Axenic cultivation of *E. histolytica*

a. **Axenic cultivation of *E. histolytica***

Several media proposed for axenic cultivation of *E. histolytica* by different workers were compared for their growth supporting property.

1. **Axenic cultivation of *E. histolytica* in modified TPS-1 medium (TPS-2)**

The trypticase-Parmede-serum-1 (TPS-1) medium of Diamond (1968) was modified by Dutta and Yadava (1972). The modified medium consisted of (final volume, 1000 ml):

- Trypticase (BBL) .... 15.00 gm.
- Parmede (Pains & Byrne) ---- 20.00 g.
- L-Cystein HCl (E.Nerck) ... 2.00 g.
- D-Glucose ............ 5.00 g.
- Na Cl .............. 5.00 g.
- KH₂PO₄ .............. 0.600 g.
- K₂HPO₄ (anhydrous) ... 1.00 g.
- Glass distilled water ... 875 ml.

The pH of this broth was adjusted to 7.2 with the help of IN NaOH. It was autoclaved at 10 lbs. for twenty minutes. The autoclaved broth was cooled to room temp. and was supplemented with 100 ml of heat inactivated, seitz filtered sterile bovine serum and 25 ml of vitamin mixture No.107 (Evans et al., 1956).
D-glucose ... ... 5.0 g.
NaCl ... ... 5.0 g.
KH₂PO₄ ... ... 0.600 g.
K₂HPO₄ ... ... 1.00 g.
Glass distilled water ... 875 ml.

The pH of this broth was adjusted to 7.0 with IN NaOH solution. It was autoclaved at 10 lbs. for 20 minutes, cooled to room temperature and supplemented with 100 ml of sterile bovine serum and 25 ml of vitamin mixture No.107. The pH of final medium was about 6.8. This medium was aseptically dispensed in 10 ml volumes in clean and autoclaved screw capped tubes.

3. **TYI-S-33 medium (Diamond et al. 1978).**

Diamond and colleagues (1978) have modified the original Diamond's medium (Diamond, 1968) by omitting Panmede and adding yeast extract and iron ammonium citrate in the modified medium designated as TYI-S-33. Composition of this medium was as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>...</th>
<th>...</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase (BBL)</td>
<td>20.0 g.</td>
<td></td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>10.0 g.</td>
<td></td>
</tr>
<tr>
<td>L-cystein HCl (E.Merck) (monohydrate)</td>
<td>1.00 g.</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.200 g.</td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>10.00 g.</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>2.00 g.</td>
<td></td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.0228 g.</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.600 g.</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.00 g.</td>
<td></td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>875 ml.</td>
<td></td>
</tr>
</tbody>
</table>
This TPS-1 medium was dispensed in 9-10 ml volumes in clean and autoclaved screw capped tubes (15x125 mm, Corning). The sterility of medium was ascertained by incubating it at 37°C for 24 hours.

Three to four days old cultures in the exponential growth phase, with a characteristic greyish white beads at the bottom were sorted out for subinoculating in fresh tubes. Each culture tube was chilled at 4°C for five minutes, for giving physical shock to amoebae, so that they dislodge from the surface of tube. Tubes were then centrifuged at 500 g for 5 minutes. The supernatant leaving 3-4 ml medium was discarded and uniform suspension of amoebae was made with pipette in the remaining volume of medium. 0.5 ml of this suspension was inoculated in each of the 10 ml tubes containing TPS-1 medium. The cultures were incubated at 37°C and examined daily by means of an inverted microscope (10x ocular and 10x objective).

The composition of other media used for cultivation of E. histolytica are given below. Procedure for adjusting of pH, autoclaving and subculturering was same as described above for all the media.

2. TPS-1 medium (Diamond, 1968)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase</td>
<td>10.0 g.</td>
</tr>
<tr>
<td>Darmede</td>
<td>20.0 g.</td>
</tr>
<tr>
<td>L-cysteine HCl (E. Merck)</td>
<td>1.0 g.</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.200 g.</td>
</tr>
</tbody>
</table>

pH was adjusted to 6.8 with IN NaOH. This broth was autoclaved and supplemented with 100 ml of inactivated Seitz filtered bovine serum and 3 ml of vitamin Tween 80 mixture (Diamond, 1980).

**Preparation of Vitamin-Tween 80 mixture (Diamond, 1980)**

**Stock Solutions:**

a. Vitamin mixture NCTC 107
b. Vitamin B₁₂, 40 mg dissolved in water q.s. to 100 ml.
c. D.L. 6,8 - Thiocetic acid (oxidized) 100 mg dissolved in ethanol q.s. to 100 ml.
d. Tween-80, 50 g. dissolved in ethanol q.s. to 100 ml.

**Working solution:**

To 1000 ml of (a) 12 ml of (b), 4 ml each of (c) and (d) and 180 ml of water were added, for preparing the working solution of Vitamin-Tween-80 mixture.

4. Hi Media (R) Diamond Medium for axenic cultivation of *E. histolytica*

available ready made medium in powder form. This is a locally marketed by the Hindustan Dehydrated Media, Bombay, for axenic cultivation of *E. histolytica*. This medium was prepared according to the manufacturer's instructions. Briefly 45.5 g. of the dehydrated medium was dissolved in 1.90 ml of glass distilled water. Its pH was adjusted to 7.2 with IN NaOH and it was autoclaved. 100 ml of sterile inactivated bovine serum and 25 ml of vitamin mixture 107 was then added aseptically.
Cultivation from small inocula of amoebae

A comparative study of different axenic media was performed by using different inocula of amoebae. The purpose of this study was, the selection of most appropriate medium which supports the growth of *E. histolytica* from small inocula.

Healthy cultures showing sheet of amoebae at the bottom were used as the source of inoculum. Three different inocula were used for each medium.

1. Five tubes of fresh medium were subcultured from single culture tube.
2. Fifteen tubes of fresh medium were subcultured from single tube.
3. Thirty tubes of fresh medium were subcultured from single tube.

Growth of amoebae from different inocula in different media was compared by counting the yield of amoebae.

Axenic cultivation of *Naegleria aerobia* (Strain ATCC-73)

*Naegleria aerobia* was grown axenically in Nelson's medium. Bovine serum was also tried instead of foetal calf serum as a component of Nelson's medium.

Composition of Nelson's medium is following:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Panmede</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Page's Amoeba saline</td>
<td>(1000 ml) 950 ml</td>
</tr>
</tbody>
</table>
PH of the medium was adjusted to 6.5 with the help of IN NaOH. It was autoclaved at 10 lbs. for 20 minutes. 2% inactivated sterile foetal calf serum or bovine serum was added aseptically at the time of inoculation.

Preparation of Page's Amoeba Saline — (Page 1967)

A separate stock solution of each of the following component was made up by dissolving the amount given in 100 ml of glass distilled water. Final solution was made by adding 10 ml of each stock solution to enough glass distilled water to make one liter.

- NaCl - 1.20 g.
- MgSO₄ 7H₂O - 0.04 g.
- CaCl₂ 2H₂O - 0.04 g.
- Na₂HPO₄ - 1.42 g.
- KH₂PO₄ - 1.36 g.

Nelson's medium thus prepared was dispensed in 5–6 ml volumes in 15 mx125 mm corning screw capped tubes for the purpose of culture maintenance. For mass cultivation medium was dispensed in 100 ml volumes either in roux's bottles for stationary growth or in 500 ml flasks for agitated growth in gyroshaker. After dispensing the medium, it was incubated at 37°C for 24 hours to ascertain the sterility.

Sub-cultivation of N. aerobia was done as described for E. histolytica.
Axenic cultivation of *A. culbertsoni* was done in Balamuth's medium (1975).

Its composition is as follows:

- Protease Peptone  ...  ...  20.0 g.
- Yeast Extract  ...  ...  2.0 g.
- Glucose  ...  ...  18.0 g.
- Mg SO$_4$·7H$_2$O  ...  ...  0.098 g.
- Ca Cl$_2$  ...  ...  0.045 g.
- Sodium citrate  ...  ...  1.00 g.
- Fe (NH$_4$)$_2$ SO$_4$  ...  ...  0.196 g.
- KH$_2$PO$_4$  ...  ...  0.330 g.
- Na$_2$ HPO$_4$  ...  ...  0.325 g.
- Glass distilled water  950 ml.

pH of this broth was adjusted to 6.5 with the help of IN NaOH. Final volume was made to 1000 ml. This was autoclaved at 10 lbs. for twenty minutes.

For the purpose of culture maintenance medium was dispensed in 9-10 ml volumes in corning cotton plugged sterile tubes, but for mass cultivation it was dispensed in 100 ml volumes either in roux's bottles for stationary growth or in 500 ml flasks for agitated growth. All these were incubated at 37°C for 24 hrs to ascertain the sterility of medium.

Sub culture of *A. culbertsoni* was done in the same way as described for *E. histolytica*. 
Preparation of *E. histolytica* Antigen

Four to five days old cultures showing exponential growth of amoebae were chilled at 4°C for 5 minutes. This physical shock dislodged the amoebae from glass surface. Tubes were centrifuged at 1000 g and except for a small volume (0.2-0.5 ml) at bottom, the supernatant was discarded. Sedimented amoebae from each tube were pooled together with chilled normal saline and washed thrice in the same in a refrigerated centrifuge. A haemocytometer count of amoebae was made and their concentration was adjusted to a known value. These amoebae were subjected to ultrasonication in Soniprep 150 (MSE Instruments, England) with amplitude needle at 14 for 7-8 minutes at 4°C. Aliquots of sonicated suspension were checked for intact amoebae under microscope. Ultrasonicated suspension was centrifuged at 4000 g for 30 minutes at 4°C. The opalescent supernatant solution was collected while the sediment was discarded. This supernatant was dispensed in small aliquotes and lyophilized under negative pressure and temperature in Virtis lyophilizer.

The protein contents of each batch of antigen was determined by the technique of Lowry et al. (1951).

Preparation of *A. culbertsoni* and *N. aerobia* antigen

Five to seven days old cultures in Roux bottles, kept stationary and 3 to 4 days old cultures in flasks,
kept in a shaker (250 rpm) showing exponential growth were harvested. Amoebae were dislodged from the glass surface by repeatedly jetting of medium on the walls with the help of a pipette. Medium containing amoebae was collected in 250 ml centrifuging bottles. It was centrifuged at 600-700 g. for 10 minutes at 4°C. Supernatant was discarded and sedimented amoebae were pooled with chilled normal saline. Rest of the procedure was same as described for E.histolytica.

**Fractionation of whole Amoebic Antigen on Sephadex G-200 column**

**Preparation of Histolyticin**

About 45×10⁶ amoebae (trophozoites) obtained through axenic cultivation of E.histolytica in the TPS-2 medium were suspended in approximately 15 ml saline and homogenised in the potter-elvagen. Homogenate was centrifuged at 4000 G for 30 minutes at 4°C and the soluble supernatant portion was collected (18 ml).

The supernatant was concentrated by freeze-drying and protein of histolyticin was estimated by method of Lowrys et al. (1951).

**Preparation of Sephadex G-200 column**

Sephadex G-200 column of 2.5x 35 cm measurement was preequilibrated with tris HCl buffer, pH 7.5, 0.05m. Flow rate of column was fixed to about 6.5 ml /hour (nearly 1 ml/10 minute) Histolyticin was applied to the
top of column and elution started with tris HCl buffer
(pH 7.5, .05 m), 5 ml for each fraction were collected.

Proteins of different fractions collected were
estimated by reading their optical densities at 280 nm
extinction. Bovine serum was taken as a standard.

Indirect Haemagglutination Test for serological
activity and skin reaction test was performed to compare
reactivity for the cellular immune response of different fractions.

For characterisation of fraction by IHA test four
concentrations (40 µg/ml, 20 µg/ml, 10 µg/ml and 5 µg/ml)
of test peaks I, II and III were made.

Glutaraldehyde fixed cells (2.5% suspension, PBS 7.2)
were suspended in equal volumes of 1:40000 dilution of
tannic acid in PBS 7.2. Cells were tanned for thirty
minutes at 4°C with slight rotation. Tanned cells were
washed thrice with PBS 7.2 and finally suspended to 2.5%
in PBS 6.4. Antigen fractions were diluted in PBS 6.4
as the dilutions given above (40 µg, 20 µg, 10 µg, 5 µg/ml).
Two ml of each of the above antigen concentration and
2 ml of tanned SRBC's were mixed together and incubated
at 37°C for 30 minutes with gentle stirring for sensitization
of cells with antigen. After sensitization cells were
sedimented and washed once with diluent (1% Normal rabbit
serum in PBS 7.2) and suspended to original volume. The
standard control of sera was made using SRBC's sensitized
with optimum dose of whole antigen. Plates were incubated
at room temperature for two hours.
<table>
<thead>
<tr>
<th>Sera</th>
<th>IHA titers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Chhotey (1189)</td>
<td>4096</td>
</tr>
<tr>
<td>2. Md. Asif</td>
<td>4096</td>
</tr>
<tr>
<td>3. Jai Jai Ram</td>
<td>4096</td>
</tr>
<tr>
<td>4. Maya Chand</td>
<td>1024</td>
</tr>
<tr>
<td>5. Narayan</td>
<td>4096</td>
</tr>
<tr>
<td>6. Md. Baksh</td>
<td>4096</td>
</tr>
<tr>
<td>7. Ayodhya Prasad</td>
<td>4096</td>
</tr>
<tr>
<td>8. B-93</td>
<td>4</td>
</tr>
</tbody>
</table>

Different fractions of *E. histolytica* antigen at different sensitizing concentrations were tested against sera No. 1-3 and B-93 included in control plates.

Guinea pigs were sensitized in two groups of 8 animals each for applying skin reaction test with different fractions of whole *E. histolytica* antigen. One group was sensitized with 10 µg SE and other with 200 µg of whole antigen. Skin test was applied by challenging the sensitized guinea-pigs with different concentrations of *E. histolytica* fractions (2 µg, 1 µg and .5 µg/ml). Reactivity of fractions was compared on the basis of appearance of differential indurations at injection sites.

**Experimental Induction of Specific immune response in guinea pigs against pathogenic amoebae.**

Albino guinea pigs of either sex, weighing 200-250 gms each, obtained from animal house of Central Drug Research Institute, Lucknow, were employed for experimental
induction of immune response against three pathogenic amoebae \textit{E. histolytica}, \textit{N. aerobia} and \textit{A. culbertsoni}.

The antigen preparation made from axenic strains of \textit{E. histolytica} NIH-200, \textit{N. aerobia} ATCC-73 and \textit{A. culbertsoni} A-1 were used for sensitization. Route of administration of antigen was in the foot pads. Eight groups of four animals each, were used in the study for sensitization with three amoebic antigen. Animals of group I to VI were sensitized with different amounts of different amoebic antigen protein (0.2 ml per animal). The antigen (10-2000 \(\mu g\)) was emulsified in an equal volume (0.2 ml) of Freund's complete adjuvant (FCA) and injected equally in the four foot pads. Animals of group VII received adjuvant alone (0.2 ml per animal) and those of group VIII were given normal saline (0.1 ml per foot pad).

\textbf{The protocol used for sensitization of guinea pigs}

\textbf{Doses of antigen in 0.2 ml saline (emulsified with 0.2 ml FCA)} \textit{E. histolytica} antigen

\begin{itemize}
  \item 10 \(\mu g\)
  \item 50 \(\mu g\)
  \item 100 \(\mu g\)
  \item 200 \(\mu g\)
  \item 500 \(\mu g\)
  \item 2000 \(\mu g\)
\end{itemize}
The quantity of adjuvant in sensitizing mixture was same for each animal. Total amount of sensitizing mixture (0.2 ml antigen + 0.2 ml FCA) was equally distributed in four foot pads of each animal.

The purpose of giving different antigenic doses, was to ascertain the optimum dose for induction of immune response in experimental guinea pigs.

Immunological status of sensitized guinea pigs was monitored on different days post-sensitization by employing various immunological tests as given below:

**Immunological tests for cell-mediated immunity**

*In vivo* - Intradermal test for skin reaction

*In vitro* - Macrophage migration inhibition test

**Immunological tests for humoral immunity**

1. Indirect haemagglutination test
2. Gel diffusion precipitin test

Detailed methods for performing these tests are given below:
Tests for cellular immunity

In vivo method for demonstration of delayed type hypersensitivity

Intradermal Test for Skin Reaction:

Intradermal test was performed in sensitized guinea-pigs by challenging them with specific amoebic antigen on different days after sensitization.

Animals were shaved perfectly at the abdomen avoiding any injury. The challenging dose containing of antigen in 0.1 ml saline was injected intradermally at one spot on the abdomen of guinea pig. Four such spots were applied on each animal. All four spots were marked with a non-washable dye. Animals were read for skin test erythema and induration after 15 min., 4 hours, 24 hours, 48 hours, 72 hours and 96 hours.

Macrophage Migration Inhibition Assay:

Harvesting of peritoneal Exudate Cells from Guinea pigs

1. Guinea pigs were injected with 20 -30 ml sterile light paraffin oil, intraperitoneally 3-4 days before the peritoneal cells were to be harvested.

2. Animals were anaesthetised with ether and sacrificed by cutting the neck. This allows the guinea pig to bled completely thus reducing the contamination of peritoneal exudate with red blood cells.
3. The animal was pinned on its back and hair of abdomen were shaved and the abdomen was cleaned with spirit.

4. A mid line incision was made and skin over the abdomen was removed from the underlying fibrous tissue, reflected and pinned to the animal board.

5. The abdominal muscle was lifted with a pair of forceps and the pointed end of a scissors was inserted into it to make a small opening in the peritoneum. This procedure relieves the negative pressure in the peritoneal cavity and allows the gut to fall back instead of sticking to the peritoneum.

6. About 50 ml of Hank's balanced salt solution (HB55) containing 1 unit of heparine/ml was poured in the peritoneal cavity. The whole viscera were gently bathed with HBSS.

7. A sterile polypropylene tube with a number of perforations at the base and sides to form a cannula was placed into the peritoneal cavity.

8. A pipette was inserted into the perforated tube and the contents of peritoneal cavity were aspirated. The perforated tubes prevent the viscera from being sucked up into the pipette.

9. The steps 6-8 were repeated once more and cells collected were pooled.
10. The cells were centrifuged at 250 g. for 10 minutes.

11. The supernatant was aspirated and the cells were resuspended in 5 ml fresh medium, transferred to clean tubes and washed twice with the medium at 250 g. for 10 min. each.

12. The cells were finally suspended in 5 ml medium containing antibiotics penicillin (100 units/ml) and streptomycin (100 μg/ml) and glutamine (6.5%).

Preparation of chambers: The disposable plastic plates were used (Laxbro) chambers were rinsed with medium and a small quantity of silicone grease was applied at one spot in the bottom of wells to fix the capillaries.

Preparation of capillary tubes: The final concentration of cells was adjusted to 80-100x10⁶ cells/ml medium containing 10-15% foetal calf serum.

2. Capillary tubes were filled by capillary action and spun after sealing with plasticin. The speed of centrifuge was kept at 120 g for exactly 2 minutes at 4°C.

3. Capillary tubes were cut 1 mm below the cell liquid interface and placed immediately in the chambers. Medium was poured slowly in chambers. Antigen was added for inhibition of migration.

4. Chambers were sealed with a coverslip fixed by silicone grease.
5. The migration chambers were incubated at 37°C for about 20 hrs and the migration boundaries were drawn on a paper by projection of image with the help of Camera Lucida.

**Immunological Tests for Humoral Immunity**

**Indirect haemagglutination test:**

This test was performed according to the technique given by Krupp (1969 a,b) and Sharma et al. (1980).

**Preparation of sheep red blood cells**

Blood

Sheep was collected aseptically from the jugular vein in sterilized modified Alsever's solution in proportion of 10:12 and stored at 4°C.

After two or three days SRBC's were washed three times in phosphate buffered saline 0.15M, pH 7.2 and a 2.5% suspension of SRBC's was made in the same.

For tanning, equal volumes of 2.5% suspension of SRBC's in PBS 7.2 and working solution in (1:40,000) of tannic acid were mixed together in an Erlenmeyer's flask at 4°C for 20 minutes by gentle rotation.

After tanning the cells were washed twice with PBS 7.2 and finally diluted to a 2.5% suspension in the phosphate buffer saline pH 6.4.

Sensitization of SRBC's with three different amoebic antigens was done separately by mixing equal volumes of 2.5% suspension of SRBC's in PBS 6.4 and
optimal dilution of three antigens in PBS 6.4. It was
done at 37°C and cells were kept in suspension by gentle
rotation for 20 to 25 minutes.

Sensitized cells were washed twice with 1% normal
rabbit serum in PBS 7.2 and finally diluted to 1.5% in
the same for use in the test system.

Normal rabbit serum was examined for heterophilic
antibody against sheep SRBC's and if present it was
removed by adsorption with an equal volume of sheep
RBC's prior to use. This serum was also inactivated at
56°C for 30 minutes before use.

Test system - Disposable micro-titer plates with 96 V or
U shaped wells were used. Initially 0.05 ml of 1%
normal rabbit serum in PBS pH 7.2 was poured in each well.
Then 0.05 ml of each serum sample was added in first well
of a single row with the help of micro diluter and
serially diluted two-fold in the successive wells.
Finally 0.025 ml of sensitized SRBC's suspension was
added in each well. The plates were then covered with
the covers provided and shaken gently to mix the reactants
uniformly and were kept for 2 hours at 37°C for the
reactant to react. Results were read according to
Stavitsky et al. (1954) and the last serum dilution
showing 50% agglutination was kept as positive end-
point.
Fixation of SRBCs with glutaraldehyde (Krupp 1969b)

SRBC's stored for two or three days in the modified Alsevere's solution at 4°C were washed three times with 0.15M NaCl solution at room temperature. After the final washing, SRBC's were diluted to 2.5% suspension in 1% glutaraldehyde solution.

1% glutaraldehyde Salt solution:

25% aqueous solution of glutaraldehyde 4 ml (Riedel)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15M NaCl</td>
<td>...</td>
</tr>
<tr>
<td>0.15M Na₂HPO₄</td>
<td>...</td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>...</td>
</tr>
</tbody>
</table>

pH was adjusted to 8.2 with 0.15 M KH₂PO₄.

SRBC's were kept in suspension for 30 minutes at 4°C by gentle rotation for fixation.

Fixed SRBC's were washed with 0.15 M NaCl then with distilled water, five times each. The final 30% suspension of SRBC's was made in distilled water and stored at 4°C for further use.

Rouchartloy gel diffusion precipitin test

Clean micro-slides were warmed on a flame and smeared thinly with 2% agarose in distilled water. Then an even layer of 1% agarose was poured by slowly pipetting 3 ml of agarose solution on the slide, making it uniform with pipette tip and kept at 4°C for solidifying. Wells for antigen and antisera were punched in the agarose
layer as required. Each well was 3 mm in diameter and 5 mm apart from adjoining wells. Different patterns of wells were made depending on the number of test sera. Wells were filled with the help of pasteur pipette. A separate disposable pipette was used for each test serum. Plates were already kept in moist humid chamber so as to minimize the chances of evaporation. Antigen and antisera were filled in the respective wells thrice at the intervals of half an hour, so as to ensure adequate quantity of reactants in the test. The changed slides were kept at 4°C overnight in moist chamber and were examined after 24 hours for precipitin bands. Slides were observed for five days for the confirmation of negative results.

Staining of Gel slides

Amido black 10 B solution -

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amido black 10B</td>
<td>... 1.00 gm.</td>
</tr>
<tr>
<td>Acetate buffer</td>
<td>... 900 ml.</td>
</tr>
<tr>
<td>Pure Glycerol</td>
<td>... 100 ml.</td>
</tr>
</tbody>
</table>

Acetate buffer

M/10 Acetic acid (5.7 ml in 1000 ml) 50 ml.
M/10 Sodium acetate (136 gm. in 1000 ml) 50 ml.

After the completion of bands development, the slides were washed with physiological saline for 2 to 3 days with frequent changes daily. Thereafter slides were allowed to dry at 37°C overnight so that gel becomes
firmly attached to the slide. Then slides were washed with distilled water to make them entirely transparent and they were stained with Amido black 10B for 15 minutes. Excess stain from the background was removed by washing in the washing solution (0.2% acetic acid). Final washing was done with distilled water.

Radial quantitative immunodiffusion test

This test is performed in tripartigen immunodiffusion plates, for the quantitative determination of the immunoglobulins IgG, IgA and IgM.

Composition of the plates

Plates contain a prepared agar gel in which H-chain specific antiserum to the respective immunoglobulin is incorporated. The antiserum is produced by immunization of sheep and goats. Preservative used are sodium azide (1mg/ml), sodium P-ethyl mercury marcapto benzene and sulfonate at most 0.1 mg/ml.

Method -

1. Plate was opened and left to stand for about 5 minutes at room temperature to allow any condensation water that may have accumulated in the wells to evaporate.

2. IgA and IgM were determined using undiluted serum. Only when IgG is to be determined, the sera to be tested and the control serum used must be diluted 1:10 with isotonic saline.
3. Well No.1 was filled with 5 μl of control serum, wells from No.2 to No.12 were each filled with 5 μl of respective sera under test.

4. Plates were closed tightly and left at room temperature. Evaluation was made after a minimum diffusion time of 50 hours for IgA and IgG and 80 hours for IgM.

5. At the end of the given diffusion time, the diameter D of the precipitin rings was measured accurately to 0.1 mm using a suitable calibrated instrument.

6. The immunoglobulin concentrations related to the measured diameters were read directly from the table of reference values. The results were reliable only when the value found for the control serum applied to well No.1 lies within the confidence range taken from the table of values enclosed with each pack of the control serum. (With Hoechst Behring control serum, the confidence range is ± 15% of the immunoglobulin concentration given with each pack).

When determining IgG, the value found must be multiplied by the dilution factor 10.

If the protein concentration of serum samples diverges considerably from the normal value, the diameter of resulting precipitin ring would fall outside the assay range of the plate. In this case, the examination must be repeated, using higher or lower dilution of serum sample.
A Microzone Method for Scrum Protein

(Basic Microzone Electrophoresis System Beckman Model 152) was used.

Preparation of Reagents

1. **Beckman B-2 Buffer** (pH 8.6)

   Contents of one package were dissolved in 1 liter distilled water.

2. **Fixative/ Dye Solution**

   The contents of 30 ml bottle of Beckman Fixative/ Dye

   were diluted to 250 ml with distilled water. The relative concentrations of the components of the fixative dye solution in distilled water were

   (1) 0.2% Ponclau - S Stain (W/v)

   (2) 3.0% Trichloro acetic acid (W/v)

   (3) 3.0% Suljosalicylic acid (W/v)

3. **Rinse Solution**

   5% glacial acetic acid (V/V) was made in distilled water. It was convenient to make up about three to five liters at a time.

4. **Alcohol Rinse Solution**

   Full strength alcohol was used.

5. **Clearing Solution**

   30% reagent grade cyclohexahedron was made.

Procedure

1. Seven plastic trays were filled with following solutions:
a. 100 ml reconstituted B-2 Buffer
b. 100 ml fixative/dye solution
c. 100 ml 5% aqueous acetic acid in three plates for three successive rinse.
d. 100 ml alcohol dehydration solution
e. 100 ml clearing solution.

2. The remaining buffer solution was used to fill the cell or electrophoretic chamber up to the level mark. Fluid level was equalized on both sides of cell.

3. A cellulase acetate membrane provided with equipment was placed on top of buffer by floating with a pair of forceps without trapping any air bubble under or within the membrane.

4. When membrane was buffer saturated, it was taken out from the buffer tray and placed between two blotters and was gently blot to remove excess surface buffer solution.

5. Membrane was placed on the bridge of cell with forceps and holes in membrane were lined up with pegs provided on bridge keeping reference hole at lower left. Membrane was pushed down with forceps tip to seal securely. Precaution was taken to avoid the touching of middle portion of membrane.

6. Bridge was placed in the cell and ends of membrane were in contact of buffer. Cell cover was replaced.
7. A drop of sample was placed on the parafilm and was covered. Taking applicator in hand, white button was depressed, applicator tip was brought across the sample, barely breaking surface tension. Applicator was raised and red button was depressed.

8. Applicator was placed in position groove on cell cover. Indexed at desired sample position. White button was depressed and waited for 5 to 7 seconds, red button was depressed and applicator was washed with distilled water. Step 7 and 8 was repeated for each sample and lid was replaced.

9. Duostat was plugged into the chamber and voltage was adjusted to 250 volts. Starting current should be 4.0 to 6.0 milliamperes. After 16 to 18 minutes, power switch was turned off and cell or chamber was disconnected.

10. Membrane was now placed into Fixative/dye solution for 7 to 10 minutes. Then transferred to acetic acid and 3 successive washings were done in 5% acetic acid. Then membrane was agitated in dehydration solution for a minute and removed on a clean glass plate. It was squeezed gently to remove the excess clearing solution.

11. Membrane on glass plate was kept at 37°C overnight.

12. Membrane was pulled from glass plate and trimmed with a pair of scissors and kept in plastic envelope.
Reading of membrane

1. Power switch of densitometer (Beckman) was turned on and it was left for 15 minutes for warming up. Initial slit width was kept at 0.2 and natural density filter at 1.4 cal. Envelope containing membrane was placed into screening carriage in desired position. Zero baseline on film just ahead of the albumin fraction 85%. Span was adjusted by placing albumin fraction in beam path. Baseline zero was rechecked and pattern was read.

Stool Examination for intestinal parasites

Fresh stool samples, from the patients with gastrointestinal disorders, attending the out-patients department of Balrampur Hospital, Lucknow, were examined for aetiological agent of amoebiasis.

Three techniques were employed to examine each of the stool sample. Wet mounts in normal saline and Lugol's iodine, zinc sulphate floatation method for concentrating protozoan cysts and helminthic eggs and lastly, permanent stained smears fixed in the Schaudinn's fluid and stained with haematoxylin.

Wet mounts - Two coverglass preparations were made of each stool sample on a clean microscopic slide, one in a drop of physiological saline and the other in lugol's iodine and examined thoroughly under the microscope.
Floatation method - Zinc sulphate flotation method, described by Faust et al. (1938, 1939) was employed to concentrate the protozoan cysts and helminthic worms in stool. 33% aqueous solution of zinc sulphate (equivalent to specific gravity of 1.180) was used for floatation.

3. Permanent stained films - were prepared by smearing a representative portion of stool sample on an absolutely clean slide. This wet smear was fixed up in Schaudinn's fixative for a minimum of twenty minutes.

Schaudinn's fixative - Saturated Solution of Hg Cl₂ - 200ml (in distilled water)
95% Ethanol ... ... 100ml

Just before use, 10-15 ml of glacial acetic acid was added to above solution. The smears were stained with iron hematoxylin.

Pathogenicity of amoebae in experimental animals

1. Mice -

Albino mice, maintained at the Central Drug Research Institute, Lucknow, were used in this work. Three groups of mice of different weights viz: 10-15g, 15 to 20g, 20-25g, were compared in pathogenicity test. Young and actively multiplying amoebae (18-20 hrs. old), were suspended in sterile distilled water. The mice were anaesthetized with ether and 0.02 ml of the suspension of amoebae containing about 20,000 trophozoites, as determined by counting in haemocytometer was given
intranasally and intracerebrally to each mouse.

For control experiment, 0.2 ml of saline was administered intranasally and intracerebrally in each mouse. Very sick mice with symptoms of meningoencephalitis were killed and cultures were made with from brain tissue / E. coli on non-nutrient agar. Smear preparations were also made from brain to locate amoebae under a microscope. Mice surviving 21 days were also killed and cultures for amoebae from brain were made as above.

Albino Guinea pigs, weighing 200-250 gms, were also inoculated with the same procedure. Number of amoebae inoculated was 1x10^6 amoebae in 1 ml distilled water per guinea pig.