MATERIALS AND METHODS
3.0 GROWTH AND CHARACTERISTIC FEATURES OF SHIGELLA flexneri

3.0A Materials:
MacConkey agar, triple sugar iron agar, eosin methylene blue agar and Simmon's citrate agar were procured from Hi Media Ltd, Bombay, India.

3.0.1 Growth In MacConkey Agar
Contents:
Peptone 17g, proteose peptone 3g, lactose 10g, bile salts 1.5g, sodium chloride 5g, neutral red 0.03g, agar 15g per litre of distilled water.

Preparation:
51.5g MacConkey agar was dissolved in 1 litre of distilled water by boiling and was sterilized by autoclaving at 121°C for 20 min and poured into sterile petriplates.

Growth:
The petriplate was streaked with a single colony of Shigella flexneri, incubated at 37°C overnight and observed for growth.

3.0.2 Growth In Triple Sugar Iron Agar
Contents:
Peptone 10g, tryptone 10g, yeast extract 3g, beef extract 3g, lactose 10g, saccharose 10g, dextrose 1g, ferrous sulphate 0.2g, sodium chloride 5g, sodium thiosulphate 0.3g, phenol red 0.024g, agar 12g/litre of distilled water.
Preparation:

65g of the agar was dissolved in 1 litre of distilled water by boiling. The media was then distributed into capped test tubes and sterilized by autoclaving at 121°C for 15 min. The test tubes were allowed to set in a slanted position so that a slant and a butt of about 1 inch were obtained.

Method:

The slant was streaked with a single colony of *Shigella flexneri*. The butt was separately jabbed with the organism using the loop of the inoculating needle and kept at 37°C overnight. Observations were then made for changes in colour and for gas formation.

3.0.3 Growth In Simmon's Citrate Agar

Contents:

- Magnesium sulphate 0.2g,
- ammonium dihydrogen phosphate 1.0g, sodium citrate 2g, sodium chloride 5g, agar 15g, bromothymol blue 0.08g.

Preparation:

24.2g of the media was dissolved in 1 litre of distilled water. It was sterilized by autoclaving at 121°C for 15 min, poured into sterile petriplates and allowed to set. The plates were streaked with a single colony of *Shigella flexneri* and kept for incubation at 37°C overnight. Observations were then made for growth of the organism in the media.

3.0.4 Growth In Eosin Methylene Blue Agar (EMB Agar)

Contents:

- Peptone 10g, lactose 5g, sucrose 5g, dipotassium phosphate 2g, agar 13.5, eosin Y 0.4g, methylene blue 0.065g/litre of distilled water.
Preparation:

36 g of EMB agar were dissolved in 1 litre of distilled water and boiled. It was allowed to cool to 50°C and shaken to oxidize methylene blue (i.e. to retnore its blue colour). The floculant precipitate was dispersed and the media sterilized by autoclaving at 121°C for 15 min. The agar was poured into sterile pertriplates and streaked with a single colony of *Shigella flexneri*. It was incubated at 37°C overnight after which it was observed for signs of growth of the organism.

3.1 PREPARATION OF THE CRUDE EXTRACTS OF *SHIGELLA* SPECIES

Strains

The strains of *Shigella dysenteriae*, *Shigella sonnei* and *Shigella boydii* were procured from the National Collection Centre for Cholera and Enteric Diseases, Calcutta. *Shigella flexneri* was purchased from Microbial Type Culture Collection of the Institute of Microbial Technology, Chandigarh.

3.1 A) Materials

Nutrient agar, polymyxin B sulphate were purchased from Hi Media Ltd., India, sodium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate were purchased from SRL Chemicals Ltd., Bombay, India.

3.1 B) Solutions

3.1 B1) Nutrient Broth

Contents:

Peptone 5g, sodium chloride 5g, beef extract 1.5g, yeast extract 1.5 g.
Preparation:

13.6 g of the broth was prepared by suspending in 100 ml water. This was boiled to dissolve the media and sterilized by autoclaving at 15 lb pressure (121°C) for 15 min.

3.1 B2) **Nutrient Agar**

**Contents**

Peptic digest animal tissue 5g, sodium chloride 5g, beef extract 1.5 g, yeast extract 1.5 g, agar 15g.

**Preparation**

This agar was prepared by suspending 28 g of the above in 1000ml water and boiled to dissolve the contents. It was sterilized by autoclaving at 15 lb pressure (121°C) for 15 min mixed well and poured into petriplates.

3.1 B3) **Wash Buffer**

Sodium chloride 6.2 g

This was dissolved in 25 mM phosphate buffer, pH 7.3.

3.1 B4) **Polymyxin B sulphate** 100 mg

This was prepared in 10 ml of the wash buffer.

3.1 C) **Method**

3.1 C1) **Growth**

The four species of *Shigella* namely *S. dysenteriae*, *S. boydii*, *S. flexneri* and *S. sonnei* were individually grown in nutrient agar plates at 37°C for 18 h. A single colony of each of the species was transferred to nutrient broth. The broth was incubated overnight at 37°C with shaking.
This was used as starter culture to expand the culture under the same conditions.

3.1 C2) **Harvesting**

The cells were obtained by centrifugation of the broth at 4000 rpm for 10 min in a centrifuge at 4°C. The supernatant was discarded. The pellet was washed with wash buffer. Polymyxin B sulphate was added to the pellet in each case and warmed to 37°C for 10 min. The cell debris was discarded after centrifugation at 12000 g for 5 min at 4°C and the supernatant obtained used as the crude extract for each of the four species.

3.1 C3) **Protein Estimation**

Protein estimation of the extract of the strains of *S. dysenteriae*, *S. boydii*, *S. flexneri* and *S. sonnei* was done spectrophotometrically employing the formula:

\[
\text{Protein mg/ml} = 1.55A_{280} - 0.76A_{260}
\]

where \(A_{280}\) is the absorbance at 280 nm

and \(A_{260}\) is the absorbance at 260 nm.

3.2 A) **NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF THE CRUDE EXTRACTS OF SHIGELLA SPECIES**

3.2 A-A) **Materials**

Bromophenol blue, tetra methylene diamine (TEMED) were purchased from Sigma Chemical Company (USA). Tris buffer, Ammonium persulphate and glycine were purchased from Qualigens fine Chemicals, India. Acrylamide and Bis acrylamide were obtained from SISCO Research Labs. Pvt. Ltd., India.
3.2 A-B) **Solutions**

3.2 A-B1) **Stock acrylamide (T=30%)**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>73 g</td>
</tr>
<tr>
<td>Bis acrylamide</td>
<td>2 g</td>
</tr>
</tbody>
</table>

These were dissolved and the volume made up to 250 ml in distilled water.

3.2 A-B2) **Stock separating gel buffer or lower tris buffer**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>45.5 g</td>
</tr>
</tbody>
</table>

It was dissolved in less than 250 ml. The pH was adjusted to 8.8 with HCl and the final volume made up to 250 ml.

3.2 A-B3) **Stock ammonium persulphate**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

This was dissolved in 10 ml water and prepared fresh on the day of use.

3.2 A-B4) **Stock stacking buffer or upper tris buffer**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>15.1 g</td>
</tr>
</tbody>
</table>

This was dissolved in less than 250 ml and the pH was adjusted to 6.8 with HCl.

The final volume was made up to 250 ml with distilled water.

3.2 A-B5) **Reservoir buffer**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>28.8 g</td>
</tr>
<tr>
<td>Tris base</td>
<td>6.0 g</td>
</tr>
</tbody>
</table>

The above mentioned chemicals were dissolved in 2 litres of water.

3.2 A-B6) **Sample buffer**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>4.0 mg</td>
</tr>
<tr>
<td>Tris base</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Bromophenol blue (0.1%)</td>
<td>2 ml</td>
</tr>
</tbody>
</table>
These chemicals were dissolved in less than 10 ml distilled water and the pH adjusted to 6.8 with HCl. The final volume was made to 20 ml.

3.2 A-C) **Method**

The glass plates were thoroughly cleaned and dried. The spacers were arranged and held in place with brown tape. The glass chamber was clamped and held in an upright position.

A sufficient volume of separating gel mixture was made as follows.

3.2 A-C1) **Composition of Separating Gel (10%)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bis acrylamide</td>
<td>8.3 ml</td>
</tr>
<tr>
<td>1.5 M Tris buffer</td>
<td>6.2 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10.35 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>125 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µl</td>
</tr>
</tbody>
</table>

It was poured into the prepared chamber to a level 1 cm below the bottom of the comb. It was overlayed with water saturated butanol to generate a flat gel. The mixture was allowed to set for 0.5-1.5 h.

3.2 A-C2) **The stacking gel of 4% was prepared as follows:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3 ml</td>
</tr>
<tr>
<td>Upper gel buffer</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Stock APS</td>
<td>15 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

The stacking gel was prepared just before use and poured on top of the separating gel after washing off butanol. A comb was inserted into place and the gel was allowed to stand till it polymerized (0.5-1 h).
When the polymerization was complete, the comb and the bottom spacer was removed and the gel sandwich was installed in the apparatus. The reservoir buffer was filled in the upper and lower chambers. The buffer was cooled by circulating water through a Multi-temp circulator (Pharmacia, Uppsala, Sweden). The electrical connections were made with the power pack (Pharmacia, Uppsala, Sweden). The protein samples prepared in sample buffer were loaded into the wells. The power supply was switched on and the current adjusted to 20mA.

At the end of the run, the power supply was switched off and the gel carefully removed from the glass plates and processed for staining.

3.2 A-C3) **Staining with coomassie brilliant blue R-250**

3.2 A-C3A) **Materials**

Coomassie brilliant blue-R-250 was purchased from HiMedia laboratories Pvt. Ltd., India. Methanol was purchased from Qualigens Fine Chemicals, India. Glacial acetic acid was purchased from E. Merck (India) Pvt.Ltd., India.

3.2 A-C3B) **Solutions**

- 0.25 g of Coomassie brilliant blue R-250 was dissolved in methanol. Methanol, glacial acetic acid and water were added to give a final ratio of 5:1:5. The solution was filtered through a Whatman No. 1 paper.

- The destaining solution was prepared by mixing methanol, glacial acetic acid and water in a 3:1: 6 ratio.

3.2 A-C3C) **Method**

The gel was immersed in five volumes of staining solution for 4 h.
The stain was removed and destaining solution added. Several changes of destaining solution were made till the proteins were observed. The gel was stored in 20% glycerol.

3.2 B) **SODIUM DODECYL GEL ELECTROPHORESIS OF THE CRUDE EXTRACTS OF SHIGELLA SPECIES**

3.2 B-A) **Materials**

Sodium lauryl sulphate, β-mercaptoethanol, bromophenol blue were purchased from Sigma Chemicals, USA. Tris buffer, hydrochloric acid, ammonium persulphate and glycine were purchased from Qualigens Fine Chemicals, India. Acrylamide and bis-acrylamide were procured from SISCO Research Labs. Pvt. Ltd., India.

3.2 B-B) **Solutions**

3.2 B-B1) **Stock acrylamide (T=30%)**

Acrylamide 73 g
Bis acrylamide 2 g

These were dissolved in distilled water and the volume made up to 250 ml.

3.2 B-B2) **Stock separating gel buffer or lower tris buffer**

SDS 1 g
Tris base 45.5 g

It was dissolved in less than 250 ml. The pH was adjusted to 8.8 with HCl and the volume made up to 250 ml.

3.2 B-B3) **Stock ammonium persulphate or upper tris buffer**

Ammonium persulphate 1.0 g
This was dissolved in 10 ml water and prepared fresh on the day of use.

3.2 B-B4) **Stock stacking buffer or upper tris buffer**

- SDS 1 g
- Tris base 15.1 g

This was dissolved in distilled water. The pH was adjusted to 6.8 with HCl and the volume was made to 250 ml.

3.2 B-B5) **Reservoir buffer**

- Glycine 28.8 g
- Tris base 6.0 g
- SDS 2.0 g

These were dissolved in distilled water and the volume was made to two litres.

3.2 B-B6) **Sample buffer**

- SDS 0.92 g
- β-mercaptoethanol 2 ml
- Glycerol 4.0 mg
- Tris base 0.3 g
- Bromophenol blue (0.1%) 2 ml

These chemicals were dissolved in distilled water. The pH was adjusted to 6.8 with HCl. The final volume was made to 20 ml.

3.2 B-C) **Method**

SDS PAGE was performed as per the method of Laemmli (1970).
- The glass plates were thoroughly cleaned and dried. The spacers were arranged and held in place either by brown tape or sealed...
with agarose. The chamber was clamped and held in an upright position.

Just before use, a sufficient volume of separating gel mixture was made as follows:

**Composition of separating gel (10%)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>8.3 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>6.2 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10.1 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>125 µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>250 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µl</td>
</tr>
</tbody>
</table>

It was poured into the chamber to a level 1 cm below the bottom of the comb and was overlayed with water saturated butanol to generate a flat gel. The mixture was left to set for 0.5 - 1.5h.

The stacking gel of 4% was prepared as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3 ml</td>
</tr>
<tr>
<td>Gel buffer</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Stock APS</td>
<td>15 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

It was prepared just before use and poured above the separating gel after washing off butanol from the top of the polymerized separating gel. The comb was inserted and the gel allowed to stand till it polymerized in 0.5-1h.

When the stacking gel had polymerized, the comb, clips and the bottom spacer were removed carefully and the gel sandwich was installed in the apparatus. The apparatus was then filled with the
reservoir buffer in the upper and lower chambers. The buffer was cooled by circulating it with water through Multi-temp circulator (Pharmacia, Uppsala, Sweden). The electrical connections were made with the power pack (Pharmacia, Uppsala, Sweden).

- The protein samples to be loaded were prepared by heating with the sample buffer for 4 min in boiling water. The sample solutions each having a concentration of 0.5 mg protein/ml were cooled and loaded into the wells with the help of a gel loading tip carefully through the reservoir buffer.

- The power supply was switched on after adjustment of current to 20 mA.

- At the end of the run i.e., when the bromophenol blue had travelled to the bottom of the gel, the power supply was switched off. The gel was removed from glass plates and staining was done as given in section 3.2 A-C3.

3.3) **PREPARATION OF THE CRUDE EXTRACT OF SHIGELLA flexneri**

3.3 A) **Materials**

Nutrient broth and nutrient agar were procured from HiMedia Ltd., India. Tris-HCl, EDTA were obtained from SRL Chemicals Ltd., Bombay, India.

3.3 B) **Solutions**

3.3 B1) **Nutrient Broth**

The contents and preparation was the same as given in section 3.1 B1.
3.3 B2) **Nutrient agar**

The contents and preparation was the same as given in section 3.1 B2.

3.3 B3) **Solution 1**

- 20 mmoles of NaCl
- 10 mmoles of Tris-Cl
- 1 mmole of EDTA

These were dissolved in 1 litre of distilled water. The pH was set to 8.0.

3.3 B4) **Solution 2**

- 10 mmoles of Tris base
- 1 mmole of EDTA

These were dissolved in 1 litre of distilled water. The pH was set to 10.5.

3.3 C) **Subculturing of Shigella flexneri**

The strains were maintained in nutrient agar plates and subcultured by transferring and streaking a single colony to fresh nutrient agar plate with the help of an inoculating needle. For the long term storage of the culture, stab cultures were made and kept in the dark at room temperature.

3.3 C1) **Growth**

The growth conditions were similar to that given in section 3.1 C1.
3.3 C2) **Harvesting**

The cells were obtained by centrifugation of the broth at 4000 rpm for 10 min in a centrifuge at 4°C. The supernatant was discarded. The pellet was washed with solution 1 and centrifuged. This step was repeated again and the supnatant discarded. The pellet was incubated with solution 2 at 50°C for 10 min. It was centrifuged at 12000 rpm for 30 min and the debris discarded. The clear supernatant obtained was used as the crude toxin.

3.4 **GEL FILTERATION CHROMATOGRAPHY**

3.4 A) **Materials**

Tris-HCl and Ethylene diaminetetraacetic acid were obtained from SRL Chemicals Ltd, India. Blue dextran was purchased from HiMedia Co. Ltd, Bombay, India. Sephadex G-100 was obtained from Sigma Chemical Company, USA.

3.4 B) **Solutions**

3.4 B1) **Buffer-1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

These were dissolved in distilled water. The pH was set to 7.4 and the volume made upto litre.

3.4 B2) **Blue Dextran**

0.02 g Blue Dextran of molecular weight 20,000,000 was weighed and dissolved in 10 ml of buffer 1.
3.4 C) **Method**

- The powdered Sephadex G-100 was added to excess buffer and the gel slurry was heated in a boiling water bath for 5 hours.
- This was allowed to cool and the finer particles removed by decantation of the solution after allowing the slurry to settle.
- More buffer was added to this slurry to obtain 75% settled gel and 25% supernatant buffer.
- The column (55 x 1.5 cm) was cleaned and wetted glass wool arranged at its bottom.
- The column was mounted on a vertical stand away from direct sunlight.
- A fine tubing was attached to its nozzle and a clamp fitted in the tubing.
- The gel slurry was poured gently into the column. This was performed using a column extender.
- The clamp was opened and the bed was allowed to equilibrate. Two to three bed washings were given with the buffer held in a Mariotte flask attached to the flow regulator (Pharmacia, Uppsala, Sweden).
- To check for heterogeneities in the column and to calculate its void volume, blue dextran was used. The flow rate was adjusted to 5 ml/h.
- The buffer was allowed to drain from the bed, till it reached the bed surface without allowing the bed to dry.
- The column outlet was closed and the sample, which had been brought to room temperature was layered on the bed. The bed was drained by opening the column outlet and closed again. A small amount of buffer was added and bed was drained again. The outlet
was then closed, the column was refilled with the eluent buffer and the outlet opened again.  

The void volume was discarded and fractions of 1ml were collected.

3.4 D) **Protein estimation**

This was done by the UV-spectrophotometric method taking the optical density at 260 and 280 nm in a Beckman UV spectrophotometer (Beckman 640B, USA) employing the formula as given in section 3.1 C3.

3.5 **POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE SHIGELLA flexneri PROTEIN**

3.5 A) **Materials**

The materials were the same as given in section 3.2A-A.

3.5 B) **Method**

The method was the same as given in section 3.2A-C.  

SDS PAGE was performed as per the method of Laemmli (1970).

3.5 C2) **Staining with Coomassie Brilliant Blue R-250**

**Materials:**

The materials and methodology is given in section 3.2 C3.

3.6) **SDS-PAGE-METHOD OF SCHAGGER AND VON JAGOW (1987) FOR SHIGELLA flexneri PROTEIN**

3.6.1 A) **Materials**

Acrylamide, bis acrylamide, tris base, TEMED and sodium lauryl sulphate were procured from Sigma Chemical Company, U.S.A. Hydrochloric acid, ammonium persulphate, tricine and glycerol were procured from Qualigens Fine Chemicals, Bombay, India.
3.6.1 B) **Solutions**

All the solutions were prepared in deionized water.

3.6.1 B1) **Acrylamide-bis acrylamide mixture**

To 240 g acrylamide added 7.5g bis. The volume was made up to 15 ml.

3.6.1 B2) **Anode buffer**

12.11g Tris base was dissolved in distilled water. The pH was adjusted to 8.9 with HCl and the volume was made upto 500 ml.

3.6.1 B3) **Cathode buffer**

To 6.06g Tris base, added 8.96g Tricine and 0.5g SDS. The pH was set to 8.25. The volume was made to 500 ml with water.

3.6.1 B4) **Gel buffer**

Added 36.3g Tris base to 0.3g SDS. The pH was adjusted to 8.45 with HCl after dissolving. The volume was made upto 100 ml.

3.6.1 B5) **10% amonium per sulphate**

It was prepared fresh at the time of use.

3.6.1 B6) **Glycerol**

87% solution was prepared.

3.6.1 B7) **Sample application buffer:**

Added 2g SDS, 6.9 ml glycerol, 0.302g Tris base, 1 ml of 2- mercaptoethanol and 500 mg of bromophenol to distilled water. The pH was adjusted to 6.8 with 4 N HCl. The final volume was made to 50ml.
3.6.1 C) Method

The solutions were prepared for separating gel, spacer gel and stacking gel as follows:

<table>
<thead>
<tr>
<th></th>
<th>Separating Gel (16.5%)</th>
<th>Spacer Gel (10%)</th>
<th>Stacking Gel (4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bis acrylamide (ml)</td>
<td>11.7</td>
<td>1.8</td>
<td>1</td>
</tr>
<tr>
<td>Gel buffer (ml)</td>
<td>11.7</td>
<td>2.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Glycerol (ml)</td>
<td>4.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Water (ml)</td>
<td>7</td>
<td>4</td>
<td>8.3</td>
</tr>
<tr>
<td>APS (µl)</td>
<td>175</td>
<td>43.8</td>
<td>125</td>
</tr>
<tr>
<td>TEMED (µl)</td>
<td>17.5</td>
<td>4.4</td>
<td>12.5</td>
</tr>
<tr>
<td>Total Volume (ml)</td>
<td>35</td>
<td>8.7</td>
<td>12.5</td>
</tr>
</tbody>
</table>

The separating gel was immediately poured after addition of APS and TEMED up to a mark 5.5 cm from the top of the plate in the gel cassette. Immediately the spacer gel was added to the marked position 2.5 cm from the top of the plate. It was left to polymerize.

The stacking gel was freshly made just prior to pouring. The comb was adjusted in place, the stacking gel poured on top of the separating gel and allowed to polymerize.

The horizontal spacer was removed after polymerization. After clamping the plates to the electrophoresis chamber, the buffer chambers were filled with the cathodic and anodic solutions. The chamber was cooled to 10°C with the help of Multi-temp circulator (Pharmacia, Uppsala, Sweden). The electrodes were connected and the gel run at 20
m\(A/100\) V till the dye travelled to the other end. The plates were then disassembled and the gel proceeded for staining.

3.6.2) Diamine silver staining

The method was a modification of Switzer et al. (1979) and Hochstrasser and Merril (1988).

3.6.2 A) Materials

Ethanol was obtained from Bengal Chemicals and Pharmaceuticals, India. Glacial Acetic acid, silver nitrate, citric acid and ammonium hydroxide were procured from Qualigens fine chemicals, India.

Glutaraldehyde was purchased from Boehringer- Mannheim, Germany.

3.6.2 B) Solutions

All solutions were prepared in deionized water.

3.6.2 B1) Fixation solution

40% ethanol, 10\(\% (v/v)\) acetic acid.

3.6.2 B2) Rehydration solution

5\(\% (v/v)\) ethanol, 5\(\% (v/v)\) acetic acid.

3.6.2 B3) Glutaraldehyde solution

2\(\% (v/v)\) glutaraldehyde was prepared prior to use.
3.6.2 B4) **Silver diamine solution**

3 g silver nitrate was added to 15 ml distilled water. Separately, 5 ml of concentrated ammonium hydroxide was added to 0.75 ml of 10 M sodium hydroxide in 80 ml of water in a 1 litre measuring cylinder. The silver nitrate solution was then added slowly. The final volume was made to 375 ml. This was prepared just prior to use.

3.6.2 B5) **Reducing solution**

0.02 g of 0.0005 M anhydrous citric acid was dissolved in 100 ml water and 0.2 ml of 0.007 M formaldehyde added to this. The volume was made up to 200 ml with cold distilled water and it was prepared just before use.

3.6.2 B6) **Stop solution**

To 20 ml acetic acid, 180 ml of distilled water was added.

3.6.2 C) **Method**

All the steps were performed at room temperature with gentle shaking.

- After electrophoresis, the gel was placed in fixation solution for 1h.
- It was transferred to rehydration solution for at least 3 h.
- It was transferred to 200 ml water and washed for 5 min.
- The solution was discarded and 100 ml of glutaraldehyde was added to each tray. It was soaked for 30 min.
- The glutaraldehyde solution was discarded and the samples washed 3 times with 150 ml of water for 10 min each.
- The washing solution was then discarded and the gel was washed four times with 150 ml of water for 30 min each.
The last rinse was discarded and silver solution added instead. The gel was soaked for 5 to 30 min in this solution.

The above solution was then poured off and the gel was rinsed with water briefly followed by 3 rinses of 5-10 min each for up to 30 min.

The image was developed with citric acid/formaldehyde solution for 1-2 min till the proteins took up the stain.

The reaction was stopped with acetic acid solution for 15 min.

The gel was then washed with 150 ml water for 5 min.

It was stored in clear plastic bags.

3.7) **POLYACRYLAMIDE GEL ISOELECTRIC FOCUSING**

It was performed on a ultra thin gel (0.5mm ) cast on Gel Bond PAG film (Pharmacia, Uppsala, Sweden).

3.7 A) **Materials**

Acrylamide, bis acrylamide, TEMED, ampholytes (3-10), calibration proteins of known pi and sorbitol were obtained from Sigma Chemical Company, USA.

Glycerol was purchased from Qualigens fine chemicals, Bombay, India. Tris-HCl was purchased from SISCO research laboratories, Pvt. Ltd., India.

3.7 B) **Stock solutions**

3.7 B1) **Acrylamide, Bis acrylamide**

29.1 g of acrylamide was mixed with 0.7 g of bis acrylamide and the volume was made up to 100 ml with distilled water.
3.7 B2) **Ammonium persulphate 40% (w/v)**

It was prepared by dissolving 400 mg APS in 1 ml of water.

3.7 B3) **0.25 M Tris-HCl buffer**

3.03 g of Tris base and 80 ml of distilled water were mixed and titrated to pH 8.4 with 4 N NaOH. The volume was made upto 100ml with water.

3.7 B4) **Glycerol 87% (w/v)**

It was prepared in distilled water.

3.7 B5) **Rehydration solution**

1 0.5 g sorbitol

880 µl Ampholyte (3-10)

It was prepared in 10 ml of water.

3.7 B6) **Anodic solution**

1 M Phosphoric acid was used.

3.7 B7) **Cathodic solution**

1 N Sodium hydroxide was used.

3.7 C) **Method**

The polymerization mixture was prepared as follows:

- Acrylamide / Bis acrylamide: 2.7 ml
- Tris - HCl: 0.5 ml
- Glycerol: 1 ml
- TEMED: 10 µl
The volume was made up to 20 ml with water. Lastly, 20 μl of APS was added.

- The polymerization mixture was then added to the prepared clamped gel cassette with attached GelBond PAG film (Pharmacia, Uppsala, Sweden) with the help of a syringe to which a fine tubing was attached.
- The gel was allowed to polymerize. After polymerization, the clamps were removed and the plates removed gently.
- The gel was washed by shaking in water for 20 min.
- It was then allowed to dry.
- The dehydrated gel was rehydrated in rehydration solution.
- The gel was placed on the plate of the Multiphor apparatus (Pharmacia, Uppsala, Sweden) with the film at the bottom and the gel on its top side.
- The plate was cooled to 10°C using Multi-temp circulator (Pharmacia, Uppsala, Sweden).
- The anodic and cathodic wicks were placed in their respective positions. The samples and the calibration protein of known pl were applied at different points.
- The gel was run under the following conditions.

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Current (mA)</th>
<th>Power (W)</th>
<th>Time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>700</td>
<td>12</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>500</td>
<td>8</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>2000</td>
<td>14</td>
<td>14</td>
<td>90</td>
</tr>
<tr>
<td>2500</td>
<td>14</td>
<td>18</td>
<td>10</td>
</tr>
</tbody>
</table>
3.7 D) **Staining**

After the run, the gel was stained with coomassie brilliant blue R-250.

3.7 D-A) **Materials**

Coomassie brilliant blue R-250 was purchased from Himedia Laboratories, Pvt. Ltd., India.

Ethanol was purchased from Bengal Chemicals and Pharmaceuticals, India. Glacial acetic acid was purchased from Qualigens Fine Chemicals, India.

3.7 D-B) **Solutions**

3.7 D-B1) **Coomassie brilliant blue R-250**

It was prepared by dissolving 0.05% CBB R-250 in ethanol in an acetic, ethanol and water mixture (19:25:65 ratio v/v). CBB was dissolved in ethanol. Acetic acid was added to water and then added to the dissolved CBB in ethanol.

3.7 D-B2) **Destain I**

Acetic acid-ethanol - water were taken in a 19:25:65 ratio v/v.

3.7 D-B3) **Destain II**

Acetic acid - ethanol - water mixture in a 1:1:8 ratio v/v.

3.7 D-C) **Method**

- The gel was immersed in the stain for 4-6 h.
- This solution was discarded and the destain I added. The gel was soaked for 4 h in this solution.
- Final wash was given with destain II for 2 h.
3.7 E) **Non diamine silver staining**

This method is a modification from that of Merril et al. (1981).

3.7 E-A) **Materials**

Glutaraldehyde was purchased from Boehringer-Mannheim, Germany.

Potassium dichromate was procured from E-Merck (India) Pvt. Ltd., India.

Formaldehyde, acetic acid, methanol, silver nitrate were purchased from Qualigens Fine Chemicals, India. Sodium carbonate was obtained from Himedia Laboratories Pvt. Ltd., India.

3.7 E-B) **Solutions**

3.7 E-B1) **Fixation solution**

50 ml methanol, 10 ml acetic acid were added to 40 ml deionized water.

3.7 E-B2) **Rehydration solution**

To 10 ml methanol, 5 ml acetic acid and 85 ml distilled water were added.

3.7 E-B3) **Glutaraldehyde solution**

2% glutaraldehyde was prepared in 1 litre distilled water. It was prepared just prior to use.

3.7 E-B4) **Dichromate solution**

To 0.034 M potassium dichromate and 0.032 M nitric acid was added. It was stored in a tightly capped dark bottle.
3.7 E-B5) **Staining solution**

0.0118 M silver nitrate was prepared in distilled water.

3.7 E-B6) **Reducing solution:**

To 30 g of sodium carbonate, 0.5 ml of 37% (v/v) formaldehyde was added and the volume made up to 900 ml with distilled water. The final volume was made to 1 litre. This was prepared fresh at the time of use.

3.7 E-B7) **Stop solution**

3% acetic acid.

3.7 E-C) **Method**

The steps were performed in glass tray with gentle shaking at room temperature.

- The gel was placed in fixation solution for 1h.
- It gel was transferred to fixation solution for 10 min.
- The rehydration solution was discarded and glutaraldehyde solution was then added. It was soaked for 30 min in this solution.
- The gel was washed for 1 min in distilled water.
- This was discarded and 200 ml of potassium dichromate solution was added.
- The dichromate solution was then discarded and the silver nitrate solution added. The gel was allowed to soak for 25 min in this solution.
- The silver nitrate solution was discarded and 50 ml of sodium carbonate/formaldehyde solution was added. This was changed before it turned grey with colloidal particles. This development was allowed to proceed for 1-3 min till the proteins became visible.
- The image development was stopped by the addition of acetic acid for 5 min.
- The stop solution was discarded and the gel washed 2 times with water for 10 min each.
- The gel was stored in moist clear plastic bag.

3.8) PROKARYOTIC TEST SYSTEM

3.8.1) REC ASSAY

3.8.1 A) Materials

Nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate were obtained from Boehringer Mannheim, Germany. Sodium dodecyl sulphate and potassium dihydrogen phosphate were purchased from Qualigens Fine Chemical, India. Potassium hydroxide and disodium hydrogen phosphate were products of E-Merck, India. Mitomycin-C was obtained from Biochemical Pharmaceuticals Limited, India. Sodium phenobarbital was purchased from Loba Chemicals, India. Luria broth, Nutrient broth, glucose, calcium nitrate, ammonium sulphate, manganese chloride, ferrous sulphate, tri-sodium citrate, potassium chloride and magnesium sulphate were obtained from Hi-media Chemicals, India.

3.8.1 B) Media

3.8.1 B1) Growth Medium

Luria broth was used as the growth medium.

Contents:

Tryptone 10g, yeast extract 5g, sodium chloride 5g.
Preparation

20g of Luria broth was dissolved in 1000 ml of distilled water. It was sterilized by autoclaving at 121°C for 15 min and poured into sterile petriplates.

3.8.1 B2) Modified Schaeffer's Medium

The following components constitute the medium.

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity per litre of distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient broth</td>
<td>169.0 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Manganese chloride</td>
<td>10.8 mg</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>278 µg</td>
</tr>
<tr>
<td>Calcium nitrate</td>
<td>236 µg</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

The pH was adjusted to 7.2-7.4 and nutrient broth, glucose and agar were added last of all. The mixture was autoclaved at a pressure of $10^{5}$ Pa for 20 min.

3.8.1 B3) Minimal Salt Solution (MM)

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity per litre of distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulphate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>0.5 g</td>
</tr>
</tbody>
</table>
The solution was neutralized with potassium hydroxide and used for washing and diluting the spores. It was autoclaved at a pressure of 15 psi for 20 min.

3.8.1 B4) Buffer Solution

8 \mu M magnesium chloride and 33 \mu M potassium chloride were prepared in 190 mM phosphate buffer and pH was adjusted to 7.2.

3.8.1 B5) Cofactor Solution

The following components constituted the cofactor solution:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity per millilitre of buffer solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP</td>
<td>40.0 mg</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>20.0 mg</td>
</tr>
</tbody>
</table>

The solution was filter sterilized before use.

3.8.1 B6) Metabolic activation mixture

Cofactor solution together with liver homogenate (S9) is known as S9 mixture (S9 mix) or metabolic activation mixture or activating mixture.

3.8.1 C1) Preparation of Spores

The spores were prepared by spreading overnight broth cultures of the strains rec\(^+\) (BD 170) and rec\(^-\) (BD 224) in different sets of sterile disposable petriplates on modified Schaeffer's agar medium. The plates were incubated at 37°C for 3 to 5 days for BD 170 and BD 224 strains respectively.

The spores were scrapped up, washed once with MM and resuspended in fresh MM. They were treated with lysozyme (2 mg/ml) and SDS (1% final concentration) for 30 min.
each. The detergent was extracted out with subsequent washings with sterile distilled water and spores were stored at 4°C till use.

3.8.1 C2) **Preparation of S9**

In order to mimic the metabolic activation in vitro, rat liver homogenate (S9) was prepared. The rats were treated with sodium phenobarbital (100 mg/kg body weight in normal saline) for 3 days in drinking water and sacrificed after 3 days. The liver homogenate was prepared under sterile conditions and supernatent solution was obtained following centrifugation at 9000xg for 10 minutes.

3.8.1 D) **Methodology**

The bacterial strains *Bacillus subtilis* BD 224 (Rec−) and *Bacillus subtilis* BD 170 (Rec+) were used. These were grown overnight in Luria agar plates and subcultured and the spores prepared. To mimic the in vitro conditions, rat liver homogenate (S9) was prepared. Duplicate cultures were set up with and without activation.

2x10^6 spores and 0.3ml S9 were placed in a sterile disposable petriplate and 10 ml of 0.8% soft agar which had previously been autoclaved and kept at 43°C was poured over it. The contents were mixed uniformly. After solidification the petriplates were kept for cold incubation at 4°C for half an hour. 20 µl of test chemical (supernatant/lysate/buffer) were impregnated at appropriate plates and the plates incubated at 4°C for 12-15 h followed by incubation at 37°C for 20-24 h. Addition of S9 and cofactor solution was omitted for cultures without metabolic activation. The size of the inhibition zones was measured for the Rec-positive bacteria and compared with those of the Rec-negative. The term was expressed as Rec effect and could be
classified as (-) if there was no difference, (+) for non significant, but slightly positive or negative, (+) for positive with 3-5 mm zones.

3.9) EUKARYOTIC TEST SYSTEM

3.9.1) HeLa Cell Assay

3.9.1 A) Materials

HeLa cells were purchased from the National Centre for Cell Science (NCCS), Pune. RPMI-1640 and fetal calf serum were purchased from Sigma Chemical Company, USA. Minimum Essential Medium with glutamine was purchased from Flow Laboratories, USA. Hydrochloric acid and sodium hydroxide were procured from Qualigens fine chemicals, India. Sodium bicarbonate was obtained from HiMedia Laboratories, India.

3.9.1 B) Solutions

RPMI 1640 with L-glutamine and without sodium bicarbonate

Sodium bicarbonate: 2g
IN Hydrochloric acid
IN Sodium hydroxide

To prepare 1 litre media, 900 ml of distilled water was taken and the powder added to it with gentle stirring.

The pH was lowered to 4.0 with IN HCl to dissolve the contents. Sodium bicarbonate was then added. The pH was adjusted to 7.1 with IN NaOH. The volume was made to 1 litre with distilled water. It was sterilized by filtration through a sterile 0.22 µ filter.

MEM with L-glutamine without sodium bicarbonate
To prepare 1 litre, weighed 10.92 g MEM and added bicarbonate (2 g/L) gently. The pH was adjusted to 7.1 with 1N HCl or 1N NaOH. The volume was made up to 1 litre. It was sterilized by membrane filtration through a 0.22 μ filter.

3.9.1 C) **Tissue Culture**

3.9.1 C1) **Growth and Subculturing**

HeLa cells were grown in RPMI medium supplemented with 10% FCS in 25 mm/75 mm tissue culture flasks with canted necks in a humidified 5% CO₂ incubator at 37°C. The medium was changed routinely and a confluent monolayer of cells obtained in 7 days. For subculturing, the cells were pipetted vigorously with the help of a Pipet-Aid, centrifuged at 1000 rpm, counted on a Neubuer tissue culture flask and the volume made up with fresh medium. Fresh medium was also added to the original flask.

The cells were routinely checked under an inverted microscope.

3.9.1 C2) **Assay**

To assess the percentage of surviving cells, the spectrophotometric method of Gentry and Dalrymple (1980) was followed.

3.9.1 C2-1) **Solutions**

3.9.1 C2-2) **Buffer-A**

0.067 M PBS, pH 7.2 was prepared.

3.9.1 C2-3) **Fixative**

2% (v/v) formalin was prepared in Buffer-A.

3.9.1 C2-4) **50% ethanol**

It was prepared in distilled water
3.9.1 C2-5) **Crystal violet dye**

0.13% crystal violet was prepared in 5% ethanol - 2% formalin - 0.067 M PBS

3.9.1 C2-6) **Sterile distilled water**

This was prepared by passing distilled water through a sterile 0.22 μ filter.

3.9.1 D) **Method**

- Approximately 1.6 x 10^4 cells were seeded per well in a volume of 0.1 ml MEM supplemented with 10% FCS in a 96 - well microtitre plate (Linbro Laboratories, USA)
- The cells were allowed to adhere for 24h in a humid 5% CO₂ incubator at 37°C. The cells were exposed to 2 - fold serial dilutions of the toxin in MEM.
- Appropriate blanks were made without addition of the toxin.
- These cells were then kept exposed overnight. The toxin/medium was removed and the cells washed with buffer.
- Cells were fixed in the fixation solution for 1 min. The fixative was removed with a multi-tip multipipette (Nichyro, Japan).
- The dye was then added to the wells and staining allowed to proceed for 20 min.
- The excess stain was removed by washing with distilled water.
- The plates were then allowed to air dry. For quantitation, 50% ethanol was added to each well and the volume made upto 0.1 ml with distilled water. The concentration of the dye was determined by measuring the absorbance in an ELISA reader at 570 nm.
- The dilution of the toxin producing 50% cell detachment i.e. 50% of the dye uptake was measured.

3.9.1 E) **HEAT TREATMENT**

3.9.1. EA) **Method**

Samples of the toxin were heated on a thermal cycler (MJ Research Company, USA) and subjected to heat at 50, 55, 60, 65 and 70°C. The samples were held at each of these temperatures for 30 min. Thereafter the temperature was brought down to 37°C and then assayed for toxic activity as described in 3.9.1 D.

3.9.1 F) **EFFECT OF REDUCING AGENTS**

3.9.1 FA) **Materials**

2-mercaptoethanol and dithiotheritol were purchased from Sigma Chemical Company, USA.

3.9.1 FB) **Method**

Samples of the purified toxin were incubated for 2h with equal volume of 1 mM dithiothreitol and 0.1M 2-mercaptoethanol. This was then replaced with the fresh media and assayed for toxic activity as described in section 3.9.1D.

3.9.1 G) **EFFECT OF PROTEOLYTIC ENZYMES**

3.9.1 GA) **Materials**

Trypsin, chymotrypsin and pepsin were purchased from Qualigens Fine Chemicals Ltd., India. Proteinase K was purchased from Sigma Chemical Company, USA.
3.9.1 GB) Method

Samples of the purified protein were incubated for 2 h with equal volume 100 U of trypsin, chymotrypsin and pepsin. This was then replaced with the fresh media and assayed on HeLa cells as described in section 3.9.1D.

3.9.2) RABBIT SKIN TEST

3.9.2 A) Materials

Tris-HCl and ethylene diamine tetraacetic acid were obtained from SRL chemicals, Ltd, India. Rabbits were procured from the Institute of Microbial Technology, Chandigarh.

3.9.2 B) Solutions

3.9.2 B1) Buffer 1

   Tris-HCl 0.5 mole
   EDTA 1 mole

   These were dissolved in distilled water and the volume made up to 1 litre.

3.9.2 C) Method

   The back of the rabbits were shaved. A grid was prepared in it with a marker pen. 50 μl of the purified toxin from Shigella flexneri (F-12), 150 μl of the supernatant from Shigella flexneri, 50 μl of the buffer and 50 μl of sterile distilled water were injected in duplicate in the grid.

   The rabbits were kept for observation for any sign of redness or inflammation or blister formation routinely for a period of 7 days.