IV. MATERIALS AND METHODS

Source of data

The study was conducted at the Department of Microbiology, Sri Siddhartha Medical College and Research centre, Tumkur, (Karnataka) and the Department of Microbiology, Sri Krishnadevaraya University, Anantapur, (A.P). The study period was from June 2003 to May 2006.

Patient selection

a. Inclusion Criteria

The suspected typhoid cases of all the ages from both male and female for the culture and sensitivity test, CRP test and Buffy coat smear study. For determination of anti-O and anti-H antibodies all the above cases and human volunteers.

b. Exclusion Criteria

Patients with negative blood culture and showing the titre of anti-AH anti-BH in Widal test.
MATERIALS

1. Clinical Samples

Blood Samples

From adults 5 to 10ml of vein blood was taken aseptically from suspected cases and inoculated into 50 to 100ml of Brain Heart Infusion broth for culture and sensitivity.

From neonates 1 to 2ml of blood was taken aseptically from suspected cases and inoculated into 10 to 20ml of Brain Heart Infusion broth for culture.

Serum Samples

From adults another 5ml of the sample was collected for immunological studies such as CRP test, Buffy coat smear study and Widal test.

From neonates another 1 to 2 ml of blood sample was collected for immunological studies such as CRP test, Buffy coat smear study and Widal test. Serum samples are stored in deep freezers at -20°C until further use.

2. Isolation of Salmonella typhi

Peptone Water

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 to 7.6</td>
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</tbody>
</table>


Nutrient Broth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>-5g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>-3g</td>
</tr>
<tr>
<td>NaCl</td>
<td>-5g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>-1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>-7.2</td>
</tr>
</tbody>
</table>

Nutrient Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>-5g</td>
</tr>
<tr>
<td>NaCl</td>
<td>-5g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>-3g</td>
</tr>
<tr>
<td>Agar-agar</td>
<td>-20g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>-1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>-7.2</td>
</tr>
</tbody>
</table>

Blood Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient agar</td>
<td>-100ml</td>
</tr>
<tr>
<td>Sheep blood</td>
<td>-10 to 20 ml</td>
</tr>
</tbody>
</table>

MacConkey Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>-20g</td>
</tr>
<tr>
<td>Sodium taurocholate</td>
<td>-5g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>-1000 ml</td>
</tr>
<tr>
<td>Agar-agar</td>
<td>-20g</td>
</tr>
</tbody>
</table>

Neutral red solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% in 50% ethanol</td>
<td>-3.5ml</td>
</tr>
<tr>
<td>Lactose, 10% aqueous solution</td>
<td>-100ml</td>
</tr>
<tr>
<td>pH</td>
<td>-7.5</td>
</tr>
</tbody>
</table>
Brain Heart Infusion Broth

- Calf brain infusion: 200g
- Beef Heart Infusion: 250g
- Peptone: 10g
- NaCl: 5g
- diSodium phosphate: 2.5g
- Dextrose: 2g
- Distilled Water: 1000 ml
- pH: 7.4 ± 0.2

Wilson and Blair Bismuth Sulphite Media

- Bismuth ammonia-citrate scales: 30g
- Sodium Sulphite: 100g
- diSodium Hydrogen phosphate: 100g
- Glucose: 50g
- Distilled Water: 1000 ml
- pH: 7.7 ± 0.2

Muller-Hinton Agar

- Beef infusion: 300 ml
- Casein hydrolysate: 17.5g
- Starch: 1.5g
- Agar-agar: 10g
- Distilled Water: 1000 ml
- pH: 7.4

3. Identification

Sugar Fermentation Medium

- Peptone (Oxoid): 15g
- Andride's Indicator: 10 ml
- Sugar to be tested: 20 mg
  (Glucose, Lactose, Sucrose, Mannitol)
- Distilled water: 1000 ml
Triple Sugar Iron Agar

Peptic digest of animal tissue  - 10g
Casein enzyme hydrolysate - 10g
Yeast extract - 3g
Beef extract - 3g
Lactose - 10g
Sucrose - 10g
Dextrose - 10g
NaCl - 5g
Ferrous Sulphate - 0.20g
Sodium thiosulphate - 0.30g
Phenol red - 0.024g
Agar-agar - 15g
Distilled water - 1000 ml
pH - 7.4 ± 0.2

Indole Test

Medium

Peptone (brand containing-sufficient tryptophan)  - 20g
NaCl - 5g
Distilled water - 1000 ml
pH - 7.4

Kovac’s Reagent

Amyl or Isoamyl alcohol - 150ml
p-dimethyl amino benzaldehyde - 10g
Conc. HCl - 50 ml.
Methyl Red Test
Medium
(Glucose Phosphate Peptone Water)
Peptone – 5g
di-Potassium hydrogen phosphate – 5g
Distilled water – 1000 ml
Glucose 10% solution (sterilized separately) – 50ml
pH – 7.6
Methyl Red Indicator solution
Methyl red – 0.1g
Ethanol – 300ml
Distilled water – 1000 ml

Voges Proskauer Test
Medium
Glucose Phosphate peptone water.
Reagent
40% KOH and 5% a-napthol.

Citrate Utilization Test

Ammonium Chloride – 1.5g
Potassium Phosphate – 1.0g
Magnesium Phosphate – 0.2g
Sodium Citrate – 3.0g
Bromothymol blue 2% – 40.0ml
Agar-agar – 20g
Distilled water – 1000 ml
pH – 6.8 ± 2
Urease Test

Peptone \(-1\text{g}\)
NaCl \(-5\text{g}\)
DiPotassium hydrogen phosphate \(-2\text{g}\)
Phenol red (1ml in 500ml aqueous solution) \(-6\text{ml}\)
Agar-agar \(-20\text{g}\)
Distilled water \(-1000\text{ ml}\)
Glucose, 10% solution, sterile \(-100\text{ml}\)
Urea 20% solution, sterile \(-100\text{ml}\)

4. Stains:

Crystal Violet Stain
Crystal Violet \(-10\text{g}\)
Absolute alcohol (100% ethanol) \(-100\text{ml}\)
Distilled water \(-900\text{ ml}\)

Dilute Carbol fuschin
Ziehl Neelson's (strong) Carbol Fuschin \(-500\text{ ml}\)
Distilled water \(-950\text{ ml}\)

Acridine Orange – R
0.1% in Distilled water.

5. Chemicals
1. Sodium Chloride
2. Glucose
3. Lactose
4. Sucrose
5. Mannitol
6. EDTA
7. Alcohol
8. Acetone
9. Iodine
10. Potassium Iodide
6. Glasswares and Others
1. Slides
2. Coverslips
3. Petriplates
4. Blood culture bottles
5. Test tubes
6. Durham’s tubes
7. Conical flasks
8. Beakers
9. Funnels
10. Glass spreaders
11. Glass rods
12. Pipettes
13. Felix tubes
14. Disposable syringes
15. Wintrobe tubes
16. Cotton Swabs
17. Gloves
18. Cotton
19. Eppendorff tubes
20. Wrapping papers
21. Filter papers.

7. Instruments
1. Microscopes – Compound Microscope and Fluorescent Microscope.
2. Weighing Machine
3. Centrifuge
4. Autoclave
5. Hot Air Oven
6. Laminar Air Flow Chamber
7. Refrigerator
8. Deep freezer
9. Incubator
10. pH meter
11. Colony counter
12. UV transilluminator
13. Inoculation loop
14. Bunsen burner
15. Spreader
16. Micropipette.

8. Antibiotic Discs (Hi Media, Mumbai)
   1. Ampicillin (AMP) – 30 µg
   2. Chloramphenicol (CHO) – 25 µg
   3. Co-trimaxazole (COT) – 25 µg
   4. Tetracycline (TRC) – 10 µg
   5. Ceftriaxone (CFT) – 30 µg
   6. Cefuroxime (CFX) – 30 µg
   7. Ciprofloxacin (CFC) – 30 µg
   8. Ofloxacin (OFC) – 30 µg
   9. Nalidixic acid (NAD) – 30 µg
   10. Amikacin (AMK) – 30 µg
   11. Gentamycin (GTM) – 30 µg
   12. Amoxicillin (AMX) – 25 µg

9. Antiserum for serotyping (Central Research Institute,Kasoli, India.)
   O – antisera;
   Polyvalent – O
   9 – O, Group – D.
   H – antisera;
   Polyvalent – H
   d – H (Salmonella typhi)
   Vi – antiserum.
Salmonella agglutinating sera for Serotyping of *Salmonella typhi* were obtained from Murex Biotech Ltd., Central Road, Temple Hill, Dartford DAI, 5LR, U.K., supplied by Span Diagnostic Ltd., G.I.D.C., Sachin, Surat, India.

10. Phage typing

The confirmed isolates were sent to the National *Salmonella* Phage Typing Centre at Lady Hardinge Medical College, New Delhi for Phage typing.

11. Biotyping

- Arabinose
- Dulcitol
- Xylose

12. CRP Test

This test was done by latex agglutination method by using kit supplied by HUMATEX CRP from Human Gerellschiftfur Biochemica and Diagnostica mbh.

13. Buffycoat Smear Study

It was done by Brooks GF etal. Method. (Brooks etal, 1973).

14. *Salmonella* Antigen

*Salmonella* anti-O and anti-H antigens.

15. Control strains

*E. coli* ATCC 25922 and *Salmonella typhi* NCTC-786.
METHODS

1. Processing of Blood Samples

Two blood samples taken from each patient at the time from different sites for culture and sensitivity. Blood is obtained by using sterile precautions. First, the site was cleaned with 10% povidine-iodine solution and left for 1 to 2 minutes. Then the site was cleaned with 70% alcohol. By wearing sterile gloves, blood was drawn from the sites using a sterile needle and syringe. The sites chosen were anticubital fossa or forearm vein.

5 to 10 ml of the blood from adults and 1 to 2 ml of blood from neonates, were inoculated into 50 to 100 ml of BHI broth for adults and 10 to 20 ml of BHI broth for neonates.

Blood culture bottle were incubated at 37°C overnight. The turbidity was observed everyday by holding the bottles against bright light. If there is a growth subculture was done on next day on Blood agar and MacConkey agar. After obtaining good growth, it was plated on selective medium, Wilson and Blair Bismuth Sulphite agar (Wilson and Blair, 1931). Identification of the colonies was made by standard colonial morphology.

When no growth occurs on plate, subsequent subcultures were done on 2
nd, 3
rd, 7
th and 11
th day. No growth was reported, only when there was no growth after one week of incubation and the incubated blood samples were not discarded as negative till 11
th day (Shaw and Mackay, 1951). The isolated colonies were subjected for identification.
2. Processing of serum samples

The blood obtained from patients and human volunteers was transferred to centrifuge tubes and centrifuged at a rate of 1000 rpm for 5 minutes. Then the serum was separated into Eppendorf tubes and preserved at -20°C in deep freezer until further use.

3. Isolation and Identification

The Mac Conkey agar and Blood agar plates were observed for the colony characteristics and subjected for standard identification procedures (Collee et al, 1996, Cruckshank 1975).

Sugar Fermentation Medium

Andrade’s indicator is prepared from 0.5% aqueous acid Fuschin to which sufficient 1 mol/ltr NaOH has been added to turn the colour of the solution yellow.

Dissolve the peptone and Andrade’s indicator in 1 litre of water and add 20g of the sugar to be tested include Glucose, Lactose, Sucrose, Mannitol. Distribute 3 ml each in standard test tubes containing an inverted Durhams tube. Sterilize by tyndallization that is steaming at 100°C for 30 minutes for three consecutive days.

Triple Sugar Iron Agar

The triple sugar iron slants with a butt were prepared and the test isolates were stabbed in the butt and streaked over the slants. The tubes were incubated at 37°C for 24
hours. The crescent shaped blackening of the medium indicates the formation of H$_2$S, which was recorded as positive.

**Indole Test**

Inoculate medium and incubate for 48 hours at 37°C. Sometimes a period of 96 hours at 37°C may be required for optimum accumulation of Indole. Add 0.5 ml of Kovac’s reagent and shake gently. A red colour in the alcohol layer indicates a positive reaction.

**Methyl Red Test**

Inoculate the liquid medium lightly from a young agar slope culture and incubate at 37°C for 48 hours. Add about 5 drops of the methyl red reagent. Mix and read immediately. Positive tests are bright red and negative are yellow. If the results after 48 hours are equivocal the test should be repeated with cultures that have been incubated for 5 days. For some organisms, incubation at 30°C for 5 days is preferable to incubation at 37°C for 2 or 5 days.

**Voges Proskauer Test**

Incubate at 37°C or 30°C for 48 hours. Add 1 ml of 40% KOH and 3 ml of a 5% solution of a-napthol in absolute ethanol. A positive reaction is indicated by the development of a pink colour in 2 to 5 minutes becoming crimson in 30 minutes. The tube can be shaken at intervals to ensure maximum aeration.
Citrate Utilization Test

Inoculate from a saline suspension of the organism to be tested. Incubate for 96 hours at 37°C.

Urease Test

Inoculate heavily over the entire slope surface and incubate at 37°C. Examine after 4 hours and after overnight incubation, no tube being reported negative until after 4 days incubation. Urease positive culture changes the colour of the indicator to purple pink. All the isolates are confirmed by slide agglutination using specific O, H and Vi-antisera.

4. Phage Typing

The confirmed isolates were sent to the National Salmonella Phage Typing Centre at Lady Hardinge Medical College, New Delhi for Phage typing.

5. Serotyping

Serotyping of confirmed isolates of Salmonella typhi was carried out by slide agglutination test. Two separate drops of saline was placed on a glass slide. Single colony of Salmonella typhi was emulsified with a loop in each drop of saline to obtain smooth fairly dense suspension. Further, to one suspension, as a control, a loopful of saline was added and mixed. To other suspension, undiluted Salmonella agglutinating sera was added and rocked gently to observe agglutination using indirect lighting over a dark background. This procedure prescribed by Cruckshank et al, 1975, was followed for all the isolates of Salmonella typhi against polyvalent-O, 9-O, group - D, polyvalent-H, d-H (S. typhi H) and Vi-antisera.
6. Biotyping

Biotyping includes the fermentation test and decarboxylase test of Arabinose, Dulcitol and Xylose.

7. Antibiotic Sensitivity Test

Kirby-Bauer's disc diffusion method (Bauer AW 1966) as per the National Committee for Clinical Laboratory Standard guidelines (NCCLS 2002) was employed to study the susceptibility pattern of the confirmed isolates against a panel of selected antimicrobial agents. Ampicillin, Chloramphenicol, Co-trimaxazole, Tetracycline, Ceftriaxone, Cefuroxime, Ciprofloxacin, Ofloxacin, Nalidixic acid, Amikacin, Gentamycin and Amoxicillin.

Preparation of media

Sterility checked Muller-Hinton agar medium and Muller-Hinton broth will be employed.

Preparation of Inoculum

The 24 hours old, 4-5 well isolated colonies will be inoculated into 5 ml of Muller-Hinton broth and incubated at 37°C for 6 hours till light to moderate turbidity developed. The turbidity thus developed will be matched with 0.5 Mac Farlands Standard.

Inoculation

The Mueller-Hinton agar sterile plates will be inoculated with the standardized inoculum of test isolate by a sterile cotton swab dipped into the inoculum tube and rotated.
firmly against the upper inside wall of the test tube to remove excess fluid. The entire surface of Mueller-Hinton agar plate will be streaked with this swab by lawn culture method.

Antibiotic discs

After the inoculum is dried, commercially obtained panel of antibiotic discs will be placed aseptically with a sterile forceps or a dispenser onto the surface of the seeded plate at least 30 mm apart. The discs are pressed gently to ensure even contact with the medium. Five antibiotic discs are accommodated in a single petridish.

Incubation

All the plates will be incubated for 16-18 hours at 37°C.

Control

The antimicrobial susceptibility pattern of the reference culture E.coli ATCC 25922 and Salmonella typhi NCTC-786 are used for comparison in the present study as control.

Reading of Zones of Inhibition:

After the completion of period of incubation, the diameter of the zone of inhibition around the disc is measured to the nearest millimeter.

Interpretation:

Zone of diameter interpretative standards are used as recommended by the National Committee for Clinical Laboratory standards. (NCCLS 2002). The respective isolate will be recorded as sensitive, intermediate & resistant to antibiotics used.
8. Determination of MIC

Micro broth dilution method as per NCCLS guidelines was employed to determine Minimum Inhibitory Concentration of the representative *Salmonella typhi* strains against Ampicillin (AMP), Chloramphenicol (CHO), Co-trimazazole (COT), Tetracycline (TRC), Ceftriaxone (CFT), Cefuroxime (CFX), Ciprofloxacin (CFC), Ofloxacin (OFC), Nalidixic acid (NAD), Amikacin (AMK), Gentamycin (GTM) and Amoxicillin (AMX) selected drugs. In all thirty representative strains from among the confirmed isolates were considered sensitive, intermediate and resistant strains of ten each were selected based on the nearest range of sensitivity and resistance. As per the NCCLS guidelines the MIC range was taken to prepare the highest dilution factor and two-fold dilution were made in a series of 15 of 5 ml test tubes.

i. Preparation of stock solution

The highest range of all the drugs used in general was 512 µg/ml. 1024 mg pure form of drug was dissolved in 1000 ml suitable diluents to obtain 1024 µg/ml.

ii. Preparation of inoculum

The confirmed isolates of *S. typhi* were inoculated in 5 ml of sterile nutrient broth taken in different test tubes and incubated at 37°C for 6 hours till moderate turbidity was developed. The turbidity was matched with 0.5 ml Mac Farlands turbidity standard (Mac Farlands, 1907 and Brown 1919)

iii. Inoculation

0.5 ml of Mueller-Hinton broth was added in 15, 5 ml test tubes. 0.5 ml working antibiotic solution was added in the first tube. From the first tube 0.5 ml was transferred
into the second tube further, in this manner it was serially diluted in two folds. 0.5 ml of the inoculum was added in each tube.

iv. Incubation and Interpretation of results

After inoculation the tubes were incubated at 37°C for 18 hours. The incubated tubes were observed for the lowest concentration of the drug that inhibits the growth of the organism by visual inspection of turbidity.

v. Control

Reference strain *E.coli* ATCC 25922 was used in order to have a comparative measure.

9. CRP Test

This test was done by latex agglutination method using kit supplied by Human Diagnostic Company.

A qualitative determination (Screening test)

<table>
<thead>
<tr>
<th>Bring latex reagent, controls and serum samples to room temperature. Mix the latex reagent. Carefully prior to use to suspend the latex particles completely</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette/drop onto separate cells of the slide</td>
</tr>
<tr>
<td>Serum sample</td>
</tr>
<tr>
<td>Control serums positive, bottle 2, red cap</td>
</tr>
<tr>
<td>Control serum negative, bottle 3, green cap</td>
</tr>
<tr>
<td>CRP latex reagent, bottle 1, white cap</td>
</tr>
<tr>
<td>Onto all samples and control cells</td>
</tr>
</tbody>
</table>
Mix with separate sticks and spread the fluid over entire area of the particular cell.

Tilt the slide back and forth for 2 minutes so that the mixture rotates slowly inside the cells.

At the end of the 2 minutes read results under bright artificial light.

Interpretation of results

Distinct agglutination indicates CRP content of more than 6µg/l in the non-diluted serum specimen. Sera with positive results in the screening test should be retested by the titration test.

Semi quantitative test:

Dilute specimens with glycine NaCl buffer.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>CRP (µg/l is non-diluted specimen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 + 1 (1 : 2)</td>
<td>12</td>
</tr>
<tr>
<td>1 + 3 (1 : 4)</td>
<td>24</td>
</tr>
<tr>
<td>1 + 7 (1 : 8)</td>
<td>48</td>
</tr>
<tr>
<td>1 + 15 (1 : 16)</td>
<td>96</td>
</tr>
<tr>
<td>1 + 31 (1 : 32)</td>
<td>192</td>
</tr>
</tbody>
</table>

Continue test as described in part A

Interpretation of results:

Rend the titre in the last dilution step with visible agglutination and multiply the titre with the conversion factor 6 (Human's Humatex CRP is standardized to detect CRP concentrations in non-diluted serum samples of approximately 6 µg/l or higher to get the result in µg/l.
Positive and negative control sera are to be used with each series. The positive control should show a distinct agglutination within 2 minutes. The negative control should show a smooth suspension without visible agglutination even after 2 minutes.

10. Buffycoat smear study by Brooks Method

This is one of the rapid methods for diagnosis of septicemia. 1-2 ml of blood should be collected in a bottle containing EDTA at a concentration of 2-2.5 mg/ml. As an anticoagulant and centrifuged at 3000 rpm for 30 minutes. Smears are prepared from separated Buffycoat and stained with acridine orange examination of smear is done under oil immersion using fluorescent microscope. Bacteria if present stained bright orange colour and polymorphonuclear leucocytes appear yellow green. Smear can be considered positive if two or more organisms are observed intracellularly or extracellularly. Atleast 100 microscopic fields should be examined taking 3 minutes. (Brooks et al. 1973).

11. WIDAL TEST: (TUBE DILUTION METHOD) (Coolee et al., 1996).

1. Take the patients’ serum in a serial dilutions against each of the different Salmonella suspensions. For each series use seven small (7 x 1 cm) test tubes, six for six serum dilutions and the seventh for a saline, non-serum control.

2. Place 0.4 ml saline (0.85 % NaCl) in each of tubes 2-7. Make up a 1 in 15 dilution of the patient’s serum in saline and with a fresh graduated pipette add 0.4 ml of the diluted serum to each of tubes 1 & 2. Tube 2 will then contains 0.8 ml of serum diluted 1 in 30.
3. Mix the fluid in tube 2 by pipetting up and down several times, then transfer 0.4 ml into tube 3. With the same pipette similarly mix the 0.8 ml contents of tube 3 and then transfer 0.4 ml to tube 4. Repeat the process to tube 6. From which after mixing, discard 0.4 ml. Each tube then contains 0.4 ml fluid, tubes 1-6 containing serum dilution of 10, 20, 40, 80, 160 and 320 and tube 7 only saline.

4. With a fresh pipette, then add 0.4 ml bacterial suspension to each tube, starting at tube 7 and working backwards to tube 1. The serum dilutions in tubes 1-6 are now 30-960.

5. With a capillary pipette, transfer the mixtures to narrow agglutination tubes, starting at tube 7 and working backwards to tube 1.

6. Incubate agglutinations for 2 hr at 37°C and read after standing on the bench for half an hour. Incubate O agglutinations for 4 hour at 37°C and read after refrigeration overnight at 4°C. Use a water bath for incubation. Read the tests by viewing them under a good light against a dark background the large flakes of H- agglutination are easily visible with the naked eye, but ax2 magnifying lens should be used to detect the small granules of O agglutination after raising these by rotating the tube.