CHAPTER 3

MATERIALS AND METHODS

A. LABORATORY EVALUATIONS OF CEFTRIAXONE TO SHIGELLA SPECIES

The organisms used are the shigellae of all groups and vary in types obtained mostly from the Department of Medical Sciences, Ministry of Public Health and some were obtained from the rectal swab of the patients who were studied.

1. Antimicrobial Susceptibility Tests

Using the disc agar diffusion method[1],[2] with the following details.

1.1 Medium and preparation of plates

Medium : Mueller Hinton Agar (Difco, control no. 660370) was used. The following shows the ingredients per litre.

Beef, Infusion from	300	gm
Casamino Acids, Technical	17.5	gm
Starch	1.5	gm
Bacto - Agar	17	gm

To rehydrate the medium, suspend 38 grams in 1000 ml cold Purified Water, USP, distilled or deionized water, and heat to boiling to dissolve the medium completely. Dispense into flasks and sterilize in the autoclave for 15 minutes at 15 pounds pressure (121° C). Avoid excessive heat during rehydration or sterilization.

Final pH is 7.3 ± 0.1

In the preparation of plates, 20 ml of sterile Mueller Hinton Agar pH 7.2 - 7.4 was dispensed into sterile glass petridishes of 90 mm diameter with the same brand and lot to produce the uniform thickness of the agar. The agar was allowed to harden on a flat level surface. The plates were dried for 1 hour at 37° C.

1.2 Discs

The antibiotic discs tested were the following

- Ampicillin 10 µg/disc (BBL) (Lot.No.205029)
- Cephalothin 30 µg/disc (BBL) (Lot.No.007003)
- Chloramphenicol 30 µg/disc (BBL) (Lot.No.001000)
- Trimethoprim/Sulfamethoxazole (TMP/SMX)
  1.25 mg/23.75 g per disc (BBL) (Lot.No.107077)
- Tetracycline 30 µg/disc (BBL) (Lot.No.102015)
- Ceftriaxone (Rocephin®) 30 µg/disc obtained from The F. Hofmann - La Roche & Co. ltd.

1.3 Preparation of the Inoculum

Seventy-seven isolates of Shigella of all groups were separately inoculated into 1 ml of sterile nutrient broth in tubes and incubate for 4 - 6 hours at 37° C. Then standardize the inoculum to match a 0.5 turbidity standard of Mac Farland when comparing the tubes against a white background with a contrasting black line.

1.4 Preparation of test plates

Spread 0.1 ml of standardized Shigella suspension (described in 1.3) over the agar surface (from 1.1) in several directions.

After that the discs of antibiotics were placed, left in room temperature for 30 minutes, then incubate at 37° C for 16 - 18 hours.

1.5 Interpretation of the test results

The diameters of the inhibition zones of the antimicrobials were measured with a sliding calipers with an acuracy of nearest 0.1 mm. The end points were taken as the area showing no visible growth that could be detected with the unaided eye. Faint growth or tiny colonies near the edge of the inhibition zones were ignored if they were presented. The zone-size interpretative chart of Kirby-Bauer[16,17] was used as shown in the table 20.

Table 20 Zone-size interpretative chart[16,17]

Antimicrobial agents	Disc potency (µg)	Diameter of zone of inhibition (mm)		
		Resistant	Inter-mediate	Susceptible
ampicillin, when testing Enterobacteriaceae	10	≤ 11	12-13	≥ 14
cephalothin	30	≤ 14	15-17	≥ 18
chloramphenicol	30	≤ 12	13-17	≥ 18
neomycin	30	≤ 12	13-16	≥ 17
tetracycline	30	≤ 14	15-18	≥ 19
Trimethoprim/Sulfamethoxazole	1.25/23.75	≤ 10	11-15	≥16
ceftriaxone[56]	30	≤ 12	13-15	≥16

2. Determination of Minimal Inhibitory Concentration (MIC)

The agar dilution method was used[18]. It was performed by incorporating the antimicrobial agent into an agar medium just before it is poured onto a petridish. Many isolates of Shigellae were spot - inoculated simultaneously on to a series of petridishes containing vary concentrations of ceftriaxone, then the results were read, all the materials and methods were described as follows.

2.1 The test medium

Mueller - Hinton agar (see title 1.1 p 53) was prepared in 500 ml flasks and sterilized at 121° C under 15 pounds per square inches pressure for 20 minutes.

2.2 The antibiotic diluent

It was the sterile phosphate buffer pH 7.0 as would be described in the title no. (4.4.1) on page 62.

2.3 Preparation of the antimicrobial dilutions

They were prepared at the concentrations ten times that desired in the final test, as follows.

2.3.1 0.2439 gm of ceftriaxone working standard was dissolved in sterile 0.1 M phosphate buffer pH 7.0 to make 100.0 ml suhution in a volumetric flask, this will obtain a 2000 µg/ml solution.

2.3.2 12.8 ml. of the aboye solution was diluted with 7.2 ml. of the same sterile buffer to make 1,280 µg/ml solution. Then it was further diluted to make a series of two fold dilutions of ceftriaxone containing 0.078, 0.1563, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1,280 µg/ml.

2.4 Prepatation of test plates

The agar medium was melted and allowed to cool to 45° to 50° C in a water bath. Then 18 ml of it was transferred to each sterile test tube which contains 2 ml of each ceftriaxone dilution. The 10 ml Cornwall continuous pipette (reorder no. 3056, Division of Becton-Dickinson and company, Rutherford, New Jersey, 07070, USA) was used.

The tubes were mixed thoroughly but gently and the agar was poured into the sterile 90 mm plates and allowed to harden on a flat level surface.

The agar plates would give the final ceftriaxone concentrations of 0.0078, 0.0156, 0.0313, 0.0625, 0.125, 0.250, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0 and 128.0 µg/ml.

At least one control plate, containing Mueller - Hinton agar without antimicrobial, was prepared for every series of dilutions and in every different group of bacteria tested.

The plates were freshly prepared in the day the experiment was done.

2.5 Preparation of the inoculum

103 isolates of shigellae from every group varying in types obtained from stools of Thai patients from many parts of Thailand were prepared and standardized in the same manner as described in title number 1.3 (page 54)

The control strains of S. aureus ATCC6538-P and S. lutea ATCC 9341 were prepared in the same way.

2.6 Inoculations of agar plates

The inoculum-replicating apparatus[18] was used. About 19 standardized shigellae suspensions were transferred to the appropriate wells in each seed plate containing 21 reservoirs, and the control strains of Staphylococcus aureus ATCC 6538-P and Sarcina lutea ATCC 9341 were placed to the other 2 wells in every plate.

An aluminium replicating device[18] (the hand-held multi-point plate inoculator) was dipped into the wells of inoculum in the seed plate and then the inoculum suspensions were spotted onto the previously dried surface of each antimicrobic-containing plate, by touching the ends of the inoculators on the agar surface. The plates were incubated at 37° C for 16 to 20 hours .

There were 1 or 2 control plates without antimicrobic which were inoculated as a control in every set of tests .

2.7 Reading of test results

The agar dilution plates were examined for growth, after incubation. First, the control plates without antimicrobic were checked to be sure that each test strain was capable for providing adequate growth. Then the remaining plates were examined to determine the minimal concentration of drug required for inhibition of growth. The end points were judged when there was a definite dense film of growth in the next plate of lesser concentration. The control strains end points were checked in each set of tests to confirm the reproducibility of the test.

B. CLINICAL STUDY

1. Drug used

Ceftriaxone sodium injection (Rocephin(R)), obtained from The F. Hoffmann-La Roche & Co.Ltd. Each vial contains 1 gm. of ceftriaxone sodium in lyophilized form. Lot number : Pt 3395 H 03 E 0731 Expiration date : 8-83

1.1 Method of Reconstitution and route of administration.

1.1.1 For intravenous injection

add 10 ml. of sterile water for injection and shake gently to make a clear solution and inject direct intravenously or by intravenous infusion.

1.1.2 For intramuscular injection

add 3.5 ml. of 1 % Lidocaine for injection and shake gently to make a clear solution.

1.2 Dose of ceftriaxone

Single dose of 50 mg per kilogram body weight of ceftriaxone was given intravenously or intramuscularly.

2. Criteria for Selecting the Patients

2.1 Children of age not more than 12 years of both sexes with a history of fever, bloody mucous stools, tenesmus and symptoms suggestive of shigellosis with no any other complications and gave positive shegella stool cultures before treatment were selected to study.

2.2 The patients did not receive any other antibacterial

drug during the course of observation.

2.3 Number of patients to be studied was not less than 20 cases.

2.4 Most of them were the in-patients which were admitted in Bamrasnaradura Infections Hospital for at least 5 days (or until the symptoms were improved.

3. Other Treatment

When the patients were first admitted, all of them received the oral electrolyte solution which contains the basic electrolytes as in the following formula :-

Sodium Chloride	3.5	gm
Potassium Chloride	1.5	gm
Sodium Bicarbonate	2.5	gm
Glucose	20.0	gm
Water to	1000.0	ml

This will give Na^+ 90, K^+ 20, Cl^- 80, HCO_3^- 30 and glucose 110 milli-mol per litre.

The quantity of electrolytes given depends on the degree of dehydration and frequency of defecations.

The half strength of the above solution was used if the patients were under 6 months old.

Most of the patients received paracetamol syrup for fever until their body temperature had returned to normal.

4. Determination of patients' serum levels of ceftriaxone after injection.

The method used was the microbiological assay of antibiotics which was adapted from the USP mothod[19] and the materials and methods were set out as follows.

4.1 The assay medium : the antibiotic medium no. 2 (Bacto - Penassay Base Agar, Difco, control no. 659790) was used and contains the following ingredients per liter.

- Bacto - Beef Extract 1.5 gm
- Bacto - Yeast Extract 3 gm
- Bacto - Peptone 6 gm
- Bacto - Agar 15 gm

To rehydrate the medium, suspend 25.5 grams in 1000 ml. cold distilled water and heat to boiling to dissolve the medium completely.

The medium is dispensed in 500 ml conical flasks with cotton wool plugs and sterilized at 15 pounds pressure (121° C) for 15 minutes. The final pH was 6.5 ± 0.1.

4.2 The assay microorganism : Sarcina lutea ATCC 9341 The microorganism is maintained and grown on the nutrient agar slants and had been subcultured every 2 weeks and kept at 4 - 10° C

The culture must be freshly inoculated on an antibiotic medium no. 2 (mentioned above) slants and incubated at 37° C for 16 - 18 hours before each experiment. They are washed out with sterile NSS and adjusted to give a turbidity that will permit 25 % light transmission.

4.3 Preparation of plates

Sterile glass petridishes of 90 mm diameter were used and were of uniform size, They were chosen from the same lot of the same brand (Pyrex).

One ml of the standardized inoculum was added to each 100 ml of the assay medium which had been melted and cooled at 45-50° C, the flasks swirled to obtain a homogenous suspension, 15 ml of the seeded medium were added to each sterile petridish, and allowed to harden on a flat level surface. Then, a sterile cork borer with 7 mm diameter was used to press upon the hardened agar to make 6 sharp circles to give holes after removing the agar within the circles with a sterile needle.

4.4 Preparation of the Standard

The working standard was obtained from the F. Hofmann-La Roche & Co. ltd. as disodium salt crystaline powder of ceftriaxone with the potency of 820 µg/ml, It was kept in tightly closed vials which are placed in the desiccator over silica gel and stored in the refrigerator.

The standard solution was freshly prepared on the day on which the experiment was done by the following method :-

4.4.1 Preparation of the diluting solution

Sterile phosphate buffer pH 7.0 was used in standard and sample dilutions, It was prepared with the following formula :-

0.1 M Phosphate buffer pH 7.0

A. Dibasic sodium phosphate 5.934 gm

Distilled water to make 500 ml

B. Monobasic sodium phosphate 4.600 gm

Distelled water to make 500 ml

300 ml of A are mixed with 200 ml of B, which gave a buffer solution of pH 7.0

4.4.2 Dilution of the standard

1. 0.2439 gm of Ceftriaxone working standard was dissolved in sterile 0.1 M phosphate buffer pH 7.0 to make 100.0 ml solution in a volumetric flask. This gave a 2,000 µg/ml solution, the solution 1.

2. The solution 1 was further diluted with the sterile 0.1 M phosphate buffer pH 7.0 to make a series of two fold dilutions of ceftriaxone in a range from 1.6 µg/ml to 25.6 µg/ml as follows, using aseptic technique.

no. of solution	proportion of Standard Solution to buffer solution	final concentration, µg/ml
(2)	1.28 ml of solution 1 + 98.72 ml of buffer pH 7	25.6
(3)	5 ml of solution 2 + 5 ml. of buffer pH 7	12.8
(4)	5 ml of solution 3 + 5 ml. of buffer pH 7	6.4
(5)	5 ml of solution 4 + 5 ml. of buffer pH 7	3.2
(6)	5 ml of solution 5 + 5 ml. of buffer pH 7	1.6

4.5 Preparation of the samples

The sera were obtained from the patients at 1, 8 and 24 hours (and some other times eg 2, 5, 48 hours in some cases) after administration of the drug and store in the freezer under - 20° C until the time of assay. The sera had to be diluted to give the appropriate size of zone diameter, but their final concentrations had to be within the standard curve concentrations(1.6 - 25.6 µg/ml). This was done by making various experimental dilutions of the sera each time blood was drawn after injection.

The results obtained were the following :- Sera of the 1^{st} and 8^{th} hour after injection needed to be diluted to make 1 : 15 dilution. Sera of the 24^{th} to 48^{th} hour after injection needed to be used in the undiluted form.

4.6 Assay Procedures :-

4.6.1 Preparation of the standard plates

In each experiment, one set of standard curve determination plates was included. It contains 4 sets of standard

dilutions with the triplicate plates, leaving one dilution to be the reference solution in every plate of assay to check the variations of the zone diameters.

Every inoculated agar plates from 4.3 had 6 holes, 3 of them were filled with 50 µl of one standard dilution and 3 holes with the reference dilution using alternate holes.

The volume of the solution added to each hole was accurately measured and transferred with the 50 µl micro-pipette (Centaur Sciences, Inc. 180 Harvard Avenue Stamford, CT 06902)

The plates were left at room temperature for 1 hour to allow the antibiotic to diffuse into the medium. Then, they were incubated at 37° C for 16 - 18 hours. The diameters of the inhibition-zones (produced by the varied concentrations of the standard dilutions) were measured with a sliding caliper to get the greatest possible accuracy of 0.1 mm.

4.6.2 Standard curve determination

The average standard values in each dilution were corrected with the corrected values obtained from the differences of the average reference zone in each set from the overall average reference zone of the experiment (C).

The corrected standard values were plotted on two cycle semilogarithmic paper, using the concentrations in µg/ml as the ordinate (logarithmic scale) and the diameter (in mm) of the inhibition zone as the abscissa. The straight line obtained from the

graph was used as the standard for calculating the sample potencies which will be mentioned below.

4.6.3 Preparation of the sample plates :

Each sample, the serum dilutions of each venepuncture after injection, was filled into 3 holes per plate with the 3 alternate holes filled with the reference standard solution, Three plates were used for each sample to give triplicate plates,

The frozen sera were thawed at room temperature and diluted as described in 4.5, then they were treated in the same manner as the standard dilution as described in 4,6,1

4.6.4 Calculations of the sera concentrations of Ceftriaxone

The average zone diameters of the serum dilution and the reference standard dilution on the three plates were used. If the average zone diameter of the sample is larger than that of standard, add the difference between them to the reference concentration diameter of the standard response line, and vice versa. From the response line,read the concentration corresponding to these corrected values of zone diameters. Then the exact sera concentrations were obtained by multiplying the above values with their dilution factors.

5. Method of clinical evaluation

The clinical evaluation was based on the following criteria after one dose injection of ceftriaxone.

5.1 The reduction of body temperature (pyrexia) after receiving ceftriaxone along with paracetamol.

The criterion was set that the patient had a fever if the body temperature was over 38° C. The body temperature was recorded every four hours during hospitalization by the nurses.

5.2 The stool characteristics observed were, whether they were mucous, bloody, loose or watery or not. And to observe when they became normal i.e. became formed stools.

5.3 The frequency of defecations. It was set that if the number of defecations per day did not exceed 3 times it was normal.

5.4 Other gastro-intestinal tract symptoms such as nausea, vomiting, gastric distension had been also observed to see the effects of the drug on them.

5.5 Other systemic symptoms such as degree of dehydration etc.

These clinical symptoms were evaluated for the number of days needed to overcome them within the 5 days of observation in the hospital.

6. Determination of presence of shigella in rectal swab after ceftriaxone administration

This was done by the Clinical Microbiology Laboratories, Department of pathology, Bamrasnaradura Infectious Diseases Hospital.

The rectal swabs or the stool swabs were taken from the patients once a day in the morning and were inoculated bed side by direct plating on the Salmonalla-Shigella agar (SS agar) (Gibco) and/or Mac Conkey agar and were incubated at 37° C for 18 - 24 hours. Then the pale, little colonies were picked up and inoculated by streaking and stabbing into the Triple Sugar Iron agar (TSI agar) slants. They were incubated at

37° C for 18-24 hours. Then the cultures with acid butt and alkali slants were produced and produced no hydrogen sulfide gas (H_2S) were considered to contain the suspicious organisms. These cultures were tested preliminarily by slide agglutination tests with the Shigella antisera subgroup A,B,C and D. When they gave positive results, these then were confirmed by biochemical tests as follows.

Differentiation of Shigellae, Salmonellae and related organisms

Test or substrate	E. coli	Shigella	Salmonella	Arizona	Citrobacter
lysine decarboxylase	d	-	+*	+	-
Indol	+	+ or -	-	-	d
Motility	+ or -	-	+	+	+
Urease	-	-	-	-	d
Lactose	+	-**	-	d	(+) or +
Sucrose	d	-**	-	-	d
Citrate (Simmon's)	-	-	+,*** d	+	+, d
malonate	-	-	-	+	d

*S. paratyphi A is lysine negative

**Shigella sonnei usually ferment lactose and sucrose slowly

***S. typhi, S. paratyphi A and some rare biotypes fail to utilize citrate.

The results of the test were recorded for presence of Shigella everyday after treatment with Ceftriaxone for at least 5 days or until the patients were discharged.