthy persons. Bentonite flocculation test was positive in 92.3% liver abscess cases, 86.1% amoebic dysentery cases and 84.4% amoebic hepatitis cases. Among the controls 6% gave false positive test. The specificity of this test was comparable with the IHA and IFA tests. These workers consider a titre of 1:4 as diagnostic for amoebiasis. Vinayak et al. (1974b) also reported that the results of bentonite flocculation test were comparable to that of IHA test.

4. COMPLEMENT FIXATION (CF) TEST

Pioneering attempts to devise an amoebic complement fixation test were made by Izar (1914), Mage (1920) and Scalas (1921). But it was not until Craig (1927, 1929) undertook serious and orderly study of this test that it was shown to possess diagnostic value in amoebiasis. Craig employed amoebic antigens prepared by extracting twenty-four to forty-eight hour old cultures of E. histolytica (in association with bacterial flora) with absolute alcohol. He emphatically expressed his preference for using human haemolytic system to any other system. The complement fixation test involves the detection of specific antigen–antibody interaction as demonstrated by
'fixation' of complement in the presence of a haemolytic system. One of the chief difficulties in developing and standardizing such a test is the preparation of a suitable antigen. Craig's antigens were not strong enough to permit much dilution and the difference between antigenic unit and the anti-complementary unit was small. Craig (1937) indicated that his test detected acute cases of amoebiasis as well as carriers of *E. histolytica* with and without symptoms. He also reported 100% sensitivity of the test and stated that the test gave no false positive results. Craig's findings have been evaluated by a number of workers during the subsequent years. Thus Magath and Meleney (1940) used antigens prepared from cultures of *E. histolytica* made relatively free of concomitant bacteria and starch, lysed with glass-beads on a shaking machine and extracted with alcohol at room temperature for one week. Although the test gave a distinctly higher percentage of positive results in cases of acute amoebiasis, the accuracy reported previously by Craig (1927, 1929, 1937) could not be approached. There were instances in which in spite of evident invasion of tissues by *E. histolytica*, the complement fixation reactions were negative. Thus a serious doubt on the sensitivity of the test was cast by Magath and Meleney (1940) who thought that a considerable improvement in the antigens was necessary before this test could be adopted. Over the years after this, various workers devoted themselves to prepare adequate antigens for this test. Thus Fulton et al. (1951) prepared antigens from saline suspension of *E. histolytica* cultures completely washed free of bacteria and starch and filtered through a gradocel membrane of 700 μm porosity. Heinz et al. (1956) Wells (1956), Buonomini and Kicciardi (1958) and Kessel et al. (1965) prepared antigens by ultrasonication,
alcoholic extraction, cryolysis and mechanical rupture of amoebae respectively. Magauda-Borzi and Pennisi (1960) described various methods for extracting antigens of *E. histolytica*. Extractions were made by cryolysis, with alcohol, trichloracetic acid, deoxycholate and phenol. Phenol-extracted antigen was shown to give best results in complement fixation test. Various workers have reported conflicting results regarding the sensitivity and specificity, and hence the utility of this test in the serodiagnosis of amoebiasis. Dolkart *et al.* (1951), McDearman and Dunham (1952), Kenney (1952), Heinz *et al.* (1956) Kessel *et al.* (1965), and Kenney and Illes (1968) found this test to be of some utility in detecting the symptomatic cases of extraintestinal amoebiasis. On the other hand Elsdon-Dew and Maddison (1952), Lippi *et al.*, (1952), Dolkart (1953), Villari and Digilio (1955), and Wells (1956) have reported that this test is of limited application as a serological tool.

Krupp and Powell (1971) found CF inferior to IHA and immuno-diffusion tests for diagnosis of clinically invasive amoebiasis. CF test was considered as least sensitive and it gave positive reaction with only 65% of the cases, as against 94% and 95% positive test with IHA and immuno-diffusion respectively. Felgner *et al.* (1973) found CF test useful for diagnosing amoebic liver abscess cases, but even patients harbouring non-pathogenic amoebae gave false positive CF test. Robinson (1972) reported that CF test was completely negative with sera of patients showing asymptomatic amoebiasis, but it was valuable for diagnosing cases of invasive amoebiasis.
5. INDIRECT FLUORESCENT ANTIBODY (IFA) TEST

Fluorescent antibody technique was employed for the diagnosis of amoebiasis by Jeanes (1964, 1966). The test was positive in all the eight patients suffering from amoebic liver abscess. The titres varied from 1 in 64 to 1 in 16,384. In the control sera from patients suffering from miscellaneous non-amoebic disorders, the maximum titre obtained was 1 in 16.

Use of 'Huff' strain of *E. histolytica* as antigen in diagnosing amoebic patients with indirect fluorescent antibody test was evaluated by Goldman (1966), 'Huff' strain was found to show too low sensitivity than a 'classic' virulent strain of *E. histolytica*. Studies by Beltran et al., (1965), Boonpucknavig and Hainn (1967), Coudert et al., (1967, 1968), Ambroise - Thomas and Truong (1969) Hernandez, et al. (1969), Jeanes (1969) and Yu and Loo (1970) only show the value of this test in the clinical diagnosis of amoebiasis. Sensitivity of the test varied from 93% to 100% for amoebic liver abscess cases, and from 73.1% to 94% for the intestinal amoebiasis cases. Hepatic amoebiasis cases usually gave very high titres which varied from 1:256 to more than 1:1024. Non-specific reactions with this technique are negligible and can be avoided by absorbing the antisera. Agarwal et al. (1971) reported that the test was highly specific as it did not give false reaction with sera from healthy cases. Ambroise - Thomas and kien Truong (1972) found this test positive in 100% cases of hepatic amoebiasis which gave titres ranging from 1:100 to 1:256000, 59% positive with sera from amoebic dysentery cases who gave titres of 1:50 or higher, and only 25% positive with chronic amoebiasis cases, post-amoebic colitis and asymptomatic cases. Follow-up studies with hepatic amoebiasis cases showed that
IFA titres fell rapidly in 2 months and after 12 months the titre was usually negative or 1:50 to 1:100. Norcott (1973) also found that titres of IFA test which were highest in hepatic amoebiasis, fell to 1:64 or lower in 2 months after cure. IFA test was found to give positive reaction (titre of 1:100 with good fluorescence) in 98% of sera from extra-intestinal amoebiasis cases, 78% of intestinal amoebiasis cases with haematophagous trophozoites, and 35% of asymptomatic patients with cysts. One of the limitations of this test is low positivity among cases of asymptomatic amoebiasis. The titres dropped rapidly after about 6 months of successful treatment. The IFA test can also be successfully used on blood samples dried on the filter paper discs. Cox and Nairn (1974) reported the successful use of freeze-dried amoebae, which had been reconstituted and stored at 4°C for a year, in the IFA test. The stored amoebic antigen gave same titres as the fresh amoebae when tested against the same serum a year later. Mizgireva and Yumaevea (1976) used different strains of \textit{E. histolytica} for IFA test and reported that the test showed a sufficiently high specificity for diagnosis of invasive amoebiasis cases. These workers have emphasized that all the strains of \textit{E. histolytica} do not give equally bright fluorescence when used as antigen in IFA test. Only these strains which give the brightest fluorescence in primary screening should be used as antigen for diagnostic purposes.

A brief review of the immunofluorescence studies presented above and those summarized in Table II, show that different laboratories use different diagnostic titres for diagnosing amoebic cases. Although the test is of considerable help for diagnosis, a common standard for different laboratories should be established so that the test can be used reliably.
TABLE II. SUMMARY OF THE RESULTS OBTAINED BY VARIOUS WORKERS WITH IPA TEST (Figures in parenthesis indicate percentage of positive cases).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Maximum IP titer obtained in various groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maddison et al. (1968)</td>
<td>1 in 16, 1 in 4096, 1 in 256, 1 in 64</td>
</tr>
<tr>
<td>Jeanes (1969)</td>
<td>1 in 16, 1 in 1024</td>
</tr>
<tr>
<td>Agarwal et al. (1971)</td>
<td>1 in 50, 1 in 500, 1 in 200, 1 in 100</td>
</tr>
<tr>
<td>Ambroise-Thomas and Kien Truong (1972)</td>
<td>1 in 40, 1 in 2896, 1 in 128</td>
</tr>
<tr>
<td>Ray et al. (1974)</td>
<td>1 in 512, 1 in 32, 1 in 16</td>
</tr>
<tr>
<td>Stamm et al. (1976)</td>
<td>1 in 25600, 1 in 500, 1 in 138, 1 in 1024</td>
</tr>
<tr>
<td>Mithal et al. (1978)</td>
<td>1 in 64, 1 in 32, 1 in 16</td>
</tr>
<tr>
<td>Lilution of FITO-conjugate</td>
<td>Maximum IP titer obtained in various groups</td>
</tr>
<tr>
<td>Employed</td>
<td>1 in 50, 1 in 200, 1 in 100, 1 in 50</td>
</tr>
<tr>
<td>Natural</td>
<td>1 in 16, 1 in 4096, 1 in 256, 1 in 64</td>
</tr>
<tr>
<td>Acute amoebic dysentery/colitis</td>
<td>1 in 64, 1 in 32, 1 in 16</td>
</tr>
<tr>
<td>Chronic amoebic dysentery/colitis</td>
<td>1 in 512, 1 in 32, 1 in 16</td>
</tr>
<tr>
<td>Amoebic liver abscess</td>
<td>1 in 25600, 1 in 500, 1 in 138, 1 in 1024</td>
</tr>
<tr>
<td>Hepatomegaly/ portal hepatitis</td>
<td>1 in 64, 1 in 32, 1 in 16</td>
</tr>
<tr>
<td>Carriers</td>
<td>1 in 50, 1 in 200, 1 in 100, 1 in 50</td>
</tr>
<tr>
<td>Reference</td>
<td>Limitation of the results obtained in various groups</td>
</tr>
</tbody>
</table>

TABLE II. SUMMARY OF THE RESULTS OBTAINED BY VARIOUS WORKERS WITH IPA TEST (Figures in parenthesis indicate percentage of positive cases).
Soluble Antigen-Fluorescent Antibody Test.

The possibility of using soluble antigens in the fluorescent antibody test was first suggested by Paronetto (1963). Chief advantage of the test over conventional indirect fluorescent antibody test is the objectivity of the fluorometric reading of the test. The test has been markedly improved and employed in serodiagnosis of various parasitic infections such as schistosomiasis (Toussaint and Anderson, 1965; Sadun and Gore, 1967), *Trypanosoma cruzi* infections (Toussaint et al. 1965), and human filariasis (Luxbury and Sadun, 1967). The test consists in saturating the cellulose-acetate membrane discs with the optimal dilution of soluble antigen, drying up these discs in a dessicator, followed by antisera. The tests are read on a fluorometer.

Gore and Sadun (1968) successfully employed this technique for serodiagnosis of amoebic patients, using a commercial amoebic antigen prepared from axenically growing *E. histolytica*. They studied a total of 52 sera from amoebiasis patients. Of these 35 were confirmed cases of amoebiasis while 17 others belonged to the undefined category. In general high titres (1:256 to 1:1024) were observed in the sera from patients with extra-intestinal amoebiasis. A high degree of sensitivity was also observed when this test was employed with sera from patients with amoebic dysentery and from asymptomatic individuals, (the titres ranging from 1:64 to 1:1024). Highly reproducible results were obtained when same immune serum was tested with same lot of antigen and same fluorescent-antiglobulin at six different times. The test was negative in 96% of 152 control sera and in the remaining 4% the titres were low (up to 1:32).
6. ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme linked immunosorbent assay (ELISA) test recently developed by Bos and Van den Lijk (1976) and Bos et al., (1976) for serodiagnosis of invasive amebiasis, provides quantitation of specific antibodies being obtained by enzyme-labeled anti-immunoglobulin in antigen-coated polystyrene tubes or micro-plates. Antigen prepared from axenic *E. histolytica* is used for coating polystyrene tubes, and the antigen is then reacted with test serum (antibody). The antigen-antibody coating in the tube is then reacted with horseradish peroxidase conjugated to sheep antihuman immunoglobulin. The amount of enzyme peroxidase remaining in the tubes after washing provides a measure of the amount of specific antibodies in the serum by colouring a substrate. The sensitivity of the test is superior to that of the indirect fluorescent antibody test. Sera of amoebic liver abscess cases give titres up to 1:10,000 and those of intestinal cases give lower titres. Among asymptomatic carriers only 50% cases showed positive ELISA test but the titres were very low (1:20 to 1:500). The test is highly recommended for seroepidemiological studies.

II. DETECTION OF *E. HISTOLYTICA* ANTIGEN AND ANTIBODIES IN LIVER ABSCESS PUS, INFECTED TISSUES AND FECAL SAMPLES

1. PRECIPITIN TEST

Gel-diffusion precipitin test was employed by Mahajan et al., (1974a) for the detection of amoebic antigen in liver pus aspirated from six cases of amoebic liver abscess. The pus was centrifuged and the supernatant was tested by counter-immunoelectrophoresis against an amoebic antiserum made in rabbits. Within 1 hr, 2-4 precipitin lines developed. In the controls pyogenic pus was used but no lines developed. This technique is very valuable in establishing the diagnosis of amoebic liver abscess cases.
For detection of antiamoebic antibodies in liver pus, Mahajan et al., (1975b) employed counter-immunoelectrophoresis technique. Antigen prepared from trophozoites of *E. histolytica* was reacted against the pus sample in agar-gel. Out of 15 pus samples tested, antibodies against *E. histolytica* were demonstrated in 11 samples (73.7%). Sera of these 15 liver abscess cases were also tested and 14 gave positive precipitin lines. This test is valuable in those cases which do not show amoebae or amoebic antigen in their pus.

2. **Immunofluorescence Test**

Methods have been employed for detection of trophozoites of *E. histolytica* by immunofluorescence techniques from faecal samples, infected intestinal tissues and from experimentally produced hepatic abscess.

Antiserum produced experimentally in rabbits against trophozoites of *E. histolytica* is tagged to fluorescein-isothiocyanate, and used for the direct staining of the trophozoites of *E. histolytica* from faecal samples, infected tissues or culture. Among studies which have been carried out recently, Nayebi (1971) produced antisera against Shirazi and K9 strains of *E. histolytica* in rabbit, and after tagging with the immunofluorescent label, it was used for staining *E. histolytica*. Shirazi strain showed good fluorescence when stained with its unabsorbed antisera, or with the same serum absorbed with strain K9. It was found to give very little fluorescence when stained with its homologous serum absorbed with Shirazi strain or with K9 antisera whether it was unabsorbed or absorbed with any amoebae. These authors have suggested that the two strains of *E. histolytica* differ antigenically.
Detection of amoebae in tissues from patients with intestinal amoebiasis, by indirect immunofluorescent method has been reported by Hoffmann and Miller (1975). Using 1:10 dilution of the hyperimmune serum and FITC staining for 16 hrs, the amoebae could be detected in 30 specimens of tissues from patients, but amoebae could be shown in only 5 of 15 autopsy specimens because these tissues had undergone decomposition and autolysis. Parelkar et al., (1971) used fluoroscan (an antihuman globulin conjugated with fluorescein isothiocyanate) for staining trophozoites in hamster liver abscess. They have used paraffin/embbeded and sectioned liver tissue for the detection of amoebae which were treated with high titre serum from amoebic patients before staining with fluoroscan. Parelkar and Stamm (1973) demonstrated trophozoites of E.histolytica directly from the faecal samples, as well as after fixing the amoebae with polyvinyl alcohol. Non-specific fluorescence was avoided by faecal absorption of the antiserum. They employed sera from patients with a high titre in immunofluorescence test for indirect staining, and the fluorescein-isothiocyanate used was diluted 1:50 before use. Although maximum fluorescence was observed at pH 8.0 they considered pH 7.1 as optimal because it reduced the non-specific fluorescence which occurred at pH 8.0. They have emphasized that amoebae which are difficult to detect in the faecal samples or in haematoxylin stained preparations, can be readily observed by fluorescence microscopy.

III. DETECTION OF COPROANTIBODIES

Davies (1922) was the first to demonstrate the presence of antibody in intestinal lumen or in the stool. Burrows et al.,(1947) found that the experimental infection with cholera vibrios in the guinea pig was modified by prior
infection or by immunization with vaccine given parenterally.

It was observed that this modification was associated with the occurrence of agglutinating antibody in the feces. They named this fecal antibody as the "coproantibody". Later, it was demonstrated that a major proportion of the coproantibody was being locally produced in the intestine as a response to enteric cholera infection in man (Freter and Gangarosa, 1963). Recently, Blecka (1978) has demonstrated elevated levels of IgA in the feces of patients suffering from various parasitic infestations.

Detection of coproantibody in the intestinal amebiasis was first reported by Shaalan and Baker (1970). Among 35 cases of chronic amebic dysentery they studied, coproantibody could be detected in the saline stool extracts of only 12 patients (34.3%). They employed complement fixation test (CFT), using an antigen prepared from the alcoholic extract of the stools of patients with acute amebic dysentery. The saline stool extracts were shown to be non-antigenic, and having neither complement-like, hemolytic nor anti-complementary effects. Among 15 non-amebic (control) patients, none was found to be positive with CFT for coproantibody. Shaalan and Baker (1970) attributed the low positivity rate obtained in their study to the poor reactivity of immunoglobulin IgA in the complement fixation reactions.

In a study of complement fixation test vis-a-vis indirect haemagglutination (IHA) test, Mahajan et al. (1972) demonstrated that the latter detected the presence of coproantibodies in a larger percentage of cases in as much as 36 percent cases showing a negative CFT were found to be positive with IHA test. They employed an antigen prepared from a strain of *E. histolytica* growing with *Klebsiella aeruginosa*, by repeated differential centrifugation to minimize the bacterial contamination and then
subjecting the trophozoites to ultrasonic disruption. Among 76 cases of intestinal amoebiasis, 81.5% were found to be positive for coproantibodies with IHA. With complement fixation test, presence of coproantibody could be detected in only 30 out of 64 (46.8%) cases of intestinal amoebiasis.

IV. CELL-MEDIATED IMMUNOLOGICAL METHODS FOR \textit{E. histolytica}

Cell-mediated immunity (CMI) plays an important role in microbial and parasitic infections (Scully, 1972). The CMI response is mediated primarily by lymphoid cells rather than by humoral antibody. Cellular immunity can be transferred by lymphoid cells and not by immune serum. Two important parameters of cellular immunity namely (1) delayed dermal hypersensitivity (skin reaction) and (2) antigen induced lymphocyte transformation (blastogenesis) have been applied for the clinical diagnosis of amoebiasis.

1. INTRA-DERMAL TEST (SKIN TEST)

Earliest attempts to devise an intradermal test for diagnosis of amoebiasis were those of Scalas (1923) and Spector (1932): Scalas (1923) reported this test to possess a fair degree of specificity; subsequent workers (Spector, 1932, Secret, 1952, Leal, 1953; Kum, 1958, Kistic, 1959), however, reported conflicting results, though a correlation between positive intradermal test and active amoebiasis was evident in the studies carried out by Leal (1953), Kum (1958) and Kistic (1959). Diagnostic value of the skin-test could not be properly assessed because of the differences in the amoebic antigen employed which was prepared from either infected tissues or stools and was sometimes subjected to drastic treatment like heating at 100°C as done by Kum (1958).
Maddison et al. (1968) reported that 75 per cent of the patients with amoebic liver abscess gave an immediate type of skin-reaction. Similar results were obtained by Savanat and associates (1973) who employed antigen prepared from axenic *E. histolytica*. In an experimental study, Lunde et al., (1969) had demonstrated the skin-test potency of the axenic *E. histolytica* antigen in sensitized guinea pigs. This antigen was shown to be specific in nature as no cross-reaction could be elicited with antigen prepared from protozoan (toxoplasmin).

Kretschmer et al., (1972) gave intradermal injections of 0.1 ml histolyticin (water soluble extract of axenic *E. histolytica*) to 51 cases of hepatic amoebiasis, 17 cases of intestinal amoebiasis and 50 control cases having other abdominal diseases. Immediate positive intradermal reaction was observed in 78, 76 and 64% of the cases and delayed positive in 57, 47 and 20% of the cases respectively. They have suggested that positive delayed reaction in 20% control cases might be due to past or subclinical amoebic infections.

Savanat et al., (1973b) carried out skin test with antigen of axenic *E. histolytica* and recorded immediate type of reaction which developed as a wheal at 15-20 min after inoculation of antigen. The reaction was positive in 92% cases of hepatic amoebiasis and in 82% of the cyst-passers and 30% of non-cyst passers. All healthy children gave negative reaction. No data on the appearance of delayed-type of reaction is presented in this study. The skin test compared well with the results of immunoelectrophoresis test on these patients. They have also reported that skin test reactivity takes longer to develop after the onset of clinical symptoms, but once it has developed, it persisted longer than the precipitins.
Miller and Scott (1970) compared the efficacy of intradermal reaction and IHA test in six groups of subjects. Group A which included 23 acute amoebic dysentery cases, gave a positive skin test in all cases (100%) (either direct or indirect or both) whereas only 70% (16 cases) were positive by IHA test. Group B included 21 cases with history of amoebic dysentery or liver abscess which were given treatment during the previous 30 months, and skin test was positive in 20 cases and IHA in 17 cases. Group C included 24 school children from Leon Lake Indian Reserve where clinical amoebiasis is common, and 19 children showed positive skin test and 14 gave positive IHA. All the children were asymptomatic at the time of testing. In the remaining three groups (D-F) which were non-amoebic healthy (controls), both intradermal and IHA tests were negative. Positive skin test in asymptomatic school children (group C) suggests that tissue invasion can occur without clinical disease symptoms. Cysts passers who do not show clinical amoebiasis (in group D), gave negative skin test.

Meerovitch and Scott (1973) reported that intradermal injection of histolyticin in patients with low IHA antibody titre, could raise the IHA titre sufficiently high in 7 out of 11 cases and cause confusion in the interpretation of serological tests.

2. LYMPHOCYTE TRANSFORMATION

The specific blast formation in human lymphocytes from cases of amoebiasis can be initiated by exposing the lymphocytes to an extract of \(E.\) histolytica antigen. The blast formation takes place when lymphocytes and antigen of \(E.\) histolytica are incubated for 3-4 days, and the degree of blast formation can be measured quantitatively by the uptake of thymidine by blast cells. Savanat et al.
(1973a) studied blast formation in 24 amoebic patients (23 cases of hepatic amoebiasis and 1 case of amoebic cervicitis) as shown by $^{3}H$-thymidine up-take and found positive test in 23 cases (95%). Out of 25 non-amoebic controls (15 healthy and 10 other diseases), the test was negative in 24 cases (one case which gave positive test was suffering from malaria). Ortiz-Ortiz et al. (1974) also found blast formation after antigenic stimulation as a sensitive test for the diagnosis of invasive amoebiasis. Harris and Gray (1976) also found positive blast formation in cases of invasive amoebiasis as shown by thymidine up-take. Out of 23 patients tested, 21 gave positive reaction.

V. PREPARATION OF *E. HISTOLYTICA* ANTIGEN

Following the development of techniques for the successful mass cultivation of axenic *E. histolytica* strains (Diamond 1968), attempts were made to prepare and standardize the amoebic antigens for the purpose of serodiagnosis. Thompson et al. (1968) prepared amoebic antigens from the two strains of axenically growing *E. histolytica*. The two antigens did not show any significant difference in their serological reactivity in IHA, Gw and CF tests and in the opinion of those authors (Thompson et al. 1968) the axenic antigens from either strain used, or from the two strains pooled could be of broad usefulness in the detection of amoebic antibodies. Standardized as a dry powder, this antigen upon reconstitution contained about 1.8 mg/10 x 10$^6$ amoebae. The lyophilized antigen could be stored at room temperature for 4 to 5 weeks without any deterioration; when stored at -20°C, its performance was, after six months, as well as of freshly prepared antigen. Exposure to 37°C and 56°C-60°C for periods upto 8 hours did not cause loss of potency of the antigen in the lyophilized
state. However, the reconstituted antigen showed a pronounced loss in serologic reactivity after storage at 4°C for 73-84 days. Thompson et al. (1968), thus, recommended that the amoebic antigens should routinely be stored in the lyophilized state in the cold.

Lunde and Diamond (1969) analysed antigens prepared from several strains of axenically growing B. histolytica and B. histolytica-like amoebae. They reported a significant variation in the protein contents of the different strains of amoebae. In terms of mg protein/10⁶ amoebae, the value for strain HB 301: NIH was 0.8-1.2 mg; for HB-9, 1.8 mg; for F-22 1.7 mg and for 200: NIH 0.9-1.2 mg. The corresponding mg N/10⁶ amoebae for these strains were 0.196 mg - 0.174 mg, 0.105 mg, 0.111 mg, and 0.14 mg, 0.23 mg respectively. The Laredo strain contained, on average, only 0.15 mg protein per 1 x 10⁶ amoebae (0.029 mg N/10⁶ amoebae). Antigen prepared from Laredo strain showed a markedly low serologic reactivity in IHA test. A comparison of the immunoelectrophoretic patterns of the antigens prepared from different strains also indicated that the Laredo strain contained fewer antigenic components than those of the typical strains of B. histolytica growing at 37°C. It is, therefore, necessary to employ typical strains of B. histolytica for the preparation of antigen for immuno-diagnosis. Improved method has been only recently developed for axenic cultivation of B. histolytica (Singh et al., 1973, 1974), which will be useful for obtaining greater yield of axenic amoebae. Lutta (1976) has described methods for initiating axenic cultures from small inocula of amoebae and 800 to 1200 fold growth of axenic amoebae has been achieved. These methods are now being used for the large scale cultivation of axenic B. histolytica in this Institute, for the preparation of standard lyophilized B. histolytica antigen.
VI. ANTIAMOEBIC ACTIVITY OF THE SPECIFIC ANTISERA.

Cole and Kent (1953) were the first to study the influence of experimentally raised specific antisera on the trophozoites of *E. histolytica*. They raised anti-amoebic sera in rabbits inoculated repeatedly with suspensions of a strain of *E. histolytica* grown in association with *Trypanosoma cruzi* and observed amoeba-immobilizing activity of the antisera. They found that the number of immobilized amoebae increased directly with the concentration of serum but varied considerably with time, maximal immobilizing effect being observed after 20-30 minutes, with subsequent gradual regain of activity by the amoebae. Experimental antisera was also demonstrated to markedly reduce the phagocytic index of *E. histolytica* (Shaffer and Balsam, 1954). Immune sera from patients with current or past histories of amoebiasis was shown by Nakamura (1959) to exert growth inhibitory effect on *E. histolytica*. Such inhibition of growth varied from 19.0 to 59.8 per cent. When amoebae maintained temporarily free of bacteria in a Kinger solution-coagulated egg-horse serum medium (Nakamura, 1955) fortified with the growth factors, ribose-5-phosphate, adenosinetriphosphate and sodium thio-glycollate, were used for evaluating the action of antiamoebic antisera on the growth of amoebae, significant inhibition of growth of amoebae (67.6 per cent) was obtained with rabbit antisera raised by intravenous immunization. When the rabbit was immunized by subcutaneous route, the inhibition of growth of amoebae, obtained with the antisera was 31.1 per cent. In view of the inhibitory action of antisera on *E. histolytica*, it was believed that the antisera contained a component responsible for the inhibition of growth of amoebae in vitro. Nakamura (1959) concluded that it was not clear from his study if the specific antibody actually possessed an amoebicidal or
amoebostatic property or merely interfered with the metabolic and growth processes of the amoebae. The inhibitory effect, however, was not complete since amoebae were able to overcome slowly the inhibitory action of antibodies.

In recent past, several workers have studied the effect of immune serum or the gamma globulin fraction isolated from immune serum on the trophozoites of \textit{E. histolytica}. Thus, Torre \textit{et al.}, (1973) demonstrated inhibitory effect of human immune serum as also of the gamma globulin isolated from the same serum on the growth of cultures of \textit{E. histolytica}. Chevez \textit{et al.}, (1973) reported that 90 per cent of the trophozoites were lysed within one hour of their contact with the immune serum. Sepulveda \textit{et al.} (1973) found that unlike gamma globulin isolated from the immune serum, the commercial gamma globulin did not exert cytolytic effect on the trophozoites of \textit{E. histolytica}. Ahmad and Eisati (1976) studied the interaction between humoral antibodies and axenic \textit{E. histolytica} and reported that in order to obtain a complete inhibition of growth of amoebae, about 30 per cent concentration of the immune serum worked effectively. Recent studies in our laboratory have demonstrated that when high titre antiserum was used there was a complete lysis of amoebae at 10 per cent concentration of the antiserum (Sharma \textit{et al.}, 1978). The inhibitory action of the antiserum was found on both homologous and heterologous strains of amoebae.

In the present study, following serodiagnostic techniques have been evaluated:

(i) Indirect haemagglutination test for coproantibodies,
(ii) Indirect haemagglutination test for serum antibodies.
(iii) Gel-diffusion precipitin test,
(iv) Immuneelectrophoresis test,
(v) Counter immunoelectrophoresis, and
(vi) Indirect fluorescent antibody test.

In addition, influence of specific antibody on the trophozoites of *E. histolytica* in vitro, experimental induction of delayed hypersensitivity to *E. histolytica* were also studied.