MATERIALS AND METHODS
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3.1 Studies with the whole biomass of parent and mutant Bacillus thuringiensis strains

At Vector Control Research Centre, the mosquitocidal activity of two strains of B. thuringiensis var. israelensis (Balaraman et al., 1981; Balaraman and Hoti, 1987) have been reported. They are the wild type strain VCRC B17 and the mutant of B17 called VCRC MB24. The present study was undertaken to ensure their safe use in the environment.

Parallel toxicity studies on the parent and mutant Bacillus thuringiensis var. israelensis (Bti) strains were conducted. The two Bti strains namely VCRC B17 (wild type) and its mutant VCRC MB24 were mass cultured in a 100 litre fermentor using NYSM medium. Sampling was carried out at frequent intervals from the 20th hour of inoculation to check for sporulation and crystal formation. The cells were harvested after complete sporulation by spinning at 10,000rpm, lyophilised and stored at 4°C. The Colony Forming Units (CFUs) of these preparations was found to be $2 \times 10^{14}$ per mg for VCRC B17 and $2 \times 10^{12}$ for VCRC MB24. These data were used for the dose calculation and feeding the experimental animals.

3.1.1 Determination of LD50 with rats

An attempt was made to determine the LD50 values for both parent and mutant Bti strains. Such studies can also indicate the probable target organs of the toxins and their specific effect and provide guidance on the doses to be used in more prolonged studies.
LD50 test was carried out according to the method of Pizzi et al. (1950) in animals of both sexes because of their differences in susceptibility.

Adult albino rats of Wistar strain of an inbred colony were used for this experiment. Animals were divided into six different groups each consisting of 10 animals. Group-I consisted of control animals which were treated with saline and the animals in Group-II to Group-VI were, after overnight fasting, fed orally with 10mg, 100mg, 1gms, 2gms and 4gms per Kg body weight of the whole biomass of the parent and mutant strains respectively using a stomach tube. Body weight and food intake were checked before and during the experimental period. The animals were maintained for 14 days and closely observed for mortality or development of any toxic symptoms and behavioral changes. At the end of the experimental period the animals were sacrificed and gross checking of the vital organs for any abnormality was carried out.

3.1.2. Acute Toxicity studies (Single exposure studies)

This involves a single administration of the test samples and observing for toxic symptoms for a period of 21 days. Acute toxicity tests were done following the guidelines given by H.D. Burges (1981). He suggests that single heavy dose not exceeding 5gms per Kg body weight by oral and $10^7$ spores per rat by parenteral routes can be used to assess the acute toxicity. Based on this, acute toxicity tests were carried out by treating the rats with lyophilised biomass of mutant and parent Bti strains through different routes.

Routes of Administration: For determining acute toxicity the test samples were administered through different routes like oral, intraperitoneal and dermal.
Animals: Adult albino rats of both sexes of Wistar strain of an inbred colony were used for this experiment.

3.1.2.1 Acute oral

A 21 days oral toxicity study was performed with albino rats (4 months old) of both sexes. Sixty rats (30 male & 30 female) were separated into 3 groups, each consisting of 10 males and 10 females. The animals were observed for a period of 10 days before start of the experiment under standard animal house conditions with regular checking of food intake and body weight. After the initial period of observation the test animals in group 2 and 3 were fed orally with 2ml saline containing $2 \times 10^8$ cells of parent and mutant respectively with the aid of a stomach tube. Control animals were fed with an equal volume of saline.

The animals after the treatment with the test samples were observed daily for mortality, signs of illness, behavioral changes etc. for 21 days. During the experimental period, food intake and animal weights were recorded weekly to determine food intake/day/rat and weight gain/week/rat. At the end of the experimental period both control and test animals were sacrificed and grossly checked for pathological or toxicity symptoms in the vital visceral organs.

3.1.2.2 Acute intraperitoneal

Sixty rats (30 male and 30 female) of the same age group (4 months old) were isolated and kept under observation for food intake and body weight for a week. They were then divided into three equal groups, Group-I served as control and Group-II and III were treated with parent and mutant respectively. A dose of $2 \times 10^8$ cells in 0.5ml of saline was injected intraperitoneally into the
test animals and the control animals received an equal volume of saline. After treatment the animals were maintained for 21 days during which they were observed daily for signs of illness or mortality. There was a regular checkup of food intake and body weight. These data were recorded to determine the food intake/day/rat and the weight gained/week/rat. After the expiry of 28 days the experiment was terminated by sacrificing the animals. The vital organs were grossly checked for pathological lesions.

3.1.2.3 Acute dermal

Substances that come in contact with the skin may be absorbed through the skin in amounts sufficient to produce systemic toxicity at a site remote to the site of application. Acute dermal toxicity test gives an estimation of the hazard caused by a compound when it is absorbed exclusively through the skin and no other route of entry is involved.

Sixty rats (30 male and 30 female) of the same age group (3 months old) were isolated and kept under regular observation for food intake and body weight for a week. The animals were then separated into three equal groups. Group-I served as control, Group-II and III served as test animals.

The fur (hair) on the flanks and stomach of the animals (both control and test) were shaved off on either side for an area of 2cm², 24hrs prior to the application of the test sample. The skin on one side was abraded by drawing a hypodermic needle in straight parallel lines over the entire shaved application area. A suspension of 0.25 ml containing 2x10⁶ cells of parent was applied closely to the shaved skin of each of the animal in Group-II. In the same way Group-III animals were treated with 2x10⁶ cells of mutant. After treatment the treated area was covered with an
impervious plastic sheet to prevent evaporation and drying of the sample. The control animals were treated in the same way with an equal volume of saline. After 24hrs the plastic sheet was removed and the application site was sponged with a moist cloth to remove the unabsorbed test material and observed closely for development of any toxicity symptoms like erythema or oedema. Again after 72hrs, the application site was checked in the same way. The animals were maintained for 14 days and closely observed for any behavioural changes, signs of toxicity or mortality. At the end of the experiment all the animals were sacrificed and grossly checked for pathological changes in the vital organs and the data were recorded.

3.1.3 Mucous membrane irritation test

Accidental contact between pesticides and mucous membranes may occur as a splash or unintended ingestion. For these reasons it is of utmost importance to be able to evaluate the mucous membrane irritancy potential of these materials.

Ten male albino rabbits with body weights ranging from 1.5 to 2.0 kg were isolated and kept under observation for 7 days for normal food intake and maintenance of body weight. Two animals served as control and the remaining eight animals were divided into two groups, each consisting of four rabbits. They were treated orally with the cells of parent and mutant strains respectively at a concentration of 0.5gms in 2.0 ml of sterile water using syringe and 20 gauge needle with a rounded tip. The throat of the animal was rubbed externally to induce swallowing. The control animals were given an equal volume of sterile distilled water. The animals after treatment were left for overnight fasting. Half of the animals were sacrificed at 24 and 48hrs after dosing. The oesophagus and stomach were opened and observed for signs of irritation, mucous
membrane sloughing, ulceration and necrosis, etc. and the scoring was done according to Draize's (1959) procedure.

3.1.4. Short term oral Toxicity studies with rats

Short term toxicity studies involve repeated administration usually on a daily or 5 times/week basis over a period of 10% of the life span namely 3 months in rats. Accordingly, a 90 days oral repeated exposure study with both parent and mutant strains was performed in rats.

Adult albino rats of both sexes, (Wistar strain of an inbred colony) were used for this experiment. Rats in two different sets were divided into 4 groups consisting of 20 animals each (10 male and 10 female). Group I served as control. The animals in Group II, III and IV were fed with 3 different doses of the test materials (VCRC B17 & VCRC MB24) namely $10^6$, $10^9$, $10^{10}$ CFUs respectively, in 1.0ml of saline using a feeding tube. Animals of group I were identically treated with 1.0ml of saline. Food intake and weight gain of the animals were checked weekly throughout the experimental period. Animals were killed by decapitation at the end of the experimental period i.e., on day 91 of the treatment period. Before decapitation, body weight of each animal, bleeding time and clotting time were recorded.

Blood was collected directly from the jugular vein into tubes containing heparin solution for haematological examination and without anticoagulant for serum separation which was used for biochemical estimations.

The liver, heart, kidney, adrenal, brain, spleen and testes or ovary were removed and weighed individually and the organ/body weight ratio was calculated.
Small pieces of stomach and intestine were fixed in 10% formal saline for histopathological examination. The tissues were processed by routine manual process with 3 changes in absolute alcohol for dehydration and 1 change in xylene for clearing and 3 changes in paraffin wax. They were finally embedded in paraffin wax. Sections of tissues were cut using a rotary microtome and affixed to clean microslides with Mayer's albumen adhesive. The slides were placed on the hot plate maintained around 50°C and the sections were arranged properly with mounted needles. The slides were stained with eosin and haematoxylin and stored in dust-free slide boxes.

The various haematological parameters such as haemoglobin, RBC, WBC, platelet count, differential count of WBC, bleeding time and clotting time were estimated using standard procedures (Dacie and Lewis, 1975).

The serum that was separated was used for analysing various biochemical parameters, which are as follows:

i. The sodium and potassium levels by flame photometry.

ii. The serum glucose level by ortho toluidine method (Sasaki and Matusi, 1972)

iii. The serum cholesterol level by using ferric chloride reagent (Zak, 1977).

iv. Total protein by the method of Lowry et al. (1951).

v. Urea level by using diacetyl monoxime colour reagent (Natelsoo, 1957) and

vi. The serum levels of enzyme alkaline phosphatase was assayed by the method of Kind and King (1954) and transaminases SGOT and SGPT using the method of Reitman and Frankel (1957).
3.1.5 Mutagenicity by Chromosomal aberration test

Chromosomal aberration test is one of the short-term screening tests for detecting mutagenicity of a test material. The production of chromosomal aberrations was studied following the method of MacGregor and Varley (1983). According to this method, the somatic chromosome preparations are made from bone marrow of animals 24hrs after the administration of the test materials as the mitotic cycle in the tissue is completed in 20-24hrs. The chemical agents, which cause chromosomal aberration (damage) are known as "Clastogens" and this aberration involves major damage to chromosomes and hence to the DNA.

In this test, Male albino rats (Wistar strain of an inbred colony) weighing between 175-190gms were used in this experiment. Rats were divided into 5 groups (each group consisting of 3 animals) and placed separately in polypropylene cages and were provided with pelleted diet and water ad libitum. Group I served as control and two different doses namely, 1mg and 10mg cells of parent strain homogenised in 1ml of sterile distilled water was administered intraperitoneally to the animals in groups II and III respectively. Groups IV and V were treated in the same way with the mutant strain. The control group of animals were administered with distilled water. Animals were sacrificed after 24hrs and 1mg Colchicine in 1ml distilled water (to arrest mitotic division at metaphase stage) was administered intraperitoneally 1.5hrs prior to sacrifice. The femur bones were extricated and the bone marrow was flushed out with physiological saline. The cells were centrifuged and treated with hypotonic (75mM) KCl at 37°C for 30 minutes. Then cells were centrifuged and fixed in methanol/acetic acid (3:1) fixative. Slides were prepared with a good spread of metaphase plates by dropping the cells on ice-cold glass slides. The slides were dried immediately by keeping them on a warm plate and stained.
with 5% Giemsa for 5 minutes. The slides were washed with distilled water, dried and screened for chromosomal aberrations such as gaps, breaks, exchange, acentric fragments, ring etc.

### 3.1.6 Carcinogenicity by Microsomal degranulation test

Microsomal degranulation test is a rapid, reliable and inexpensive method for the detection of the carcinogenicity of environmental pollutants. As it has been shown by Butler (1966) and Jagota and Dani (1981), that ribosomes detach from rough endoplasmic reticulum (RER) on administration of carcinogens to animals, it was suggested by Williams and Rabin (1971) that measurement of degranulation of RER would provide an useful index to the carcinogenic potential of test compounds.

In this study, liver microsomes of rats treated with mutant and parent strains were used for the detection of carcinogenicity. The liver provides a good model system for the study of carcinogen induced degranulation for two reasons: firstly, it is a rich source of rough EPR and secondly, it has a metabolic capacity required to generate active forms of carcinogen from precursors. A direct method of monitoring ribosome loss is by estimating RNA/Protein and RNA/Phospholipid ratios of membranes. These ratios will decrease in degranulated membranes due to the loss of RNA with the ribosomes.

Male albino rats (Wistar strain weighing 120-150gms each) were divided into seven groups each consisting four animals. Group I served as control. Group II and III animals served as positive control and were administered orally with 20 and 40mg Dianizidine in 0.5ml DMSO respectively. The animals in Groups IV, V, VI and VII were fed orally with two different doses
i.e. 20 and 40mg of parent and mutant respectively for one month. The control animals were treated the same way with 0.5ml DMSO. At the end of the experimental period the animals were sacrificed and the livers isolated.

Four grams of sliced rat liver was washed thrice with 0.225M Sucrose-Tris (S-T) buffer (0.225M of Sucrose and 25mM Tris at pH 7.5), minced and homogenized with 10ml of S-T buffer in a Potter-Elvehjem apparatus at 4°C using a motor driven teflon pestle rotating at 5000 rpm for 20 minutes. The supernatant was treated with an equal volume of 8mM CaCl₂ and stirred using a magnetic stirrer for 30 minutes and centrifuged at 5000 rpm for 20 minutes at 4°C (Kamath and Ananth Narayan, 1972). The precipitate (microsomal fractions) obtained was weighed and used for the estimation of RNA, Protein and Phospholipids.

RNA was estimated according to a modified method of Munro and Fleck (1966) after the original method of Schmitt and Thannhausar (1945).

Lipids were extracted by the method of Folch et al. (1957). Phospholipids were estimated according to the method described by Ames (1966).

Protein concentration was estimated by the method of Lowry et al. (1951).

From the above values RNA/protein and RNA/Phospholipid ratio of the rat liver microsomes were calculated based on which the percent degranulation was determined.
3.2 Studies with the intact and solubilized Insecticidal Crystal Proteins (ICPs)

Earlier reports have stated that the crystal toxin when solubilized has haemolytic and cytotoxic effects. Hence experiments were designed to look for the effect of solubilized ICPs of Bti strains, VCRC B17 and VCRC MB24 in vivo and in vitro at molecular level on biomembranes in comparison to that of intact ICPs.

Purification of the crystal toxin: The cultures of parent and mutant were grown at 30°C in NYSM medium. Smears were taken at 6hrs intervals from the 24th hour of inoculation and checked under the microscope for sporulation and crystal formation. The process was continued until most of the cells had lysed and parasporal bodies are released (4 to 5 days). The spores and parasporal bodies were harvested by centrifugation at 12,000 rpm for 20 minutes at 4°C and washed thrice in cold double distilled water. Parasporal bodies were then isolated by isopycnic centrifugation at 25,000g in a sorvall SS34 rotor for 3hrs in 50% (wt/vol) Sodium Bromide gradient (Ang & Nickerson, 1978). With this method crystals are buoyant at the top of the centrifuge tube, whereas spores and spore-crystal aggregates are formed into a pellet at the bottom of the tube. The top crystal layer was collected, washed with cold double distilled water three times, checked for purity, lyophilised and stored at -70°C.

Solubilization of the Crystal: The lyophilised crystals were solubilized (2mg/ml) in 0.05M NaOH (pH 12) for 5hrs at room temperature (Bulla et al., 1979). The pH of the resulting mixture containing soluble crystal protein plus insoluble crystal residue, was lowered to pH 8.0 by using 0.5M Tris HCl Buffer (pH 8.0) and the mixture was clarified by centrifugation and preserved at 4°C until use.
Protein estimation: The protein content of intact and solubilized insecticidal crystal proteins (ICPs) of both the Bti strains was determined by Lowry's method (1951).

3.2.1 Tests with Human erythrocytes in vitro

Effect of intact and solubilized ICPs of both parent and mutant Bti strains on erythrocytes of normal human blood samples in vitro was determined by three different tests, viz., determination of lipid peroxidation, Osmofragility test and estimation of membrane bound enzymes.

3.2.1.1 Determination of lipid peroxidation

The principal elements of biological membranes are phospholipids and proteins. Phospholipids which contain unsaturated fatty acids are vulnerable to lipid peroxidation damage. Lipid peroxidation (LPO) is a form of oxidative degeneration of polyunsaturated lipids. The cytotoxic effect of inclusion proteins has been established by several workers and hence, the effect of solubilized ICPs from parent and mutant Bti strains on erythrocyte membrane lipid was studied by the modified method of Stocks and Dormandy (1971). The test was carried out by incubating the RBC's with two different concentrations of intact and solubilized ICPs of mutant and parent Bti strains corresponding to 50 and 100μg protein and then drawing 0.2ml of the sample at hourly intervals and then estimating the level of Malondialdehyde (MDA) released.

In a test tube, 0.2ml of 1% RBC was suspended in 0.8ml of Phosphate buffered saline. To this 0.5ml of 10% TCA was added, followed by 2.0ml of 1% thiobarbituric acid (TBA) and 0.075ml of 0.1mol EDTA. The contents of the test tubes were then mixed and kept in a boiling
water bath for 15 minutes, cooled to room temperature, centrifuged and the absorbance of the supernatant was read at 532nm using a spectrophotometer. Each test sample had its own blank tube which was not boiled. Subtraction of absorbance of unboiled test tube from boiled test value eliminated any increase in absorbance due to non-TBA reactive material (if any) in blood. EDTA was added to chelate any iron or other metal in the extract which otherwise can initiate lipid peroxidation during boiling and may result in falsely elevated TBA reactivity.

The effect of ICPs on the erythrocyte lipid peroxidation was expressed in terms of nanomoles of MDA released.

3.2.1.2 Osmotic fragility test

The haemolytic activity of the ICPs was evaluated by performing osmotic fragility test as described by Dacie (1960). The test was performed after incubating the fresh human blood sample with intact and solubilized ICPs of mutant and parent strain at a concentration of 100μg protein for 60 minutes.

Stock solution of buffered Sodium Chloride (10%): This was prepared by dissolving 90gms of Sodium Chloride, 13.65gms of Disodium Hydrogen Phosphate and 2.43gms of Sodium Dihydrogen Phosphate in distilled water and made upto one litre.

NaCl (1%): One hundred ml of the stock solution was diluted to one litre. Further dilutions giving solutions equivalent to 0.9, 0.8, 0.7, 0.65, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, 0.3, 0.2 and 0.1gms% NaCl were made and stored at 0°C.
A volume of 0.05 ml of blood was added to 5.0 ml volumes of the range of hypotonic solutions and immediately mixed by inverting several times. The tubes were allowed to stand at room temperature for 30 minutes, then remixed and centrifuged for 5 minutes at 1200–1500 g. The amount of lysis in each tube was compared with that in the 100% lysis tube (0.1g% NaCl) at 540 nm. The supernatant from the 0.9gms% NaCl tube was used as the blank. The percentage lysis was plotted against Sodium Chloride concentration and the degree of lysis was recorded in the form of a fragility curve.

3.2.1.3 Determination of membrane bound enzymes

Human erythrocytes were isolated and washed 4 times with PBS, (pH 7.4) and then incubated with the solubilized ICPs of parent and mutant, corresponding to 50 and 100 μg protein for 1 hr. The effect of solubilized ICPs was compared with that of intact ones by incubating RBC's with intact crystals corresponding to 100 μg protein.

The erythrocyte membrane was isolated according to the procedure of Dodge et al., (1967). The packed cells were haemolysed by treating them with a hypotonic buffer (pH 7.2) in the ratio of 14:1 (buffer:cells) overnight. Ghosts were sedimented in a high speed centrifuge at 20,000 g at 4°C for 40 minutes. The supernatant was decanted carefully and the ghost button was resuspended by swirling and washed 3 times with phosphate buffer pH 7.4 till the supernatant after the last wash was either pale pink or colourless.

The pellet of the erythrocyte membrane was suspended in 0.32M sucrose solution and homogenized to get 1% homogenate. 0.1 ml aliquotes of this preparation were used for the
estimation of protein and to determine the activity of membrane bound acetyl cholinesterase and Na⁺K⁺ATPase.

3.2.1.3.1 Estimation of Acetylcholinesterase

Acetylcholinesterase is a specific enzyme that catalyses the hydrolysis of a neurotransmitter acetylcholine yielding acetate and choline and the activity of this enzyme was determined following the procedure given by Hestrin (1949).

The acetyl group reacts with alkaline hydroxylamine to form acetyl hydroxamate. This hydroxamate then reacts with ferric chloride in acid medium to form a coloured complex which can be read at 540nm.

Reagents:

0.01M Acetylcholine chloride.
1.0M NaCl
1.0M MgCl₂
0.5M Tris-HCl buffer, pH 7.5.
0.2M EDTA
2.0M Hydroxylamine hydrochloride
3.5M NaOH

Mix the reagents 2.0M Hydroxylamine hydrochloride and 3.5M NaOH in 1:1 v/v proportion before use.

6.0N HCl
0.37M ferric chloride in 0.1 M HCl
To 0.1ml of erythrocyte homogenate, added 0.13ml of NaCl, 0.02ml of MgCl₂, 0.05ml of Tris, 0.002ml of EDTA, 0.2ml of acetylcholine chloride and incubate for one hour at 37°C. A control was also set up simultaneously which had all the reagents except the enzyme.

After incubation, the reaction was arrested by adding 2.0ml of alkaline hydroxylamine (reagent No 8) to all the tubes followed by the addition of the enzyme to the control tubes. This was by the addition of 1.0ml each of hydrochloric acid and ferric chloride to all the tubes and the colour developed was compared with that of standard acetylcholine chloride (0.01M) at 540nm.

The enzyme activity was expressed in terms of micromoles of acetylcholine chloride hydrolysed/min/mg protein.

**Na⁺K⁺ATPase:**

Activity of this enzyme was determined following the method of Bonting (1970).

**Reagents:**

- **Tris-HCl buffer**: 184mM, pH 7.5
- **MgSO₄**: 50mM
- **KCl**: 50mM
- **NaCl**: 600mM
- **EDTA**: 1mM
- **ATP**: 40mM
- **TCA**: 10% solution
To 1.0ml of Tris buffer, 0.2ml each of the reagents 2, 3, 4, 5 and 6 were mixed so that the final volume of 2.0ml contained 92mM Tris buffer, 5mM MgSO₄, 60mM NaCl, 5mM KCl, 0.1mM EDTA and 4mM ATP. After 10 minutes equilibration at 37°C, the reaction was started by the addition of 0.1ml of RBC membrane. The assay medium was incubated for 15 minutes at 37°C and at the end of the incubation period the reaction was stopped by the addition of 1.0ml of 10% TCA.

The amount of inorganic phosphate liberated from ATP was estimated by the method of Fiske and Subbarow (1925). The enzyme activity was expressed in terms of micromoles of phosphate liberated per minute per mg of protein.

3.3 Primary skin irritation test on Rabbits

Primary irritants are those substances which produce localised damage at the point of contact as cytotoxic action, which may histologically be observed as an inflammatory response.

This test was carried out in albino rabbits following the procedure of Draize et al., (1944) to evaluate the effect of solubilized ICPs of parent and mutant on intact and abraded skin sites.

Adult male albino rabbits weighing 1.5 to 2.0kg were used for this study. The animals were divided into 6 groups, each with 3 animals. Group I served as control and was treated with saline; Group II animals were treated with 0.5% formalin and served as positive control. Animals in group III and IV were treated with the intact and solubilized ICPs of parent and those in V and VI were treated with intact and solubilized toxin preparations from mutant strain respectively at a dose level corresponding to 100µg protein. Prior to application of the test material the hair from
the flanks of rabbits were removed for one square-inch area and the skin on one side was abraded with blunt hypodermic needle. This abraded skin application sites are used to ensure that the chemical contacts non intact skin. It is important to evaluate how such skin sites will react to the chemical irritant. On each site designated as abraded skin, a total of four aberrations (one inch long) were made on the shaved skin with the help of a blunt needle. Care was taken to ensure that only stratum corneum was disrupted and scratches were not deep enough to produce bleeding.

The test materials were applied to the one square inch area of both intact and abraded sites of the test animals. The entire trunk of the body was wrapped loosely with an impervious plastic sheet which was tapped in place to allow circulation of air. The control and positive control animals were also treated the same way. The animals were immobilised in a restrainer for 24hrs and then the impervious wrapper and covering bandages were removed. The application sites were sponged with damp cloth and observed for development of erythema and oedema at 24 and 48hrs. The readings were tabulated from which the Primary Irritation Index (PlI) was calculated according to Draize (1959) technique. Primary Irritation Index can be defined as the average sum of erythema and oedema for all sites on all rabbits. Indices below 3 are designated as slightly irritating. Indices from 3 to less than 5 are moderately irritating and indices of 5 or more are severely irritating.
Tabulation of Skin Responses by Draize Technique:

<table>
<thead>
<tr>
<th>Response</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema and eschar formation</td>
<td></td>
</tr>
<tr>
<td>No erythema</td>
<td>0</td>
</tr>
<tr>
<td>Very slight erythema (barely perceptible)</td>
<td>1</td>
</tr>
<tr>
<td>Well-defined erythema</td>
<td>2</td>
</tr>
<tr>
<td>Moderate to severe erythema</td>
<td>3</td>
</tr>
<tr>
<td>Severe erythema (beet red) to slight eschar formation (injury in depth)</td>
<td>4</td>
</tr>
<tr>
<td>Oedema formation</td>
<td></td>
</tr>
<tr>
<td>No oedema</td>
<td>0</td>
</tr>
<tr>
<td>Very slight oedema (barely perceptible)</td>
<td>1</td>
</tr>
<tr>
<td>Slight oedema (edges of area well defined by definite raising)</td>
<td>2</td>
</tr>
<tr>
<td>Moderate oedema (raised approximately 1mm)</td>
<td>3</td>
</tr>
<tr>
<td>Severe oedema (raised more than 1mm extending beyond application site)</td>
<td>4</td>
</tr>
<tr>
<td>Maximum Erythema + Oedema</td>
<td>8</td>
</tr>
</tbody>
</table>

3.4 Mutagenicity by Ames test

This test was performed following the method of Ames et al. (1975). In this *in vitro* test, histidine negative mutants of *Salmonella typhimurium* were used as sensitive indicators of DNA damage. Unlike mammals, these bacteria lack the enzyme systems for metabolising foreign compounds to
electrophilic metabolites capable of reacting with DNA. The bacteria were treated with the test compound in the presence of a post-mitochondrial supernatant known as "S9" mix (microsome fraction) prepared from the livers of rats.

**Bacterial strains:** Two Histidine deficient (His') tester strains of *Salmonella typhimurium* TA 100 and TA 1535 (obtained from Ames laboratory, University of California, USA) were used for the assay. The bacterial cultures were inoculated from the frozen permanent stock cultures to 10ml of nutrient broth and incubated overnight in a shaker.

**Preparation of rat liver S9:** Six male rats (150–175gms) were injected (IP) daily with Phenobarbitone at a dose of 50mg per kg for the induction of liver enzymes. The rats were sacrificed on the fifth day after overnight fasting. The liver was taken out aseptically and homogenised in a chilled 10M KCl solution. The homogenate was centrifuged at 9000g at 4°C for 30 minutes. The supernatant was collected and preserved at -70°C.

**Preparation of S9-mix:** The S9 obtained as described above was buffered and supplemented with the essential cofactors NADP and glucose-6-phosphate to form "S9 mix". It was prepared on the day of assay and kept sterile during its preparation and use. The ingredients of S9 mix were:

1.00M KCl
0.25M MgCl₂·6H₂O.
0.20M Glucose-6-phosphate.
0.04M NADP.
0.20M Sodium phosphate buffer, pH 7.4
S9-mix containing 10% S9 (v/v) was prepared by dispensing the appropriate volumes of the above ingredients so that the final concentration of co-factors and salts are,

- KCl 33 mM
- MgCl₂ 8 mM
- G-6-P 5 mM
- NADP 4 mM
- NaHPO₄ 100 mM

and filter sterilized using 0.22μM filter in ice. The required volume of S9 was then added and mixed well.

**Preparation of Minimal Agar media:** The composition of the minimal glucose agar media was as follows:

- Glucose 5.00 gms
- K₂HPO₄ 3.50 gms
- KH₂PO₄ 1.50 gms
- Sodium Citrate 0.25 gms
- MgSO₄.7H₂O 0.05 gms
- (NH₄)₂SO₄ 0.50 gms
- FeSO₄.7H₂O 0.0025 gms
- ZnSO₄.7H₂O 0.0025 gms
- MnSO₄.3H₂O 0.0025 gms
- CaCl₂ 0.05 gms
- H₂O 500 ml
- pH 7.2
- Agar 2.5%
Pour plate assay: Pre-incubation mixture containing 0.5ml of either S9 mix or 0.1M phosphate buffer pH 7.4, 0.1ml of 1/100 diluted bacterial culture was incubated with intact and solubilized ICPs of mutant and parent strains corresponding to 100µg protein at 37°C for 20 minutes. Following incubation, 2ml of molten top agar containing biotin and trace amount of histidine (0.6% agar in 0.5% NaCl solution containing 0.05mM L-histidine HCl and 0.05mM biotin) was added to the pre-incubation mixture and poured on to the minimal glucose agar plates. 0.02mg each of Benzo(a)pyrene in DMSO with S9 mix and Sodium azide without S9 mix were used as positive controls. The plates were incubated at 37°C for 48hrs.

Confluent lawn of S. typhimurium microcolonies were seen throughout the surface after 72hrs. In the case of mutagenic transformation, large size (0.2-0.6mm), opaque, elevated revertant (his+) colonies of wild type S. typhimurium were observed.

3.5 MUTAGENICITY BY SPERM SHAPE ABNORMALLITY TEST

Since several mutagens and carcinogens are known to produce abnormal sperms such as double headed, giant sized, calyx, round, spear, banana, and amorphous types, this in vivo test was carried out in rats and the sperms were observed for any possible abnormalities on treatment with the solubilized toxin preparations of parent and mutant.

Adult male wistar rats of same age group were used for the study. The animals were divided into 3 groups with 5 animals each. Group I served as control. Group II and III animals were injected intraperitoneally with 1.0ml of solubilized ICP preparations (corresponding to 100µg protein) of parent and mutant strains respectively. The control animals were treated with saline in the same way. The animals were kept under observation and were sacrificed after 24hrs
of treatment. Immediately the cauda epididymis was removed from the animal and placed in 0.9% saline. It was minced with scissors and then left undisturbed for 30 minutes for the diffusion of spermatozoa. The spermatozoa were spread on microscopic slides, air dried and fixed in absolute methanol and then stained with 1% eosin yellow. Three hundred spermatozoa from each animal were examined for abnormalities.

3.6 Effect on the rat intestinal Brush Border Membrane (BBM) vesicles in vivo and in vitro

For in vivo studies, 3 months old male rats in different groups were treated orally with solubilized ICP preparations of parent and mutant at a concentration corresponding to 100μg protein/ml regularly for 30 days. Control animals were fed with an equal volume of distilled water.

Preparation of Brush Border Membrane vesicles from rat small intestine: Brush border membrane was prepared from rat intestinal mucosa according to the method of Kessler et al. (1978). All the operations were carried out at 4°C. Fasted rats were killed by decapitation and the small intestines were removed and washed with ice cold saline (0.9% NaCl). The intestine was everted and the mucosa was scrapped using glass slide. This was homogenized in a waring blender for 2 minutes with 50mM mannitol in 2mM Tris-HCl buffer, pH 7.1 to obtain 1% homogenate. The homogenate was filtered through a fine nylon mesh and to this, solid CaCl₂ was added to a final concentration of 10mM. The suspension was stirred for 15-20 minutes and centrifuged at 3000g for 15 minutes. The pellet was discarded and the supernatant was centrifuged at 27000g for 30 minutes. The pellet obtained was resuspended in 60mM Tris/HCl, pH 7.1 and centrifuged again at 27000g for 30 minutes. The pellet obtained from 1g intestinal mucosa (3.0-3.5mg protein) was suspended in 10ml of 10mM Tris/HCl buffer, pH 7.1 and aliquoted to 1ml for enzyme assay.
For *in vitro* studies, the BBM vesicles were prepared from normal rats by the above said procedure and incubated with intact and solubilized ICPs from the parent and mutant at a concentration of 100µg protein/ml of BBM vesicles and plain buffer was added to control tubes. The tubes were incubated at 37°C for 30 minutes and used for the assay of following enzymes.

3.6.1 Estimation of γ-Glutamyl transpeptidase:

This enzyme catalyses the reversible transfer of γ-glutamyl groups to amino acids or peptides or to water.

The enzyme was assayed according to the method of Rosalki and Rau (1972).

**Reagents:**

1. **Substrate:** 30.3mg of L-γ-glutamyl p-nitroanilide/10ml. The substrate was sparingly soluble and was dissolved by warming to 50°C-60°C. The substrate solution was used within 2hrs of its preparation.

2. **Tris-HCl buffer:** 0.1M, pH 8.2

3. **Glycyl glycine:** 13.2mg of glycyl glycine was dissolved in 10ml of water. This was used as a second substrate.

4. **Acetic acid:** 10%

5. **Standard:** 13.8mg of p-nitroaniline (recrystallised) in 100ml of distilled water

A quantity of 0.5ml of enzyme was added to the incubation mixture containing 0.5ml γ-glutamyl p-nitroanilide, 2.2ml of glycyl glycine and 1.0ml of buffer. After incubation for 30
minutes at 37°C the reaction was terminated by the addition of 1.0ml of 10% acetic acid. The amount of p-nitroanilide liberated in the supernatant was calculated based on the difference in the optical density at 410nm between samples, with and without the substrate. The substrate incubated in the absence of enzyme under the same conditions was used as a reference blank. Optical densities of solutions of p-nitroaniline in the range 0.005-0.02 micromoles served as standard curve for arriving at the quantity of the product formed.

The activity of the enzyme was expressed as micromoles of p-nitroaniline liberated/min/mg protein.

3.6.2 Estimation of Alkaline phosphatase

This enzyme was assayed as described by Thambi Dorai and Bachhawat (1977) by using p-nitrophenyl phosphate as the substrate. The assay mixture contained 0.5 micromol of the substrate, 50 micromol of Tris-HCl buffer pH 9.0, 0.5 micromol of MgCl₂ and the enzyme in a total volume of 0.5ml. Incubation was done at 37°C for 15 minutes. The reaction was stopped by adding 2.5ml of 1M NaOH. The yellow colour developed due to the release of p-nitrophenol was measured at 405nm. One unit of the enzyme is defined as the amount of enzyme required to liberate 1 micromol of p-nitrophenol from p-nitrophenyl phosphate in 15 minutes under the assay conditions. The specific activity was expressed as units per mg protein.

3.6.3 Estimation of Na⁺K⁺ATPase:

The activity of this enzyme was determined following the method of Bonting (1970).
3.6.4. Estimation of Disaccharidases

The activities of Sucrase and Lactase were determined by the method of Dahlqvist (1984). 30 microlitres of membrane preparation was added to equal volume of 56mM substrate in 100mM sodium maleate buffer, pH 6.0 and incubated at 37°C for 60 minutes. The reaction was stopped by adding 900 microlitre of Tris-glucose oxidase reagent. The reaction mixture was mixed well and incubated at 37°C for 30 minutes and the colour developed was read at 450nm. For blank, enzyme, tris-glucose oxidase reagent and buffered substrate were added in that order, mixed well and incubated at 37°C for 30 minutes.

One unit of disaccharidase activity is the amount of the enzyme that hydrolyses 1 micromole of the substrate/minute/mg protein.

3.7. Effect on the enzymes of central nervous system of rats

There are reports suggesting that the central nervous system of rats was vulnerable to concentrations of $10^6$ and $10^7$ cfu of *B. thuringiensis* var. *israelensis* (Siegel et al., 1987). Hence the *in vitro* effect of intact and solubilized crystal preparations of parent and mutant strains on the two important enzyme systems, namely, Acetylcholinesterase and ATPases that are involved in the neuro transmission of the central nervous system in rats was studied.

Three months old albino rats were used for this study. The brain was dissected out and homogenised in ice cold Sucrose-EDTA (0.8M:1mM) solution using Potter Elvehjem homogeniser fitted with a teflon pestle to prepare a 1% homogenate. All operations were carried out at 4°C. The homogenate was incubated with intact and solubilized ICP preparations of mutant
and parent Bti strains at a concentration of 100μg protein/ml for 15 minutes at 37°C and used for 
the assay of Acetyl cholinesterase, Na⁺K⁺ ATPase, Ca²⁺ATPase and Mg²⁺ATPase activities.

3.7.1 Estimation of Na⁺K⁺ ATPase

The activity of this enzyme was determined following the method of Bonting (1970).

Reagents:

- Tris-HCl buffer : 184mM, pH 7.5
- Magnesium sulphate : 50mM
- Potassium chloride : 50mM
- Sodium chloride : 600mM
- EDTA : 1mM
- ATP : 40mM

A volume of 1.0ml of tris buffer and 0.2ml each of the reagents 2, 3, 4, 5 and 6 were 
mixed together such that the final volume of 2.0ml contained 92mM Tris buffer, 5mM MgSO₄, 
60mM NaCl, 5mM KCl, 0.1mM EDTA and 4mM ATP. After 10 minutes equilibration at 37°C, 
reaction was started by the addition of 0.1ml of 1% brain homogenate. The assay medium was 
incubated for 15 minutes at 37°C and at the end of the incubation period the reaction was stopped 
by the addition of 1.0ml of 10% TCA.

The amount of inorganic phosphate liberated was estimated by the method of Fiske 
and Subbarow (1925). The enzyme activity was expressed in terms of micromoles of phosphate 
liberated per minute per mg of protein.
3.7.2 Estimation of Ca\(^{2+}\)ATPase

The activity of this enzyme was estimated according to the method of Perry (1955) with minor modifications.

Reagents:

- Tris-HCl buffer: 25 mM, pH 8.0
- Calcium chloride: 50 mM
- ATP: 10 mM

To the reaction medium which contained 0.1 ml of CaCl\(_2\), ATP and buffer, 0.1 ml of 1:20 diluted enzyme was added and incubated at 37\(^\circ\)C for 15 minutes. The reaction was arrested by the addition of 0.5 ml of 10% TCA. The amount of inorganic Phosphate liberated was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as micromoles of phosphate liberated/minute/mg of protein.

3.7.3 Estimation of Mg\(^{2+}\)ATPase

The activity of the enzyme was estimated by the method Ohinishii et al. (1982).

Reagents:

- Tris-HCl buffer: 375 mM, pH 7.6
- Magnesium chloride: 25 mM
- ATP: 10 mM
The assay was initiated by the addition of 0.1ml of 1:20 diluted homogenate to the incubation medium containing 0.1ml of water and 0.1ml of each of the above reagents. Incubation was carried out at 37°C for 15 minutes. The reaction was terminated by the addition of 0.5ml of 10% TCA. The liberated inorganic Phosphate was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as micromoles of phosphate liberated/minute/mg of protein.

3.7.4 Estimation of Acetyl Cholinesterase

The activity of acetyl cholinesterase in brain was assayed following the method of Hestrin (1949).

Statistical Tests Used

Analysis of Variance (ANOVA), Z-test and Student's t-test were used to test the significance of values accordingly.