CHAPTER 3
MATERIAL AND METHODS

CHEMICALS/ REAGENTS

All the chemicals used in the present study were of molecular biology grade obtained from various suppliers. They are listed below.

Agarose (Sigma, USA), Bromophenol blue (Sigma, USA), Tris (hydroxymethyl) Aminomethane acetate (Sigma, USA), Ethidium bromide (Sigma, USA), 2-Mercaptoethanol (Sigma, USA), TEMED (ICN,Biochemical USA), Protein molecular weight marker (Bangalore Genei, India), Formamide (SRL Research Chemicals, India), Congo red (Sigma, USA), dNTPs (Bangalore Genei, India) Taq polymerase with PCR buffer (Hi-Media), Methanol, Acetic acid, Commassie Brilliant blue R-250 (Sigma USA ), Glycin (RM 199), Trypan blue (RM 263), saffreinine Certified AR.(RM 129)

MEDIA

The following media were used for bacteriological study –

Nutrient Agar Cat No:(M 001)(Hi- Media, India), Luria Broth Cat No:(M 575)(Hi- Media, India), Soyabean Casein Digest Cat No:(M 290)(Hi-Media, India), Salmonella shigella Agar Cat No:(M 108)(Hi- Media, India), Yersinia selective Agar Cat No: (M 843 ) YSA , Supplement (CIN ) (FD 034) (Hi- Media, India), MacConkey Agar Cat No:(M 843)(Hi-Media, India), Tryptic Soya Broth Cat No: (M 323)(Hi-Media, India), MacConkey Broth Cat No: (M 007)(Hi-Media, India), Blood Agar Base 2 Cat No: (M 834A)(HiMedia, India), Nutrient Broth Cat No: (M 002)(Hi-Media, India), Xylose Lysine Deoxycholate Agar (XLD Agar) (M 031) (H-Media, India) Motility Test Medium (M 260)(H-Media, India), Urea Agar Base Christensen (M 111) (H-Media, India), Brain Heart Infusion Agar (M 211)(H-Media, India), Brain Heart Infusion Broth (M 210) (H-Media, India) and Cary and Blair Transport media(M 202) (HiMedia, India). The media were prepared and plated before use following the manufacturers' instructions.
Material and Methods

KITS

Gram staining kit (Hi-Media), Biochemical characterization, Hi25 Enterobacteriaceae Identification Kit (KB 003) (Hi-Media), Genomic DNA isolation mini kit (Qiagen, India), Modified Lowry Protein Assay Estimation Kit (PIERCE Biotechnology, Rockford), Laemmli Buffer Stock (SERVA Electrophoresis), Urea Agar Base (BBL, USA), Motility Test Medium (BBL, USA).

CONTROL STRAINS OF BACTERIA

Control strains of *Yersinia enterocolitica* (IP 28205 & IP 28206) were maintained in the laboratory. Control strain of *Salmonella typhimurium* (ATCC 13311) was obtained from Central Research Institute (CRI), Kasauli, (Himachal Pradesh). The strains were characterized morphologically and biochemically on the basis of standard tests. This positive control strain was also grown on BHI supplemented with sorbitol at 37°C for 24 hrs. The strains were stored in 30% glycerol & 70% Nutrient agar at 4°C and -80°C.

SAMPLE SOURCE

Bacteriological investigations were performed in the following samples to harvest *Y. enterocolitica*.

1. Stool samples of diarrhea patients from slums in and around Chandigarh, India.
2. Pig throat swabs obtained from slaughter houses, Industrial area, Chandigarh, India.
3. Pork meat obtained from butcher shops in and around Chandigarh, India.
4. Drinking water (Chandigarh Municipality supply), Chandigarh, India.

PERMISSIONS AND ETHICAL CLEARANCE

Administrative approval was taken from the authorities before commencement of the research work. Medical Superintendent, General Hospital Sector-16 Chandigarh, India approved sample collection from primary health centers & dispensaries in and around Chandigarh (Annexure 1). Pig throat swab
sample collection from slaughter house was permitted by Commissioner, Municipal Corporation, Chandigarh (Annexure 2). The animal ethical clearance was obtained from the Institutional Animal Ethics Committee, Panjab University, Chandigarh.

Patient selection:

Diarrhea Patients (3 or more times watery stool per day) visiting Primary Health Centres and private registered medical practitioners in slum colonies in and around 5 kms of Chandigarh from January 2002-December 2004 were considered for the study. The stool samples were collected from villages nearby Chandigarh namely Khudda Lahora, Khudda Jassu, Dhanas, Dadumajra and Manimajra with population of 3467, 1438, 3055, 3396 and 5521 respectively as per Census of India 2001 (Annexure 3). The stool samples were also collected from Ward Number 5 with a total population of 57977(Table 1).

Exclusion criteria for sample collection

The patients on antibiotics were excluded from the study for taking stool samples. The criterion for the selection of the patients was as per the ethical standards.

The stool samples were collected in Carry and Blair transport media and immediately transported to Department of Experimental Medicine & Biotechnology, PGIMER, Chandigarh, India and processed as follows:

**ISOLATION OF Yersenia enterocolitica from stool samples:**

In order to improve the chances of recovering pathogenic *Y. enterocolitica* following enrichment procedures were used

1) Cold Enrichment (Pai et al., 1979)

2) Alkaline treatment (Ratnam et al., 1983).

3) A number of enteric media designed for the isolation of enteric bacteria were evaluated for the isolation of *Yersinia* (Head et al., 1982; Harmon et al., 1983; Schiemann 1979)
Material and Methods

TABLE 1: Details of Patients from which stools samples were collected for isolation of *Y. enterocolitica*

<table>
<thead>
<tr>
<th>Villages from where samples are collected</th>
<th>Total population</th>
<th>Sample taken</th>
<th>Age groups (years)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt; 5</td>
<td>6-18</td>
</tr>
<tr>
<td>Khudha Lahora</td>
<td>3467</td>
<td>52</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>Khudha Jassu</td>
<td>1438</td>
<td>25</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Dhanas</td>
<td>3055</td>
<td>49</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>Dadumazara-1</td>
<td>3396</td>
<td>57</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>Manimazara</td>
<td>5521</td>
<td>120</td>
<td>72</td>
<td>35</td>
</tr>
</tbody>
</table>

Slum area

<table>
<thead>
<tr>
<th>Ward 5</th>
<th>Khumhar colony</th>
<th>57977</th>
<th>69</th>
<th>40</th>
<th>22</th>
<th>7</th>
<th>48</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Janata colony</td>
<td></td>
<td>58</td>
<td>38</td>
<td>16</td>
<td>4</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Bhaskar colony</td>
<td></td>
<td>65</td>
<td>46</td>
<td>10</td>
<td>9</td>
<td>35</td>
<td>30</td>
</tr>
</tbody>
</table>

*Total population is taken from Census of India, 2001, issued by N.S. Bhaduria, Assistant Registrar General, Chandigarh (Annexure 3).

Inclusion criteria: Patient passing watery stool for more than 3 times a day.
Exclusion criteria: History of antibiotic consumption.

Cold enrichment (Pai et al., 1979)

The stool samples were subjected to cold enrichment in PBS (1/15 M, pH 7.6) for 21 days at 4°C.

Alkali treatment (Ratnam et al., 1983) to exclude other organisms of family Enterobacteriaceae

*Y. enterocolitica* tolerates short exposure to weak alkali better than other members of the family Enterobacteriaceae. Alkaline treatment was performed by the method of Ratnam et al. (1983). Briefly, the cold enriched samples were mixed with 0.5% KOH in 0.5% NaCl for 10-15 seconds followed by streaking on MacConkey agar (MA) and other enteric media (XLD & SS agar) and incubated for 24hrs-48hrs both at 37°C and 22°C. Subculture was done from the cold enriched sample on enteric media (XLD & MacConkey) on 7th, 14th, and 21st day. The sample which did not show any growth till 21st day was discarded.
Colony identification:

All the suspected colonies of *Y. enterocolitica* from MA, XLD and SS Agar were cultured to get the pure growth & subjected to identification procedure.

Identification & confirmation

Identification of *Yersinia enterocolitica* suspected colonies having morphology typical of *Y. enterocolitica* were subcultured on media for further characterization, the following tests were done for the presumptive identification of the *Yersinia*:

1) Gram staining
2) Catalase and Oxidase tests
3) Motility test
4) Christensens urea agar test

Gram staining

All colonies were subjected to Gram staining with Gram staining kit obtained from Hi Media Laboratories, Mumbai, India following the below mentioned procedure:

Procedure

1. Thin smears were prepared, dried in air and fixed by gentle heating.
2. The smears were then flooded with Gram's Crystal Violet (S012) for one minute.
3. These were washed with water and flooded with Gram’s Iodine (S013) for one minute.
4. The smears were again washed with water and decolorized with Gram’s Decolourizer (S032) until no further violet colour comes off.
5. These were then washed in water and counter stained with 0.5% Safranin (S027) for about one minute.
6. After counterstaining, the smears were again washed with water, dried and finally observed under oil immersion objective.
7. Colonies showing gram -ve bacilli were subcultured for further characterization.

**Catalase & Oxidase tests**

The catalase & oxidase tests were performed for rapid screening of Enterobacteriaceae.

Catalase test was performed by adding two drops of 10% H$_2$O$_2$ on a colony taken over glass slide with the help of a toothpick. Positive catalase test was recorded by appearance of bubbles within 20-30 seconds of addition of H$_2$O$_2$.

The oxidase test was performed by filter paper strips soaked in 1% N', N', N', N' - tetramethyl -- phenylenediamine dihydrochloride prepared in distilled water. The classified colony of *Y. enterocolitica* was scrapped by a toothpick and rubbed on the filter paper. The oxidase test was considered positive on appearance of blue black colour within 10-15 seconds.

**Motility Test**

Motility test medium was used for the detection of motility of gram-negative enteric bacilli by using standard kits as per manufacturer’s protocol. Two tubes of motility media were inoculated. One tube was kept at 22 °C and the other at 37 °C for 48-72 hours. Bacterial motility was observed directly by examining the tubes following incubation. Growth was found to spread out from the line of inoculation when the organism was motile. Highly motile organisms provided growth throughout the tube. Growth of non motile organisms occurred only along the stab line.

**Composition of BBL Motility Test Medium**

- **Beef** ........................................ 3.0g
- **Pancreatic Digest of casein** ...... 10.0g
- **Sodium Chloride** .................. 5.0g
- **Agar** ....................................... 4.0g
Material and Methods

The final volume was made to 1 litre with distilled water.

Procedure

The tubes were inoculated with pure culture by stabbing the centre of the column to greater than half the depth. The test tubes were incubated for 24-48 hours at 37 °C and 22 °C in anaerobic atmosphere.

Limitation of the Procedure

Many organisms fail to grow deep in semi solid media in test tubes. In that case, inoculating media in petriplates may be advantageous.

Urease hydrolysis activity, estimated by Urea Agar Base method

Bacteria identified in Enterobacteriaceae family were screened for Y. enterocolitica by analyzing urease hydrolysis activity using Urea Agar Base (BBL, USA). Urea Agar base media was used for the differentiation of organisms on the basis of urease production.

When organisms utilize urea, ammonia is formed during incubation which makes the reaction of the media alkaline, producing a red pink colour. Consequently, urease production may be detected by the change in the phenol red indicator.

Composition of BBL Urea Agar base

Pancreatic Digest of Gelatin ............ 1.0 g
Dextrose ........................................ 1.0 g
Sodium Chloride .......................  5.0 g
Potassium Phosphate ..................  2.0 g
Urea ............................................ 20.0 g
Phenol Red ................................. 12.0 g

The final volume was made to one litre by adding distilled water.
Material and Methods

Preparation of Urea Agar Base Concentrate (10X)

1. 1.7 g of granulated agar was added to 100 ml of purified water. The solution was heated with agitation and boiled for one minute.

2. It was dispensed in 9 ml aliquots into tubes and autoclaved at 121 °C for 15 minutes.

3. The agar was cooled to 45-50 °C. One tube of the concentrate was allowed to come to room temperature. One ml of the concentrate was added to each 9 ml of cooled agar solution and mixed thoroughly.

4. The tubes were allowed to cool in a slanted position to allow the formation of slants with deep butts.

5. The samples of the finished product were tested for performance using stable, typical control structures.

6. The agar was inoculated with a heavy inoculum (2 loopfuls) of growth from an 18 to 24 hours pure culture (TSI Agar or other suitable medium) by streaking back and forth over the entire slant surface. It was taken care not to stab the butt since it served as a colour control.

7. The reaction was observed every day for a total of six days.

8. The production of urease was indicated by an intense pink-red colour throughout the slant. The extent of the colour indicated the rate of urea hydrolysis.

9. No colour change meant a negative reaction. The agar medium in this case remained pale yellow to buff.

Bio-Chemical Characterization by using Hi25 Enterobacteriaceae Identification Kit (KB 003)

Biochemical characterization of thus obtained Yersenia enterocolitica was performed by Hi25 Enterobacteriaceae Identification Kit (KB 003) (Hi- Media, India) as per manufacturer’s instructions. Here 25 biochemical tests were
performed to further confirm the isolated Y. enterocolitica strains. The results were compared with the reference index given in Annexure 4.

Introduction

Hi25 Enterobacteriaceae Identification Kit (KB 003) KB003 is a comprehensive test system that can be used for the identification of gram negative Enterobacteriaceae species. Organisms belonging to Enterobacteriaceae gram negative, oxidase negative and nitrate positive rods are the most frequently isolated bacteria from clinical specimens. Hi 25 identification kit can be used for screening pathogenic organisms from urine, enteric specimens and other relevant clinical samples. It can also be used for validating known laboratory strains. The complete list of organisms that can be identified with this system is given in the identification index provided with the kit (Appendix-4).

Principle

Each Hi 25 kit is a standardized colourimetric identification system utilizing thirteen conventional biochemical tests and eleven carbohydrates utilization tests. The tests are based on the principle of pH change and substrate utilization. On incubation, organisms undergo metabolic changes which are indicated by a colour change in the media that is either visible spontaneously or after addition of a reagent. Oxidase test is performed separately using oxidase reagent disc provided with the kit.

Procedure

1. Preparation of inoculum

   - The organisms to be identified were isolated on a common medium like Nutrient Agar (M001/M127) or a differential medium like MacConkey Agar (M082)
   - A single isolated colony was picked up and inoculated in 5 ml BHI Broth and incubated at 37°C for 4-6 hours until the inoculum turbidity is ≥0.10D at 620nm or 0.5 McFarland standard.
Material and Methods

- Oxidase test was performed on the organism to be tested by using oxidase disc (DD018) provided with the kit. A well isolated colony was picked up and rubbed on a single oxidase disc. Positive reaction was indicated by development of deep purple colour within 10 seconds. Colour change in 10-60 seconds indicated a delayed positive reaction and the colour development after 60 seconds or no change in colour indicates a negative reaction.

- The result was noted in the result entry Datasheet. Oxidase test must be performed as it is an integral identification system. it must be performed to differentiate Enterobacteriaceae from other gram negative rods

Sources of error:

- Erroneous false negative results may be obtained if the inoculum turbidity is less than 0.10D

- Results are more prominent if an enriched culture is used instead of suspension

2. Inoculation on the strip

- The kit was opened aseptically by peeling off the sealing foil.

- Each well was inoculated with 50 μl of the above inoculum by surface inoculation method or by stabbing each individual well with a loopful of inoculum

3. Incubation

- The strips were incubated at a temperature of 35±2 °C for 18-24 hours.

Interpretation of results

- The results were interpreted as per the standards given in the identification index. Addition of reagents wherever required was done at the end of incubation period.
Material and Methods

Important points to be taken into consideration while interpreting the result

1. All reagents were brought to the room temp. before using.

2. In case of carbohydrate fermentation test, some microorganisms show weak reaction. In this case the reaction was recorded + and incubated further for 48 hours. Orange colour after 48 hours of incubation was interpreted as a negative reaction.

3. In case of Lysine and Ornithine decarboxylation, sometimes incubation up to 48 hours was required.

4. At times organisms give conflicting results because of mutation or due the media used for isolation, cultivation and maintenance.

5. The identification index has been compiled from standard references and results of tests carried out in the laboratory.

Precautions:

- Precautions were taken while handling the clinical samples and pathogenic bacteria and potentially pathogenic bacteria and handled accordingly.
- Aseptic conditions were maintained during inoculation and handling of the strips.
- Reagents should not come in contact with skin, eyes or clothing.

Identification and confirmation:

Following tests were done for the identification and confirmation of the *Yersinia enterocolitica*:

1. **ONPG**
   - A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect β-galactosidase activity.
   - Original colour of the media was colourless.
   - Positive reaction gave yellow colour.
Material and Methods

- Negative reaction showed no change in colour

2. Lysine decarboxylase
- A full loop of previously isolated pure Y. enterocolitica cultures was used to detect Lysine decarboxylation reaction.
- Original colour of the media was olive green.
- Positive reaction gave purple colour.
- Negative reaction gave yellow colour

3. Ornithine decarboxylase
- A full loop of previously isolated pure Y. enterocolitica cultures was used to detect ornithine decarboxylation reaction.
- Original colour of the media was olive green.
- Positive reaction gave purple colour.
- Negative reaction gave yellow colour

4. Urease
- A full loop of previously isolated pure Y. enterocolitica cultures was used to detect urease activity.
- Original colour of the media was orangish yellow.
- Positive reaction gave pink colour.
- Negative reaction showed no change in colour

5. Phenylalanine Deamination
- 2-3 drops of TDA reagent (R036) was added.
- Development of dark green colour within one minute indicated a positive reaction.
- No change in colour denoted a negative reaction.
6. **Nitrate Reduction Test**
   - 1-2 drops of Sulphanilic acid (R015) and 1-2 drops of N,N-Dimethyl-1-Napthylamine Reagent (R009) were added
   - Immediate development of pinkish colour on addition of reagent indicated positive reaction
   - No change in colour indicated a negative reaction

7. **H₂S production**
   - A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect H₂S production
   - Original colour of the media was orangish yellow.
   - Positive reaction gave black colour
   - Negative reaction showed no change in colour

8. **Citrate utilization**
   - A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect capability of organism to utilize citrate as a sole carbon source.
   - Original colour of the media was green of citrate negative
   - Positive reaction gave blue colour
   - Negative reaction showed no change in colour

9. **Voges Proskauer's Test**
   - 2-3 drops of Baritt reagent a (R029) and 1 drop of Baritt reagent B (R030) were added
   - Pinkish red colour development within 5-10 minutes indicated a positive test
   - No change in colour or a slight change in colour (due to reaction of Baritt reagent A with Baritt reagent B) denoted a negative reaction
10. **Methyl Red Test**

- 1-2 drops of Kovac’s reagent (R008) was added
- Development of pinkish red colour within 10 seconds indicated positive reaction.
- Reagent remained pale coloured if the test was negative

11. **Indole**

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect deamination of tryptophan
- 1-2 drops of Kovac’s red reagent was added
- Original colour of the media was colourless
- Positive reaction gave red colour
- Negative reaction gave yellow colour

12. **Malonate**

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect capability of organism to utilize sodium malonate as a sole carbon source.
- Original colour of the media was colourless
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

13. **Esculin**

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Esculin hydrolisis
- Original colour of the media was cream
- Positive reaction gave black colour
- Negative reaction showed no change in colour
14. Arabinose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Arabinose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

15. Xylose Utilization

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Xylose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

16. Adonitol

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used.
- Principle was to detect Adonitol utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

17. Rhamnose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Rhamnose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour
18. Cellobiose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Cellobiose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

19. Melibiose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Melibiose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

20. Saccharose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Saccharose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

21. Rattinose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Rattinose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour
Material and Methods

22. Trehalose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Trehalose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

23. Glucose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Glucose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

24. Lactose

- A full loop of previously isolated pure *Y. enterocolitica* cultures was used to detect Lactose utilization
- Original colour of the media was red
- Positive reaction gave yellow colour
- Negative reaction showed no change in colour

Oxidase

The oxidase test was performed by filter paper strips soaked in 1% N', N', N', N' - tetramethyl - p - phenylenediamine dihydrochloride prepared in distilled water. The classified colony of *Y. enterocolitica* was scrapped by a toothpick and rubbed on the filter paper. The positive result of oxidase test was indicated by appearance of blue black colour within 10-15 seconds.
Material and Methods

Confirmation of pathogenicity of Yersenia enterocolitica by Congo red dye uptake test

The invasiveness of the Yersinia enterocolitica was tested by Congo red dye uptake as described by Statner & George (1987). Briefly, Congo red agar base was prepared from Soyabean Cassein Digest Broth to which 2% agar was added. The pH of the medium was adjusted to 8.0 and sterilized by autoclaving at 15psi for 15 minutes. Congo red dye was dissolved in 50mM autoclaved PBS (pH7.0), filter sterilized and mixed to molten agar to yield a final concentration of 50μg/ml. At this concentration, Congo red was transparent or of light orange colour. Y. enterocolitica strains were grown in Tryptic soya broth (TSB) at 23°C for 16-18 hours then diluted to obtain about 10 CFU/ml & then spread 10 μl of diluted suspension & incubated at 37°C, 22°C for 24 hrs. Y. enterocolitica was grown on nutrient agar plate and incubated overnight. The strain was streaked on Congo red dye agar plate and results were recorded after 48 to 72 hrs of incubation at 37°C.

Confirmation at national referral laboratory for Yersinia spp., The Pasteur, Institute, Paris.

Out of isolated strains of Y. enterocolitica from human diarrheal stool samples, suspected five pathogenic strains and one non pathogenic strain were sent to The Pasteur Institute, Paris in wax sealed sterile containers containing 50% nutrient agar and 50% glycerol for confirmation and serotyping.

Molecular Characterization

Genomic DNA was extracted from thus obtained pathogenic strains of Y. enterocolitica by using Genomic DNA isolation kit (mini kit) obtained from Qiagen as per manufacturer’s instructions.
Material and Methods

Reagents:

1. TAE Buffer (50X):

242 g of Tris base was dissolved in 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) was added and the final volume was made to one litre with water. The buffer was diluted 50 times before use.

2. Agarose

3. Ethidium Bromide Stock : 10 mg/ml

4. Bromophenol Blue Gel loading dye: 0.25% Bromophenol Blue, 30% glycerol in H₂O (stored at 4°C).

5 CTAB/NaCl solution:

<table>
<thead>
<tr>
<th>NaCl</th>
<th>4.1g</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW</td>
<td>100m</td>
</tr>
<tr>
<td>CTAB</td>
<td>10g</td>
</tr>
</tbody>
</table>

The water was added slowly while stirring under heating condition

Method of genomic DNA extraction from *Yersinia enterocolitica* :

1. 50 ml of Brain Heat Infusion (BHI) broth was inoculated with *Yersinia enterocolitica* and incubated at 37°C in a shaker overnight.

2. The cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C.

3. The supernatant was poured off and then the cells were washed with 10 ml of GTE ( Glucose 50mM, Tris 50mM, EDTA 10mM, pH 8.0 )

4. The bacterial pellet was resuspended in 3.6ml of GTE buffer.

5. 0.4ml of lysozyme (20 mg/ml) was added in GTE.

6. The mixture was vortexted and incubated at 37°C for 1 hour

7. 400μl of 10% SDS and 25 μl of (20mg/ml) proteinase- K were added to cell suspension and incubated at 37°C for 30 min.
Material and Methods

8. 1ml of 5M NaCl and 800μl of CTAB/NaCl were added in cell suspension, mixed thoroughly and incubated at 65°C for 15 min.

9. 5.0 ml of Tris saturated phenol was added to it and the solution was mixed by inversion.

10. It was then centrifuged at 10,000 rpm for 5 min.

11. The upper aqueous layer was collected in fresh centrifuge tube.

12. Equal volume of phenol: chloroform was added, mixed and centrifuged at 10000 rpm for 5 min.

13. The upper aqueous layer was collected and extracted with chloroform: isoamyl alcohol (24:1)

14. This upper aqueous layer was then transferred to a corex tube and 0.1 volume of 3M sodium acetate (pH 6.0) and 0.6 volume of isopropanol and then mixed properly.

15. DNA was spooled out with glass rod and washed with 70 % alcohol.

16. DNA pellet was dried completely.

17. It was dissolved in TE buffer (pH 8.0) or sterile water.

Quantification of genomic DNA

The DNA samples were diluted & quantitated using uvikon spectrophotometer (Pharmacia) at 260 nm. The purity of DNA was checked by measuring ratio of 1260 to A280. The concentration of DNA was calculated using the formula:

\[
\text{DNA conc. } \text{IN (ug/ml)} = \frac{A_{260} \times 50 \times \text{dilution factor}}{\text{A26 of 1 corresponds to DNA conc. Of 50 μg/ml H2O}}
\]

Agarose gel electrophoresis:

1. The open ends of the electrophoresis tray were sealed with adhesive tape. The comb was placed on the tray.
2. 0.8% agarose was prepared in electrophoresis buffer (0.5 X TBE). The mixture was heated until the agarose dissolved completely.

3. The solution was cooled to about 60°C and 2-3μl of ethidium bromide was added to it. The gel was then poured on sealed tray and left without disturbing until gel solidified.

4. The comb and the adhesive tape were removed and the tray was placed on electrophoresis tank. Enough electrophoresis buffer (0.5 X TBE) was added to cover the gel to a depth of about 1 mm.

5. The DNA samples were mixed with 6X loading buffer. The samples were slowly loaded into the wells of submerged gel.

6. The gel tank was connected with electrical leads so that the DNA migrates from cathode towards anode. A voltage of 5 V/m was applied and the gel was run until the tracking dye (bromophenol blue) reaches to the other end of the gel.

7. The electric current was turned off and the gel tray was taken out.

8. The gel was observed under UV transilluminator (320nm)

**PCR amplification:**

Genomic DNA isolated by above method was amplified using gene specific primers. Two set of primers were used.

1. Genus specific primers for 16s rRNA gene which is present in all strains of *Yersenia* spp regardless of virulence (Sen, 2000) were used.

   5' CGGCACGGGAAGTAGTTT 3' forward
   3' AATCGATCATCCACCCTACCG 5' reverse

2. Primer for *ail* gene which is present only in virulent strain of *Yersinia enterocolitica* (Miller et al.,1989). The primers used were

   5'TTAATGTGTACGTGAGTG3' forward
   3' CTGCGAAGTATTTATGAGG 5' reverse
Material and Methods

Primers were obtained from Bangalore Genei. PCR amplification was performed as per previously published protocol (Sen, 2000). Briefly, reactions were performed in 50μL volumes. Each 50 μL of reaction mixture contained a 400nM concentration of primers, 200μM (each) dTTP, dCTP, dATP & dGTP, 1U of Taq Gold polymerase, and 1X PCR buffer (10X supplied with the enzyme), 3mM magnesium chloride and 5mm extracted DNA. Cycling conditions consisted of an initial cycle at 95° C for 10 minutes to activate the enzyme followed by 40-45 cycles of two temperature cycling consisting of 15s at 95° C and 1 minute at 60° C. PCR products were subjected to submarine gel electrophoresis in 2% agarose containing 10mg/ml ethidium bromide in TAE buffer at 70mV for 30 minutes. Then the separated products were viewed in a Gel Documentation system (Amersha, U.S.A)

Protein estimate

The protein was measured by the method of Lowry et al. (1951). Results were expressed as mg /ml of protein.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli et al. (1970)

Reagents

1. Acrylamide solution: solution containing 30 % acrylamide and 0.8% methylene bis acrylamide
2. Stacking gel buffer: 0.2M Tris buffer (pH 6.8)
3. Separating gel buffer: 0.75 M Tris buffer (pH 8.8)
4. Ammonium persulphate solution: 10% solution in distilled water
5. SDS solution : 10 % solution in distilled water
6. Running (electrode) buffer: 0.1% SDS solution, 0.025 M Tris buffer (pH 8.3) containing 0.192 M glycine
7. Staining solution: 0.2% Coomassie brilliant blue in solution containing methanol (40%), glacial acetic acid (10%) in distilled water.
8. **Destaining solution and fixing solution:** Methanol (50%) : acetic acid (10%) in distilled water.

9. **Sample buffer:** solution containing 0.125M Tris (pH 6.8) 10% glycerol, 5% β-mercaptoethanol, 2% SDS and 0.001% bromophenol blue. The sample buffer was added to the sample solutions in equal amounts.

10. **Separating gel:**

    - Acrylamide solution: 6.6ml
    - Double distilled water: 8.09ml
    - Tris buffer (pH 8.8): 5ml
    - 10% SDS solution: 200μl
    - APS solution: 100μl
    - TEMED: 0μl

11. **Stacking solution**

    - Acrylamide solution: 1.3ml
    - Double distilled water: 6.1ml
    - Tris buffer (pH 6.8): 2.5 ml
    - 10% SDS solution: 100μl
    - APS solution: 10μl
    - TEMED: 50μl

**Experimental Procedure**

Using the spacers of 0.75 mm thickness, glass plates were sealed and then 12% separating gel was filled in them. The gel was allowed to polymerize for 30 min at room temperature. Afterwards 6% stacking gel was poured and the wells were made. The proteins were mixed in sample buffer in 1:1 ratio and then kept for 2 min in boiling water bath. The samples were then loaded in the wells made in stacking gel. Proteins were separated in 10% gel after 45 min run at 200
Material and Methods

Volts supplied from BioRad 220/20 constant voltage power supply. The gel was stained with 0.1 % Coomassie brilliant blue.

**OMP PREPARATION**

The isolated pathogenic and non-pathogenic *Yersinia enterocolitica* strains were grown at 37 °C in MA. Bacterial colonies were suspended in 10mM tris-HCl buffer (pH7.6) containing 5mM MgCl₂. Cells were washed twice in same buffer containing 2mM PMSF (Phenyl Methyl Sulphonyl Floride). Cells were then disrupted by ultrasonication using sonicator (Pharmacia). The undisrupted material was separated by low speed centrifugation (3000g x 15 minutes). The supernatant was then ultracentrifuged at 100000g for 60 minutes at 4 °C and the pellet is suspended in 1% sodium dodecyl sulphate in 10mM Tris-HCl buffer (pH7.6). After incubation for 2 hours at 37 °C in detergent, the insoluble outer membrane protein fraction was collected by ultracentrifugation at the same speed. The recovered outer membrane protein (OMP) fraction was suspended in 20mM Tris buffer containing 2mM PMSF and stored at -80 °C.

The protein content in the outer membrane protein preparation was estimated by the modified Lowry Protein Assay Estimation kit (PIERCE Biotechnology, Rockford, following manufacturer’s instructions.

The crude protein preparation was separated on SDS-PAGE using discontinuous buffer system. 10% resolving gel was prepared by pouring acrylamide solution between the plates of a gel apparatus and layered with distilled water to exclude oxygen that inhibits polymerization. Following polymerization, the staking gel of 5% was poured and comb was inserted. The gel was then allowed to polymerize for 30 minutes. The gel was attached to the electrophoresis unit. 1X running buffer diluted from stock 4X buffer containing 11.5 gm glycine, 0.8 gm SDS, 2.4 gm Tris dissolved in 200ml double distilled water (pH8.3) was added to the top and bottom chambers. The samples were prepared in 1X buffer prepared by mixing stock 10X Laemmli buffer (SERVA electrophoresis), as per manufacturer’s instructions and heated in a boiling water bath for 5 minutes. The sample containing 60 µl of protein (in each sample) was
then loaded in the individual wells along with molecular weight markers (Sigma). The gel was run at 50V for stacking of protein for 30 minutes in the stack gel, after that the gel was run at 100V till the dye front reached the bottom. The gel was then kept in the staining solution containing commassie brilliant blue 0.2 gms, methanol 50 ml, acetic acid 7 ml in 43 ml distilled water for overnight, destained and finally photographed by placing the gel on gel documentation system. The destaining solution comprised of 50 ml methanol, 7 ml acetic acid and 43 ml distilled water. The molecular weight of the proteins resolved in the gel is determined by calculation of Rf value of each band in relation to bands obtained by standard molecular weight markers (Sigma).

*Salmonella typhimurium* strain was obtained from Central Research Institute Kasauli, (HP). It was grown and maintained in Nutrient agar with glycerol throughout the study and known strain of *Yersenia enterocolitica* obtained from IMTECH, Chandigarh was grown and maintained in Nutrient agar with glycerol throughout the study. These served the purpose of control for characterization of isolated strain of *enterocolitica* from stool and other samples. All experiments performed with the isolated *Yersenia enterocolitica* were also simultaneously performed with the control strain. Results obtained from control strains and test strains of the bacteria were compared for analysis throughout the study.

**ISOLATION OF BACTERIA FROM PIG THROAT SWAB**

Pig throat swabs were collected from slaughter houses located at Industrial area, Chandigarh. During pig sacrifice in the slaughter house for commercial purpose, the throat swab of the animal was collected just after application of high voltage electric shock, a routine procedure to kill the animal. The sample was transported to Department of Experimental Medicine and Biotechnology, PGIMER, Chandigarh in TSB wet swab in PBS. Then culture and characterization protocol was followed as above, mentioned in the section dealing with isolation of bacteria from stool sample. For confirmation of results pig throat swab sample isolates were also sent to Pasteur Institute, Paris.
ISOLATION OF BACTERIA FROM PORK MEAT PIECES

Minced Pork meat pieces were purchased from two meat shops located in the previously mentioned slum colonies in and around Chandigarh. Pieces of tongue samples of pig were also purchased from the same meat shops. Samples were transported in TSB (1gm/10mL) to the Department of Experimental Medicine and Biotechnology, PGIMER, Chandigarh. In the laboratory, the samples were homogenized and overnight incubated for 16-18 hours at 22°C. Bacteria were isolated from thus prepared samples by previously described protocol. Bacteria were also attempted to be isolated from drinking water (Municipality supply) and milk samples obtained from the above mentioned slum colonies.

RABBIT ILEAL LOOP TEST (RILT)

Materials:

Adult rabbit weighing 1.5-2.0 kg, atropine-0.4mg/kg, IV states, sodium pentobarbitone 40 mg/kg, needle, thread, betadine, spirit, savlon, gauge, brufen injection, Lysol, 1% hypochloride, 2% xylocaine.

Principle

The virulence properties of Y. enterocolitica toxins is commonly checked by Rabbit Ileal Loop Test. Fluid accumulation in the loops indicate bacterial pathogenicity, that is, the diarrhea causing potential of bacteria.

Method

Healthy male New Zealand white rabbits (1.5-2.0 kg) were procured from Central Animal House, Panjab University, Chandigarh. Before experiments, the rabbits were tested for any enteric infection or clinical signs of diarrhea.

Overnight sub cultures grown at 26°C and 37°C were diluted 1:20 in fresh LB and BHI and incubated for 2hrs at 37°C. The bacteria were then centrifuged at 4000 rpm and washed once and resuspended in PBS (7.2) at a concentration of $10^8$ cfu/ml. The desired bacterial concentration was adjusted and checked by
Material and Methods

Plating serial dilution of the sample on agar and counting cfu/ml after incubation at 25 °C or 37°C for 24-48 hrs of growth.

The live bacteria cfu/ml (LD 50) doses were checked for the presence of enteric toxicity by rabbit ileal loop test as described by De and Chaterjee (1953). An adult male white rabbit was fasted for 16 hrs with free access to water, anaesthetized with sodium pentobarbital and a middle incision (3 cm long) was given to the abdomen after administration of local anesthesia (2% xylocaine). The small intestine was externalized and kept moist with normal saline during the entire operation procedure. Commencing at the distal ileum, small intestine loops were prepared in a single rabbit, separated by 1 cm inter loops. Cholera toxin (positive control), phosphate buffer saline (negative control) and test samples were injected into individual loops. These loops were then reinserted in the peritoneal cavity of the rabbit and the abdomen was closed. Rabbit was sacrificed 18 hrs after the surgery and amount of fluid accumulation per centimeter of ileum was calculated.

HISTOPATHOLOGICAL STUDIES

Histopathology sections of control (PBS), bacteria toxin and endo-toxin treated rabbit ileum were prepared. The tissue were washed, fixed in 10% formalin and then dehydrated in different grades (70%, 80%, 90%, and 100%) of alcohol. After two changes in chloroform for 30 minutes each and one change in xylene for 45 minutes, the tissues were dipped in molten paraffin wax twice for 1 hour followed by embedding in the same in Metal block. Thick sections of 4.5 – 5 μm were cut with fine blade attached to spencer microtome. Sections were stretched in hot water on albumin coated slides and stained with Delafield's Haematoxylin/Eosin Technique (H/E) (Baker, 1945) to study histology. The paraffin sections of tissue were dewaxed in two changes of xylene. The sections were downgraded through various grades of alcohol to water - 100% (3 min), 90% (3 min), 70% (2 min), 50% (2 min), 30% (2 min) and water (2 min). Then the sections were stained in haematoxylin for 15-20 minutes. They were then kept under running tap water for 15 minutes till the sections turned pink. Then the
tissues were differentiated in acid and ammonia water (one or two dips in each). They were then upgraded up to 90% alcohol by dehydrating the slides - 30% (4 min), 50% (4 min), 70% (4 min) and 90% (4 min). Then the tissues were stained with eosin (1 min or 30 sec), 90% (10 min), and 100% alcohol (10 min) and xylene (10 min). The sections were mounted in DPX & observed under different magnifications for local inflammatory changes under light microscope.