PHASE I

MATERIALS AND METHODS
Phase I of the study comprised of:

a) analysis of the crude (CAE) antigens of axenic *E. histolytica* (KCG:0986:11),
b) recognition of CAE antigens by sera from amoebic patients and control subjects,
c) detection of anti-amoebic antibodies,
d) detection of *E. histolytica* specific PEG precipitated circulating immune complexes and molecular specificity of amoebic antigen in acid-treated (dissociated) circulating immune complexes (CICs) from the serum of amoebic patients and
e) affinity purification of 58 KD amoebic polypeptide from crude amoebic extract (CAE) by rabbit anti 58 KD IgG coupled to CN-Br activated sepharose-4B.

1. **ANTIGENIC PROFILE OF CRUDE AMOEIC EXTRACT (CAE)**

1.1 Parasite and preparation of antigen: An Indian axenic isolate of *E. histolytica* (KCG:0986:11), obtained from the Kothari Centre of Gastroenterology, Calcutta was maintained axenically in Tryptone-Yeast Extract-Iron (TYI-S-33) medium (Diamond, 1978). For this purpose, the trophozoites from 48-72 hours old culture were harvested by chilling and centrifugation at 200xg for 10 minutes. These were washed thrice in phosphate buffered saline - pH 7.2 (PBS-7.2) and subjected to ultrasonic disintegration at 23 kilocycles (14 microns), with ten bursts of 15 seconds each in MSE (Measuring and Scientific Equipments, UK) ultrasonicator in ice (Vinayak et al., 1980b). The sonicated material was centrifuged at 250xg for 10 minutes at 4°C and the clear supernatant was labeled as "crude amoebic extract" (CAE). The protein content of CAE was determined by the method of Lowry (1951).

1.2 Anti CAE antibodies: Rabbits were immunized, using CAE as antigen, by giving three sensitizing doses (each of 1.5 mg of CAE proteins) at weekly intervals. The first dose of CAE was emulsified in Freund’s complete adjuvant (FCA) and was given
intra-muscularly, while subsequent two doses were emulsified in Freund's incomplete adjuvant and were given subcutaneously. One week after the last dose, the blood samples were obtained from the ear of the rabbits. The pooled anti CAE antisera was subjected to diethylaminoethyl (DEAE) cellulose chromatography to obtain the IgG fraction (Hudson and Hay, 1980).

1.3 Clinical serum samples: A total of 23 serum samples were collected from patients with confirmed amoebic liver abscess (ALA), patients with idiopathic ulcerative colitis and normal healthy subjects. A limited number of serum samples were analysed to assess the recognition of amoebic polypeptide in western blot of CAE proteins. The following criteria for diagnosis of these categories were used:

1.3.1: Patients with confirmed ALA (n = 15): These subjects had clinically enlarged, tender liver with palpable abscess and associated toxaemia, abscess demonstrable on ultrasound and aspiration of abscess yielded anchovy sauce pus which was either sterile on bacteriological examination or revealed *E. histolytica* trophozoites. These patients responded well to the specific anti-amoebic therapy, metronidazole or emetine.

1.3.2: Patients with idiopathic ulcerative colitis (n = 3): These were confirmed by sigmoidoscopy and histological changes in biopsies to have idiopathic ulcerative colitis.

1.3.3: Apparently healthy subjects (n = 5): These were aged between 20-30 years and residents of India since birth. They had no ostensible symptoms and their physical examination revealed them to be healthy. No *E. histolytica* cysts or trophozoites were found on repeated stool examination.

1.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of crude amoebic extract (CAE): SDS-PAGE was performed as described by Laemmli (1970). Briefly, the CAE protein (80 μg/lane) was subjected to SDS-PAGE in 5% stacking and
10% separating polyacrylamide gels under reducing and denaturing conditions in an electrophoretic cell (Bio-Rad Laboratories, USA) at 25 mA current for 4 hours. The resolved proteins were stained by 0.25% Coomassie blue. Molecular weight markers used were 29, 45 and 66 KD (Sigma Chemical Co. USA).

1.5 Western immunoblots of CAE: The SDS-PAGE resolved CAE proteins on polyacrylamide gels were transferred on to 0.2 μm pore diameter nitrocellulose (NCP) sheets in a transblot cell (Bio-Rad Laboratories, USA) at 200 mA current for 3 hours (Towbin et al., 1979). The sheets were cut into strips and in order to assess the transfer efficiency one of them was stained with 0.1% amido black in 45% methanol and 10% acetic acid for 5 minutes and destained in 80% methanol and 5% acetic acid. The rest of the strips containing the transferred antigens were further blocked with 3% bovine serum albumin (BSA) for 1 hour at 37°C or overnight at 4°C. Such strips were washed thrice with Tris-saline, pH 7.4 with 0.05% Tween-20 (High Media Chemicals Pvt. Ltd., India). The strips were then probed with 1:50 diluted rabbit anti CAE antibodies or sera from patients with confirmed amoebic liver abscess, idiopathic ulcerative colitis or normal healthy subjects. The NCP strips treated with anti CAE antibodies (raised in rabbits) were incubated with 1:200 diluted anti-rabbit -HRP conjugate (Lupin, India) and those treated with human serum were incubated with 1:200 diluted anti-human IgG -HRP conjugate (Lupin, India). The strips were again washed thrice with Tris-saline and reaction was developed with 3 mg of 4-chloro-1-napthol (Fluka, Switzerland) in 6 ml PBS, pH 7.2 and 5 μl of H₂O₂ as substrate. The colour reaction was stopped after 15-20 minutes with distilled water. The polypeptides that were recognised by the serum samples from ALA patients and not by the sera from control subjects were considered as the amoebic polypeptides. Their molecular weights were estimated by employing a logarithmic plot of migration of standards.
2. DETECTION OF AMOEBIC ANTIGEN IN CIRCULATION

2.1 Precipitation of circulating immune complexes (CICs) from the sera of patients using polyethylene glycol (PEG): The CICs were prepared by precipitation of the serum samples of patients with 2.5% polyethylene glycol (PEG) (molecular weight - 6000, E. Merk) (Hudson and Hay, 1980). Briefly, 150 µl aliquot of the serum was mixed with 60 µl of working solution of PEG [6 ml of 20% PEG + 3 ml of 0.2M EDTA + 1 ml of veronal buffered saline (VBS)] pH 7.6 and incubated overnight at 4°C. The samples were centrifuged at 2,000xg for 20 minutes at 4°C. The precipitates were resuspended in 2 ml of 2% PEG in 0.01M EDTA in VBS, pH 7.6 and again centrifuged at 2000xg for 20 minutes. Finally the precipitates were dissolved in 150 µl of VBS, pH 7.6 by incubation at 37°C for 1 hour.

2.1.2 Dissociation of PEG precipitated CIC: An aliquot of 50 µl of the PEG precipitated complexes after solubilization in VBS, pH 7.6 was mixed with 150 µl of glycine-HCl buffer of pH 2.8 and kept for 1 hour at room temperature (Craig and Nelson, 1984). The glycine-HCl buffer dissociated immune complexes were labeled as the ‘dissociated circulating immune complexes’ (dissociated - CIC).

3. DEVELOPMENT OF ASSAY SYSTEMS

3.1 Clinical specimens: A total of 62 serum samples were collected from cases of amoebic liver abscess, non amoebic hepatic disorders, clinically suspected cases of amoebiasis and apparently healthy subjects.

3.1.1 Confirmed amoebic liver abscess (n = 16): The criteria for diagnosis of these patients has already been mentioned under section 1.3.1.

3.1.2 Non amoebic hepatic disorders (n = 15): These included 3 patients with hydatid cyst disease confirmed by laparotomy, one of non specific hepatomegaly, 4 of viral hepatitis
(confirmed by demonstration of hepatitis B surface antigen), 6 with metastatic adenocarcinoma of liver (confirmed by fine needle aspiration cytology) and one with pyogenic liver abscess (confirmed by bacteriological examination of aspirated pus).

3.1.3 Clinically suspected cases of amoebiasis (n=11): All of these had clinically enlarged liver with palpable abscess and associated systemic toxemia, aspiration of pus could not be attempted in 7 patients and in rest, bacteriological examination of aspirated pus could not be carried out. However all showed moderate to good recovery on anti-amoebic therapy.

3.1.4 Apparently healthy subjects (n=20): The criteria for selection of these subjects has already been mentioned under section 1.3.3.

3.2 Antibody based ELISA: Micro ELISA for determination of anti-amoebic antibodies in the sera of amoebic patients: The micro-ELISA was performed basically as described by Voller et al., (1976). Briefly, the wells of polyvinyl microtitre plates (Costar Corporation, USA) were coated with 1 µg of CAE/well in 0.05M carbonate bicarbonate buffer (BCB), pH 9.6 (sodium carbonate 1.59 g/L, sodium hydrogen carbonate 2.93 g/L in distilled water) at 4°C overnight. Wells were washed with 0.15M phosphate buffered saline pH 7.2 (PBS-7.2) containing 0.05% Tween-20 (PBS-T) and were blocked with 150 µl of 1% BSA for 1 hour at 37°C. Further 100 µl of serum sample of patient, diluted 1:200 in 0.1% BSA in PBS 7.2 was added to the wells and incubated for 2 hours at 37°C. The plates were again washed with PBS-T followed by an incubation with 1:1000 diluted anti-rabbit - HRP conjugate (Lupin, India) for 1 hour at 37°C. The colour reaction was developed by the addition of 100 µl of substrate (5 mg of orthophenyl diamine (OPD) (Sigma Chemical Co., USA) in 10 ml of citrate phosphate buffer, pH 5.0 + 5 µl HzO2). The reaction was terminated by the addition of 50 µl of 6N sulphuric acid. The optical density (OD) was measured at 492 nm in an ELISA reader (Eurogenetics, Belgium).
3.3 Antigen based assay

3.3.1 ELISA for the determination of amoebic antigen specific circulating immune complexes: Amoebic antigen specific CICs were assayed in the serum of patients using micro ELISA as described by Voller et al. (1976). Briefly, the wells of ELISA plates were coated with 1:200 (3 μg/100 μl) rabbit anti CAE IgG antibodies diluted in carbonate bicarbonate buffer. Such wells were blocked with 1% BSA and after washing with PBS-T, these were incubated with 1:320 diluted PEG precipitated CICs from the serum of patient or controls for 2 hours at 37°C. Following another washing with PBS-T, such wells were incubated with 1:1000 diluted anti-human HRP conjugate (Lupin, India) for 1 hour at 37°C. The reaction was developed using OPD in citrate phosphate buffer + H₂O₂ as substrate and terminated with 6N H₂SO₄ after 5 minutes. The OD was assayed in an ELISA reader at 492 nm.

3.3.2 ELISA for the determination of amoebic antigen in dissociated CICs: The optimum dilutions of coating antigen (acid treated dissociated CICs) and detecting rabbit anti CAE-IgG antibody were determined by checker board titration. Briefly, the wells of ELISA plates were coated with 100 μl of optimally diluted acid-treated dissociated CICs (1:1600) in 0.05M carbonate buffer pH 9.6. After overnight incubation at 4°C and washing with PBS-T, the non-specific binding sites were blocked with 1% BSA. This was followed by the addition of 100 μl of rabbit anti CAE-IgG (1:2000) and incubation for 3 hours at 37°C. The wells were subsequently incubated with 100 μl of 1:1000 dilution of HRP conjugated anti-rabbit IgG (Lupin, India) and the reaction was developed with OPD + H₂O₂ as substrate. The reaction was stopped after 5 minutes and OD read at 492 nm in an ELISA reader.

3.4 SDS-PAGE analysis and Western blot of PEG precipitated CICs: The SDS-PAGE was carried out basically as described by Laemmli (1970). Briefly 80 μg of CAE and 70 μl
of PEG precipitated CIC/dissociated CIC were subjected to electrophoresis and the gels were stained with 0.25% Coomassie blue.

The resolved proteins contained in CIC/dissociated CIC on SDS-PAGE gels were transferred to nitrocellulose paper (NCP) as described by Towbin et al. (1979). After blocking the non-specific sites with 3% BSA, the nitrocellulose strips were incubated with 1:500 diluted anti-amoebic IgG and treated with HRP conjugated anti-rabbit IgG. The enzymatic reaction was developed with 4-chloro-1-napthol as substrate.

3.5 Monospecific antibodies to 58 KD amoebic antigen entrapped in CIC: 2 mg of CAE protein was run on SDS-PAGE slab gel. The polypeptide band corresponding to 58 KD molecular mass position (as determined by logarithmic plot of migration of standards) along the gel was cut and the antigen was eluted. Several runs of CAE were made on SDS-PAGE to obtain the required amount of 58 KD antigen. The eluted antigen was employed to raise anti 58 KD antibodies in rabbits by the procedure as already described (section 1.2). Each immunizing dose of 58 KD antigen contained 70 μg protein. Ten days after the last dose, the animals werebled to death and the serum was separated. The IgG fraction of the monospecific antisera was prepared by DEAE cellulose chromatography (Hudson and Hay, 1980).

3.6 Purification of 58 KD antigen

3.6.1 IgG purification by DEAE cellulose chromatography: The DEAE cellulose chromatography was performed by using the protocol of Hudson and Hay (1980). Approximately 50 g DEAE cellulose (DE-52, Whatmann, England) was mixed with 250 ml of 0.01M phosphate buffer saline (pH 8.0) and the mixture was titrated back to pH 8.0 by the addition of 1.0M HCl. After 30 minutes, the supernatant was discarded and the cellulose was washed with 0.01M phosphate buffer pH 8.0. Wet cellulose (75 g) was
mixed with 15 ml of anti-CAE antibodies or anti 58 KD antisera diluted in distilled water (1:3) and stirred thoroughly every 10 minutes for 1 hour at 4°C. Slurry was poured into a Buchner funnel, the supernatant containing IgG was sucked and the cellulose was washed thrice with 15 ml of 0.01M phosphate buffer (pH 8.0). The combined effluent containing IgG was concentrated by lyophilization. The total protein content of the concentrated material was estimated (Lowry et al., 1951). The IgG fractions of anti CAE antibodies and that of anti 58 KD antibodies was labeled as ‘anti CAE IgG’ and ‘anti 58 KD IgG’ respectively.

3.6.2 Affinity purification of 58 KD antigen: The affinity column chromatography was performed by following the method of Hudson and Hay (1980), of course, with a few modifications. In brief, 2 g of cyanogen bromide (CN-Br) activated sepharose 4B on a sintered glass funnel was washed with 100 ml of 0.1M borate buffered saline, pH 8.3. Sixty mg of rabbit anti 58 KD IgG was added to the activated swollen gel at 5-10 mg/ml in 0.1M borate buffered saline, pH 8.3 and left stirring overnight at 4°C. The beads were washed on a sintered glass funnel with 200 ml of 0.15M PBS, pH 7.2. For blocking the excess binding sites, the beads were incubated overnight at 4°C with 40 ml of 0.2M glycine in 0.1M borate buffered saline, pH 8.3. They were then treated twice with 0.1M sodium acetate buffer pH 4.0 and 0.1M borate buffered saline pH 8.3 alternatively. CAE antigen (1 mg/ml) was incubated with antibody coated beads for 2.5 hours at 4°C in a beaker with constant shaking. Beads transferred into a 5-10 ml column containing a nylon wool plug were washed with 200 ml of 0.15M phosphate buffered saline pH 7.2. Specifically bound antigens were eluted (1.5 ml) fractions with 0.1M glycine-HCl buffer pH 2.5 into test tubes containing 25 μl of 3.5M Tris (Sisco Research Lab, India) so as to neutralize the pH of eluant. Further elutions were done with 0.1M glycine-HCl buffer, pH 2.5. Optical density of each fraction was observed at 280 nm. Fractions containing the protein were pooled, dialysed against distilled water and concentrated by lyophilization.
The purity, immunoreactivity and molecular specificity of the affinity purified antigen (APA) was assessed by SDS-PAGE, ELISA and western blotting.

3.7 Assessment of purity and immunoreactivity of Affinity Purified Antigen

3.7.1 SDS-PAGE for checking the purity of Affinity Purified Antigen: 80 μg of CAE and 20 μg of affinity purified antigen was run on SDS-PAGE under reducing and denaturing conditions in an electrophoretic cell (Bio-Rad Laboratories, USA) at 25 mA current for 4 hours. The gels were stained with 0.25% Coomassie blue.

3.7.2 ELISA and western blot for checking the immunoreactivity and specificity of APA

3.7.2.1 ELISA: Micro-ELISA was performed essentially as described by Voller et al. (1976). APA (1 μg) or CAE (2 μg) per well diluted in BCB was used to coat the wells of an ELISA plate. After blocking with 1% BSA, 100 μl of 1:250, 1:500, 1:1000 or 1:2000 diluted anti 58 KD IgG and anti 58 KD antibody (whole) was incubated for 2 hours at 37°C. This was followed by incubation with 1:1000 diluted anti-rabbit HRP conjugate (Lupin, India) for 1 hour at 37°C. The reaction was developed using OPD + H2O2 as substrate and was stopped using 6N H2SO4. The OD was read at 492 nm in an ELISA reader. The antigen coated well incubated with anti CAE antibodies (raised in rabbits) was employed as positive control and the wells incubated with normal rabbit serum (NRS) or those containing no antigen were used as negative controls.

3.7.2.2 Western immuno blot: Antigens separated on SDS-PAGE were transferred onto the NCP (Micro Devices Ltd., India). After blocking the non-specific sites, the NCP strips were incubated with 1:50 diluted anti CAE-IgG antibodies or with anti 58 KD IgG antibody (monospecific) followed by incubation with 1:200 diluted anti-rabbit IgG-HRP conjugate and the reaction was developed using 4-chloro-1-napthol. Strips incubated with NRS were used as negative controls.
PHASE I
1. RESOLUTED PROTEINS OF E. HISTOLYTICA (KCG:0986:11)

The resolution of crude amoebic extract (CAE) proteins on SDS-PAGE under reducing and denaturing conditions revealed 30-35 discrete polypeptides in the gel with molecular weight ranging from 20-200 KD (Fig. 1, Lane B).

2. RECOGNITION OF MAJOR AMOEBC POLYPEPTIDES BY THE SERUM SAMPLES FROM AMOEBC PATIENTS

The probing of amoebic polypeptides transferred on to NCP with clinical serum from 15 confirmed cases of ALA showed recognition of at least 7 major polypeptides by all of them. The recognized proteins had molecular mass of 29, 38, 45, 58, 67, 89 and 95 KD. (Fig. 2, Lanes B-D). The serum samples from 3 patients with idiopathic ulcerative colitis (Fig. 2, Lane E) and 5 normal healthy subjects (Fig. 2, Lane F) failed to recognize any of the amoebic polypeptides.

3. MICRO ELISA ASSAY FOR DETECTION OF ANTIBODIES TO E. HISTOLYTICA ANTIGEN (CAE) IN SERUM SAMPLES FROM CLINICAL CASES OF AMOEbiasis

An assessment of cut off OD values in 1:200 diluted serum samples from 8 confirmed cases, each of ALA and healthy subjects, revealed that mean plus 2SD (= 0.584) OD value of sera from healthy subjects differentiated confirmed cases of ALA from healthy subjects (Table I). Thus, any serum sample from clinical cases with OD value ≥0.584 was considered to contain anti-amoebic antibodies. All the 16 cases (100%) of ALA had anti-amoebic antibodies, while they were not detected in the serum samples from 20 healthy or 15 cases of non-amoebic hepatic disorders. However, of the 11 clinically suspected cases of ALA, 3 (27.2%) were positive for anti-amoebic antibodies (Tabel II). The 16 confirmed cases of ALA had OD values ranging from 0.611 to 1.818 (mean OD = 1.009), while the values in cases of non amoebic hepatic disorders ranged
between 0.101 and 0.540 (mean OD = 0.234) and in the healthy subjects the range was from 0.119 to 0.550 (mean OD = 0.130) (Fig. 3).

4. MICRO-ELISA ASSAY FOR DETECTION OF *E. HISTOLYTICA* SPECIFIC CIRCULATING IMMUNE COMPLEXES IN THE SERA FROM AMOEBC PATIENTS

The CICs were precipitated with PEG from the sera of ALA cases and micro ELISA was performed for detecting the amoebic antigen in CIC. Mean plus 2SD (OD = 0.270) of the OD value of 8 healthy subjects was taken as the cut off as it could differentiate the confirmed ALA cases from healthy subjects (Table III). Thus PEG precipitated CICs from clinical cases with OD value ≥0.270 was considered to contain amoebic antigen. The PEG precipitated CIC samples of 14 (87.5%) out of 16 ALA cases had amoebic antigen in CIC, while the detectable level of this antigen was not seen in a 20 healthy subjects as well as 15 cases with other hepatic disorders. One (9.0%) of the serum samples from 11 clinically suspected ALA cases had detectable level of antigen specific CIC (Table IV). The OD values in the confirmed cases of ALA ranged between 0.223 and 0.833 (mean OD = 0.464), while in the one with non-amoebic hepatic disorders, these ranged from 0.006 to 0.230 (mean OD = 0.060). The range in healthy subjects was between 0.009 and 0.222 (mean OD = 0.079) (Fig. 4).

5. MICRO-ELISA ASSAY FOR DETECTION OF AMOEBC ANTIGEN IN DISSOCIATED CIC PRECIPITATED FROM THE SERA FROM AMOEBC PATIENTS

Using the same criteria of positivity, an OD value of mean plus 2SD ( = 0.435) of the specific OD value of acid treated CIC from healthy subjects was considered as the cut off OD value *i.e.*, dissociated CIC with OD values ≥0.435 was considered to contain amoebic antigen (Table V). Following dissociation of CIC with glycine-HCl, the amoebic
antigen was detected in all the 16 (100%) confirmed and one (9.0%) of the clinically suspected cases of ALA. None of the sera from healthy subjects or from patients with other liver disorders gave positive signals for the amoebic antigen (Table VI). All the confirmed cases of ALA had OD values in the range of 0.435 to 1.215 (mean OD = 0.870), while the values in cases of non-amoebic hepatic disorders were between 0.122 to 0.390 (mean OD = 0.270). The OD values in the healthy subjects ranged from 0.090 to 0.424 (mean OD = 0.235) (Fig. 5).

6. ASSESSMENT OF AMOEBIC ANTIGEN(S) ENTRAPPED IN CIC

6.1 SDS-PAGE analysis of PEG precipitated CIC: Glycine-HCl treated polyethylene glycol precipitated circulating immune complexes from the sera of ALA cases when subjected to SDS-PAGE were resolved into six polypeptides with molecular masses of 14, 29, 45, 58, 67 and 94 KD respectively (Fig. 6a, Lane B). These were always seen in the PEG precipitated CIC from the sera of 7 different ALA patients (Fig. 6b, Lane 1-6, 8 and 9), whereas very faint bands were seen in the resoluted proteins of one of the clinically suspected cases of ALA (Fig. 6b, Lane 7).

6.2 Recognition of amoebic specific polypeptides entrapped in CIC from cases of ALA: The western immunoblot of CIC polypeptides transferred on to NCP sheets and probed with anti CAE antibodies revealed three major amoeba specific polypeptides. The molecular masses of these amoebic antigens in CIC were 29, 58 and 67 KD. (Fig. 7, Lane B). Of these, the 58 KD appeared to be a major amoebic polypeptide entrapped in CIC.

7. MONO-SPECIFIC ANTIBODIES TO 58 KD ANTIGEN

Slab gels of CAE were run and the 58 KD band was cut from the gel and eluted in PBS-7.2. The protein content of the eluted antigen was measured and the eluted antigen was used to raise monospecific antibodies in rabbits. IgG fraction of the monospecific antisera was separated by DEAE- cellulose chromatography.
8. AFFINITY PURIFICATION OF 58 KD ANTIGEN FROM CAE BY AFFINITY COLUMN

The 58 KD molecule was eluted from the affinity column coupled with anti 58 KD IgG antibody under acidic conditions with glycine-HCl buffer pH 2.5 (Fig. 8). The highest concentration of eluted protein was found in fraction numbers 5 to 10. The antigen obtained after several identical elutions was concentrated by lyophilization.

9. ASSESSMENT OF PURITY AND IMMUNOREACTIVITY OF AFFINITY PURIFIED ANTIGEN

9.1 Purity of affinity purified antigen: The purity of affinity purified antigen upon resolution in SDS-PAGE was indicated by a single discrete band at 58 KD molecular mass position (Fig. 9, Lane B).

9.2 Immunoreactivity and specificity of affinity purified antigen to anti 58 KD antibodies: The immunoreactivity and molecular specificity of affinity eluted 58 KD antigen of *E. histolytica* to anti-58 KD antibodies was assessed in ELISA and western blot.

Affinity purified antigen had a high immunoreactivity to the IgG fraction of anti 58 KD antibodies (OD > 1.5) at 1:2000 dilution of anti 58 KD IgG antibodies employing 1 μg/ul of affinity purified antigen in ELISA. Crude amoebic extract gave a relatively poor reaction with anti 58 KD IgG (OD) (Fig. 10).

The immunoblots of affinity purified 58 KD antigen upon treatment with anti CAE antibodies revealed a single band at 58 KD molecular mass (Fig. 11, Lane B). Normal rabbit serum failed to recognize the APA (Fig. 11, Lane D).
Fig. 1: SDS-PAGE profile of *Entamoeba histolytica* (KCG: 0986:11) proteins. Lane A: molecular weight markers; Lane B: Crude amoebic extract (CAE).
Fig. 2: Western immunoblot of crude amoebic extract (CAE) of *E. histolytica* trophozoites probed with anti-CAE antibodies (Lane A); sera from patients with amoebic liver abscess (ALA) (Lane B-D); serum from patient of idiopathic ulcerative colitis (Lane E); pooled sera from normal healthy subjects (Lane F).
Table I: Determination of cut off OD for detection of anti CAE antibodies in the serum samples from patients

<table>
<thead>
<tr>
<th>Clinical subjects (no. of cases)</th>
<th>OD* of the patient sera</th>
<th>Actual mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean + 1SD# ≥0.437</td>
<td></td>
</tr>
<tr>
<td>Confirmed ALA cases (8)</td>
<td>8</td>
<td>0.796</td>
</tr>
<tr>
<td>Healthy subjects (8)</td>
<td>1</td>
<td>0.290</td>
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<tr>
<td></td>
<td>Mean + 2SD# ≥0.584</td>
<td></td>
</tr>
<tr>
<td>Confirmed ALA cases (8)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Healthy subjects (8)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Mean + 3SD# ≥0.731</td>
<td></td>
</tr>
<tr>
<td>Confirmed ALA cases (8)</td>
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<td></td>
</tr>
<tr>
<td>Healthy subjects (8)</td>
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<td></td>
</tr>
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# Mean OD of sera of 8 healthy subjects +1,2 or 3SD of the same.
* at 492 nm.

Table II: Detection of anti CAE antibodies in the serum samples from amoebic patients

<table>
<thead>
<tr>
<th>Disease (No. of cases)</th>
<th>Anti CAE antibodies in patient sera detected by micro ELISA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. +ve#</td>
</tr>
<tr>
<td>Amoebic liver abscess (16)</td>
<td>16(100)</td>
</tr>
<tr>
<td>Non amoebic hepatic disorders (15)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Suspected ALA cases (11)</td>
<td>3(27.2)</td>
</tr>
<tr>
<td>Healthy controls (20)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

# OD ≥ 0.584
* at 492 nm
Fig. 3: Levels of antiamoebic antibodies by ELISA in serum from confirmed ALA cases (A), non-amoebic hepatic disorders (B), suspected ALA cases (C) and healthy subjects (D). I = Mean ± SD; dotted line indicates cut off OD value (≥ 0.584).
Table III: Determination of cut off OD for detection of amoebic antigen in circulating immune complexes using anti CAE antibodies

<table>
<thead>
<tr>
<th>Clinical subjects (no. of cases)</th>
<th>OD* of the CIC of patient sera</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean + 1SD# ≥0.162</td>
<td>Mean + 2SD# ≥0.270</td>
<td>Mean + 3SD# ≥0.310</td>
<td>Actual mean</td>
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</tr>
<tr>
<td>Confirmed ALA cases (8)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>0.467</td>
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</tr>
<tr>
<td>Healthy subjects (8)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.088</td>
<td></td>
</tr>
</tbody>
</table>

# Mean OD of sera of 8 healthy subjects +1, 2 or 3SD of the same.

* at 492 nm.

Table IV: Detection of amoebic antigen in CIC from serum samples of ALA patients by polyethylene glycol using anti CAE antibodies

<table>
<thead>
<tr>
<th>Disease (No. of cases)</th>
<th>Antigen in CIC of ALA patients detected by micro ELISA</th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. +ve #</td>
<td>Range of OD</td>
<td>Mean</td>
<td>SD</td>
<td></td>
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<tr>
<td>Amoebic liver abscess (16)</td>
<td>14(87.5)</td>
<td>0.223–0.833</td>
<td>0.464</td>
<td>0.192</td>
<td></td>
</tr>
<tr>
<td>Non amoebic hepatic disorders (15)</td>
<td>0(0)</td>
<td>0.006–0.203</td>
<td>0.075</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>Suspected ALA cases (11)</td>
<td>1(9.0)</td>
<td>0.010–0.420</td>
<td>0.139</td>
<td>0.088</td>
<td></td>
</tr>
<tr>
<td>Healthy controls (20)</td>
<td>0(0)</td>
<td>0.009–0.222</td>
<td>0.092</td>
<td>0.079</td>
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</tbody>
</table>

# OD ≥0.270

* at 492 nm
Fig 4: Levels of amoeba specific circulating immune complexes (CICs) by ELISA in the sera from confirmed ALA cases (A), non-amoebic hepatic disorders (B), suspected ALA cases (C) and healthy subjects (D). I = Mean± SD; dotted line indicates cut off OD value (≥ 0.270).
### Table V: Determination of cut off OD value for detection of amoebic antigen in dissociated CIC using anti CAE antibodies

<table>
<thead>
<tr>
<th>Clinical subjects (no. of cases)</th>
<th>OD* of the dissociated CIC from patient sera</th>
<th>Mean +1SD# 0.335</th>
<th>Mean +2SD# 0.435</th>
<th>Mean +3SD# 0.535</th>
<th>Actual mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmed ALA cases (8)</td>
<td></td>
<td>0.435</td>
<td>0.535</td>
<td>0.635</td>
<td>0.740</td>
</tr>
<tr>
<td>Healthy subjects (8)</td>
<td></td>
<td>0.235</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# Mean OD of sera of 8 healthy subjects +1, 2 or 3SD of the same.

* at 492 nm.

### Table VI: Detection of amoebic antigen in dissociated CIC precipitated from serum samples of ALA patients by PEG using anti CAE antibodies

<table>
<thead>
<tr>
<th>Disease (No. of cases)</th>
<th>Antigen in dissociated CIC of patients detected by micro ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. +ve#</td>
</tr>
<tr>
<td>Amoebic liver abscess (16)</td>
<td>16(100)</td>
</tr>
<tr>
<td>Non amoebic hepatic disorders (15)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Suspected ALA cases (11)</td>
<td>1(9.0)</td>
</tr>
<tr>
<td>Healthy controls (20)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

# OD ≥0.435

* at 492 nm
Fig. 5: Levels of amoebic antigen in dissociated CICs by ELISA in the sera from confirmed ALA cases (A), non-amoebic hepatic disorders (B), suspected ALA cases (C) and healthy subjects (D). I = Mean ± SD; dotted line indicates cut off OD value (≥ 0.435).
Fig. 6a: SDS-PAGE profile of PEG precipitated circulating immune complexes from amoebic liver abscess patient (Lane B); crude amoebic extract (Lane C); molecular weight markers (Lane A).

Fig. 6b: SDS-PAGE profile of PEG precipitated CIC from different sera from ALA patients (Lane 1-6, 8,9); PEG precipitated CIC from serum of clinically suspected case of ALA (Lane 7).
Fig. 7: Western immunoblot of PEG precipitated CIC from serum of ALA patient probed with rabbit anti-CAE IgG antibodies (Lane B); molecular weight markers stained with amido-black (Lane A).
Fig. 8: Elution profile of 58 KD antigen of E. histolytica on an immunoabsorbant column.
Fig. 9: SDS-PAGE profile of crude amoebic extract (Lane A); affinity purified antigen (Lane B).
Fig. 10: ELISA reactivity of anti-rabbit monospecific IgG antibodies to affinity purified antigen (APA) or to crude amoebic extract (CAE); No Ag : without antigen; No Ab : without antibody; HIS : hyper immune serum; Whole : rabbit anti 58 KD antibodies; IgG : rabbit anti 58 KD IgG antibody.
Fig. 11: Western immunoblots of crude amoebic extract (Lane A) and affinity purified antigen (Lane B) probed with anti CAE antibodies. CAE (Lane C) and APA (Lane D) probed with normal rabbit serum.
PHASE I
Although several antibody detection based immunoassays for diagnosis of amoebiasis have been developed, each of them has its own limitations because of either low sensitivity or specificity or failure to differentiate present from past exposure to *E. histolytica*. Such a situation is related to the use of rather undefined cocktail of *E. histolytica* antigens. The present study reveals that the crude amoebic extract of Indian axenic *E. histolytica* (KCG:0986:11) trophozoites gets resolved into 30-35 polypeptides. The basic pattern of resolution of amoebic protein of KCG strain was almost similar to that observed with CAE of other amoebic strains (data not shown). With such a complex antigen of *E. histolytica*, it, therefore, is obvious that development of a specific immunoassay for confirming current ongoing amoebic disease process is rather difficult unless a defined specific molecule/epitope of an amoebic antigen is identified first and then used for immunodiagnostic purposes.

Many investigators have employed clinical sera from amoebic patients as probes to identify immunogenic antigens of *E. histolytica* in soluble whole cell amoebic protein (McGown *et al.*, 1982; Aust Kettis, 1983; Joyce and Ravdin, 1988; Shandil and Vinayak, 1990 and Ximenez *et al.*, 1993) and *E. histolytica* has been clearly revealed to be a complex mosaic of antigens. It is, therefore, very pertinent to first assess the most immunoreactive polypeptide of amoebic proteins so as to finally identify an important and relevant amoebic polypeptide.

In the present study, probing of amoebic profile (following resolution on SDS-PAGE and transfer of the same onto NCP, by clinical sera revealed that amoebic polypeptides with molecular mass 29, 38, 45, 58, 67, 89 and 95 KD were recognized by the sera from ALA patients, while none of the samples from non amoebic patients or healthy subjects could do so. This suggests that these polypeptides of amoebic protein to be possibly immunorelevant. Ximenez *et al.* (1993) have reported recognition of amoebic polypeptides having molecular mass of 62, 70, 93, 132 and 136 KD by the sera of ALA patients.
patients. These observations are at a variance as the present study failed to visualize 132 and 136 KD polypeptides in the sera of any ALA patient. Though no clear cut reasons can be assigned to these variations in results, it could be due to the difference in the methodology used for the preparation of amoebic antigen. The harvested E. histolytica trophozoites were homogenized by Ximenez et al. (1993), while in the present study, they were sonicated in an MSE ultrasonic disruptor. Even the improper transfer of these polypeptides from the gel to NCP sheet can be the cause of difference in the results of these studies. The present observations, however, are quite in agreement with those of Joyce and Ravdin (1988) and Shandil and Vinayak (1990) as these authors also failed to detect 132 and 136 KD polypeptides. Joyce and Ravdin (1988) reported specific recognition of 23, 24.5, 29, 31, 37, 43, 59, 90 and 100 KD polypeptides. Two polypeptides at 37 and 59 KD position were recognized by 100% ALA cases. Similarly, Shandil and Vinayak (1990) observed specific recognition of 29, 38, 45-67 complex 85 and 94 KD protein of the plasma membrane of E. histolytica. It, therefore, appears that 29, 38, 45, 58, 67, 89 and 96 KD amoebic polypeptides are immunogenic and, thus, would be relevant for development of a specific immunodiagnostic assay system.

In order to develop a specific immunoassay, attempt was made to assess the usefulness of antibody detection based ELISA, demonstration of amoebic antigen in circulation and finally raising of monoclonal antibodies to one of the major amoebic antigens as detected in circulation and use of such monoclonal antibody to capture the amoebic specific antigen.

During the course of amoebic infection, antibody response is mounted against a variety of amoebic polypeptides or their epitopes. Over the years, micro ELISA has become an indispensable tool to assess antibody responses. The suitability of such ELISA system for routine sero-diagnostic purposes for amoebic infection in recent years has been evaluated and reevaluated by several workers (Yang and Kennedy, 1979; Baveja et
al., 1984; Kumar et al., 1985; Gandhi, 1986; Gandhi et al., 1987; Torian et al., 1988; Sathar et al., 1988 and Vinayak et al., 1980a). The overall assessment of demonstration of anti-amoebic antibodies in clinical subjects indicate that the antibodies are frequently produced in symptomatic and invasive amoebiasis, but with variable frequency in non invasive and asymptomatic infection (Healy et al., 1974; Ambroiese, 1976; Juniper et al., 1972; Lobel and Kagan, 1979; Kagan, 1980; Vinayak et al., 1980a; Sharma et al., 1981).

The clinical significance of demonstration of antibodies in clinical subjects employing Indian axenic E. histolytica sonicated extract as antigen was reevaluated. The data of the study indicated that the serum samples from all the confirmed cases of ALA had anti-amoebic antibodies (Table I). Absence of any detectable level of anti-amoebic antibody in the sera of cases with non amoebic hepatic disorders and healthy subjects suggested high specificity of the antibody detecting assay system. It is interesting to point out that three serum samples from clinically suspected ALA (partially worked up) cases had detectable anti-amoebic antibody levels. It is rather difficult to pin point the precise reason of detectable levels of anti-amoebic antibody in clinically suspected ALA subjects. These three subjects (having anti-amoebic antibodies) may have had prior exposure to amoebae, as anti-amoebic antibodies are known to persist for years (Juniper et al., 1972; Abioye et al., 1973; Healy et al., 1974; Vinayak, 1979; Vinayak et al., 1980; Sharma et al., 1984). Although these subjects clinically responded to anti-amoebic chemotherapy, metronidazole, the clinical symptoms in these subjects could not be confirmed to be due to amoebic infection. The final diagnosis of these clinically suspected ALA cases remained obscure. Therefore it would be safe to assume that antibodies in three clinically suspected ALA cases may possibly be due to an earlier exposure to E. histolytica.

The consensus of various investigators is that mere detection of antibody in clinically suspected cases by employing CAE antigen may not help clinicians to confirm
the clinical diagnosis. The antibody analysis may be used as an adjunct to diagnosis. Several reports are available where defined antigens of *E. histolytica* have been employed to document antibody (Petri *et al.*, 1987; Ravdin *et al.*, 1990; Adb-Alla *et al.*, 1992; Shandil and Vinayak, 1990). Even with the use of defined antigens to document antibodies it does not appear to distinguish current disease process from the past exposure. The possible reason for persistence of anti-amoebic antibodies in otherwise treated clinical case is due to the fact that most of the immunoassays detect IgG isotype which has longer half life. In an endemic area for amoebiasis, the silent transmission of amoebic infection results in elicitation of some degree of humoral response. Thus, in such areas, mere detection of anti-amoebic antibodies in clinically suspected case may not confirm etiology of the clinical status. The overall clinical assessment of detectability of amoebic antibody by use of defined antigen on cocktail of antigens remains same. Thus detection of such antibody may provide help to clinicians as an adjunct only.

However, specific antibodies which recognize amoebic polypeptides in immunoblot of CAE will have some degree of clinical significance. Alternatively detection of amoebic antigen in circulation either in a free or immunocomplexed form may provide confirmatory diagnostic procedure. Since ALA infection is more or less a chronic state of infection and by the time patient lands in the hospital, he has been with the amoebic disease for a pretty long time and therefore, develop appreciable levels of antibodies. Under such situation, it is unlikely that he will have free circulating antigen. He is more likely to have amoebic antigen entrapped CIC. Detection of such CIC as well as antibodies in the sera of a clinical case of ALA would tend to suggest exaggerated antibody response resulting in availability of free anti-amoebic antibody even after a, significant quantity of the same entrapping the amoebic antigen.
In a preliminary investigations carried out, the present worker has failed to
detect free amoebic antigen in the sera of patients with amoebic liver abscess (data not
shown). This was an expected result. This observation corresponds with the study by
Mohimen et al. (1989) who have also reported that amoebic antigen is present in
circulation only in the form of antigen-antibody complex. On the contrary, Baveja et al.
(1991) have demonstrated specific amoebic antigen in circulation using ELISA and Dot
immunobinding (DIB) assay. There is no valid explanation for the difference in the data
of the present study and that of Baveja et al. (1991). This requires further confirmation.

The data of the present study, relating to the detection of amoebic specific CIC
via employing an ELISA system, wherein PEG precipitated CIC were detected by
polyclonal anti-amoebic antibodies (IgG) raised in rabbits, indicated that 14 (87.5%) of
the 16 cases of ALA, had detectable levels of amoebic antigen specific CIC. The
sensitivity of such assay system was found to be 87.5%. This observation gains support
from the earlier studies of Vinayak et al. (1986, 1990) and Gandhi et al. (1988). These
workers have also documented amoebic antigen specific immune complexes in sera of
ALA patients. The sensitivities of assay systems developed by Vinayak et al. (1990) and
Gandhi et al. (1988) and the present one are in the range of 79% to 93%. The variable
degree of sensitivities appear to be due to differences in the type of amoebic antibodies
used to capture CIC. Nevertheless, in addition to high degree of sensitivity, the assay
employed in the present study had 100% specificity, since none of the subjects with non-
amoebic hepatic disorders or healthy subjects had CIC in circulation.

In order to confirm the presence, as well as to work out molecular specificity of
the amoebic antigens entrapped in CIC, the latter were dissociated using glycine-HCl
buffer of pH 2.8. Amoebic antigen in dissociated CIC was detected via a direct ELISA
system. The results thus clearly indicated the presence of amoebic antigen in dissociated
CIC. The higher degree of sensitivity (100%) in detection of amoebic antigen in dissociated CIC as compared to amoebic antigen in undissociated CIC (87.5%) may possibly be due to enhanced binding capacity of amoebic antigen, following dissociation of CIC. One clinically suspected case of ALA also had detectable levels of amoebic antigen in dissociated CIC. This subject responded to anti-amoebic chemotherapy, metronidazole, following which the liver size regressed suggesting that this was indeed a case of ALA. The present data thus tends to indicate that the presence of amoebic antigen in dissociated CIC in the serum of even a clinically suspected case would suggest an ongoing amoebic infection and such an individual must be effectively treated.

Many investigators have made efforts to identify immunorelevant amoebic polypeptides to develop a specific diagnostic assay, but no attempt has yet been made to investigate the molecular specificity of the immune complexed amoebic antigen, which would probably be most relevant for specific diagnosis of an amoebic disease process. In order to achieve this aim, the CIC were resolved on SDS-PAGE in the present study and were found to contain six different polypeptides. Of these, three (29, 58 and 67 KD) were confirmed to be amoebic polypeptides, following recognition by amoebic antibodies raised in rabbits. It is probably the first ever attempt in this direction, although studies on identical lines for other parasitic and bacterial diseases are available. Thambiah et al. (1992) have identified and characterized a 46 KD, heat stable parasite component in the PEG precipitated immune complexes from sera of Onchocerca volvulus infected patients by western blotting. Similarly, Sinha et al. (1992) have identified a common 65 KD mycobacterial antigen in the CIC from leprosy patients (tuberculoid as well as lepromatous) by subjecting the PEG precipitated CIC to SDS-PAGE and immunoblotting. Another group of workers, following a strategy identical to ours, had identified and characterized the 50 KD and 60 KD antigens in CIC of cystic hydatid patients due to Echinococcus granulosus infection (Bonifacino et al., 1993).
A polypeptide of amoebic antigen entrapped in CIC with 58 KD molecular mass was further employed for subsequent study as this polypeptide was observed to be the major polypeptide entrapped in CIC.