MATERIALS AND METHODS

I. The enteropathogen survey of diarrheal stools

Source of specimens Rectal swabs and stool samples examined were obtained from 500 Thai diarrheal patients (adults and children) from Siriraj Hospital. Most of the specimens were collected during the acute phase of the disease.

Culture and serological methods Most fecal specimens were obtained by means of rectal swabs, although occasionally sample of freshly passed stools were submitted to the laboratory. All specimens were streaked onto the following media: Salmonella-Shigella agar, Mac Conkey agar, Brilliant Green agar, Bismuth Sulfide agar, Xylose Lysine Desoxycholate agar, Blood agar and Selenite-F broth. After overnight incubation at 37°C, the Selenite-F broth culture transfered to Salmonella Shigella agar, MacConkey agar, Brilliant Green agar, Bismuth Sulfide agar and Xylose Lysine Desoxycholate agar plates. All plates were examined after 24 and 48 hours incubation. Lactose-negative colonies were transfered to Triple Sugar Iron agar slants and subsequently to different kinds of media to determine patterns of biochemical activities.

1. Motility test Inoculate semisolid motility agar by stabbing with a 24 hours Triple Sugar Iron agar culture. Incubate the culture tubes at 37°C, 24 hours and observe growth from the

stab line (motile).

2. Urease test Inoculate culture heavily over the entire surface of the Christensen urease test medium and incubate at 37°C. Examine the interval of 2 hours, 4 hours, and 24 hours incubation. Negative tubes were observed dialy for 4 days in order to detect delayed reactions given by members of certain groups other than Proteus. Urease-positive cultures produce an alkaline reaction evidenced by a red color.

3. Fermentation test Inoculate carbohydrate media for fermentation tests (lactose, dextrose, mannitol, maltose, sucrose, dulcite and xylose) with overnight broth culture. Incubate 18 to 24 hours at 37°C. Examine for acid or acid and gas production.

4. Tests for Indol Inoculate Tryptophan broth and incubate for 48 hours at 37°C. Add 5 drops of Kovac's reagent. A deep red color indicated the presence of indol.

5. Methyl red test To 5 ml of the culture in Methyl red-Voges-Proskauer broth (MR-VP), add 5 drops of Methyl red solution. A positive reaction is indicated by a distinct red color, showing the presence of acid. A negative reaction is indicated by a yellow color.

6. Voges-Proskauer test for acetyl-methylcarbinol To 5 ml of 48 hours culture in Methyl red-Voges-Proskauer broth, add 3.0 ml of 5% alpha-napthol in absolute ethyl alcohol and 1.0 ml of 40%

potassium hydroxide solution. Shake well and allow to stand for 10 to 20 minutes. If acetylcarbinol has been produced, at the surface of the medium a bright orange red color and will gradually extend throughout the broth.

7. Citrate test Inoculate Simmons Citrate Agar. A positive test is indicated by the development of a Prussian blue color in the medium, showing that the organism can utilize citrate as a sole source of carbon.

8. Malonate test Inoculate Malonate broth with a 3 mm loopful of a broth culture. Incubate at 37°C and observe after 24 and 48 hours. Positive results are indicated by a change in the color of the indicator from green to prussian blue.

9. Phenylalanine deaminase test Inoculate Phenylalanine broth with a 3 mm loopful of a broth culture. Incubate at 37°C for 18 to 24 hours. Allow 4 or 5 drops of a 10% (W/V) ferric chloride solution. If phenylpyruvic acid has been formed, a green color developed.

10. Potassium cyanide medium Inoculate Potassium cyanide broth with one loopful (3-mm loop) of a 24 hours broth culture grown at 37°C. Incubate at 37°C and observe daily for 2 days. Positive results are indicated by growth in the presence of potassium cyanide.

11. Decarboxylase tests Inoculate lysine, ornithine and arginine decarboxylase broth (Falkow) and control portions lightly of a young agar slant culture then cover with a 4 to 5 mm layer of sterile liquid paraffin. Incubate at 37°C and examine daily for 4 days. Positive reactions are indicated by alkaline (purple) reaction the media turn yellow first due to acid production from dextrose. Most positive reaction occur in 1 or 2 days.

12. Phenol red tartrate agar Inoculate by stabbing deep into the medium with a straight wire and incubate at 37°C for 24 hours. Positive test is indicated by the development of a yellow color in the medium.

These isolates showing biochemical patterns typical of Salmonellae, Shigellae or Vibrios were definitely identified in accordance with the serological method described by Edwards and Ewing (12).

Salmonellae. Specific antisera used were:

Polyvalent-H Specific, Non-specific

Polyvalent-O Group A-G

2 - O, a - H Group A

4 - O, b - H Group B

6,7,8, - O, c - H Group C_1, C_2

9 - O, d - H Group D

3,10,15,19 - O, e - H Group E

A culture that was anaerogenic, produced alkaline slant and acid reaction in the butt, formed only a small amount of hydrogen sulfide in Triple Sugar Iron agar and failed to hydrolyze urea rapidly (2 to 4 hours) should be considered as a possible strain of Salmonella typhi and treated accordingly. A dense suspension prepared from such culture first be tested in the living (unheated) state in group D Salmonella antiserum and in Vi antiserum on a slide. After these tests were completed, the suspension was heated in a beaker of boiling water for about 15 minutes, cooled, and retested in the same antisera. Salmonella polyvalent antiserum which contained Vi antibodies was also included in the above mention tests. If the reactions of a culture were typical of Salmonella typhi on Triple Sugar Iron agar and tests for urease were negative, and if a suspension reacts as follows:

Suspension	Antisera		
	Salmonella polyvalent	Salmonella O group D (9,12)	Vi (Ballerup 029)
Living	+ + + +	-	+ + + +
Heated	+ + + +	+ + + +	-

Cultures those reacted in polyvalent Salmonella antiserum but failed to agglutinate in Vi antiserum were tested in O grouping antisera.

Other Salmonellae were identified as the above mention tests.

Shigellae Any culture that was anaerogenic, produced an alkaline slant and an acid reaction in the butt, failed to give evidence of hydrogen sulfide production in Triple Sugar Iron agar,and failed to hydrolyze urea rapidly was considered as a possible Shigella strain and treated accordingly. Dense suspensions prepared in 0.9% sodium chloride solution were examined by slide tests for agglutination in absorbed polyvalent antisera for each of the four species of Shigella (Shigella dysenteriae, Shigella flexneri, Shigella boydii, Shigella sonnei). If a suspension failed to react in one or another of the fore mentioned polyvalent antisera, it was heated in a beaker of boiling water for about 15 minutes, cooled and reteated in the same antisera. Many Shigellae possess envelope or capsular antigens which inhibit agglutination of living and unheated bacteria in O antisera. These antigens are inactivated by heat.

Vibrios A polyvalent antiserum and a single O group I antiserum containing the Inaba and Ogawa factors were used to identify the pathogenic Vibrios. Serologic identification was

accomplished by a slide agglutination test as follows. Place a loopful of physiological salt solution and a loopful of properly diluted polyvalent cholera O group I antiserum on a clean slide. Emulsify the growth from a pure subculture on agar, in the salt solution to give a milky suspension. Transfer a loopful of the suspension to a drop of antiserum and mix. The appearance of macroscopic clumping within, 1 or 2 minutes constituted a positive reaction. The saline control showed no chumping. Both Vibrio comma and Vibrio El Tor show agglutination with O group I antiserum.

Enteropathogenic Escherichia coli After incubation for 18-24 hours, plating media were examined for colonies of Escherichia coli. Portions of 1-2 colonies that appear on the blood agar were tested directly in OB antisera. If strong positive slide agglutination tests were obtained by this procedure, it may be considered presumptive evidence that one of the particular Escherichia coli serotypes was present in the specimen. The examination performed the colonies from blood agar rather than colonies from MacConkey agar plates, because it has been shown that the presence of bile salts may cause confusing agglutination reactions (2). A generous portions of the growth from each of the agar slant cultures was emulsified in about 0.5 ml of 0.9% sodium chloride solution to make a very dense suspension. Droplets of heavy suspensions were tested for agglutination on slides with droplets of Escherichia coli O and OB antisera, first as living suspensions and

again after being heated. Agglutination of a living antigen in an OB antiserum and lack of a reaction in the corresponding O antiserum are indicative of the presence of B antigen in the strain. If such a reaction occurs in one of the OB antisera, the suspension was heated at 100°C for 1 hour cooled, and retested in the indicated OB and O antisera. If the culture belongs the O antigen group, the heated antigen may be expected to react in both OB and O antisera. The antisera were prepared against the following serotypes of Escherichia coli.

O 25 : B 19	O 119 : B 14
O 25 : B 23	O 124 : B 17
O 26 : B 6	O 125 : B 15
O 55 : B 5	O 126 : B 16
O 86 : B 7	O 127 : B 8
O 111 : B 4	O 128 : B 12
O 112 : B 11	

After preliminary serological tests were completed, the biochemical reactions of the cultures were determined to make certain that they were Escherichia coli.

Proteus and other Enterobacteriaceae were identified by means of urease test and other characteristic biochemical tests.

II. Antimicrobial sensitivity test of Enteropathogens

The minimal inhibitory concentration (MIC) for each

organism isolated from topic I was determined by the plate dilution technique (2). The technique has been done by adding various concentrations of the commercially acquired antimicrobial drugs to melted sensitivity test medium. After its solidification, add one drop of a 5 hours trypticase soy broth culture by means of a modified Steers inocula-replicating apparatus (38). After 24 hours of incubation at 37°C the plate were observed for growth. The minimal concentration of antimicrobial completely inhibiting growth was considered to be the MIC.

Procedure

Medium Use sensitivity test medium dispense medium in 20 ml amounts in 150 x 25 mm screw-cap tubes.

Plates Use 100 mm in diameter glass plates.

Preparation of Antibiotic and Sulfonamide solutions

The drugs used were

Ampicillin (D - (-1) Alpha-aminobenzyl penicillin as the sodium salt), Bristol Laboratories, Syracuse, N.Y., U.S.A.

Chloramphenicol, Carlo Erba, Milano, Italy.

Kanamycin (as kanamycin sulfate solution), Bristol Laboratories, Syracuse, N.Y., U.S.A.

Oxytetracycline (as oxytetracycine hydrochloride), Borneo Laboratories, Bangkok, Thailand.

Nalidixic acid (1-ethyl-7-methyl-1, 8-naphthyridin-4-one-3-carboxylic acid), kindly supplied by Acdhon Drug, Co., Ltd. Bangkok, Thailand.

Co-trimoxazole (Septrin), kindly supplied by Burroughs Wellcome Co. London, England.

Neomycin (as Neomycin sulfate) Upjohn, kindly supplied by Acdhon Drug, Co., Ltd., Bangkok, Thailand.

Colimycin, Atlantic Laboratories, Division of Atlantic Trading Co., Ltd.

Furazolidone (Furatin, Furoxone), China Chemical Eaton, U.S.A. kindly supplied by Acdhon Drug, Co., Ltd. Bangkok, Thailand.

Erythromycin (Ilosone), Eli Lilly, Sydney, Australia.

Prepare solution of antimicrobial drugs to meet the required concentrations:

Solution A	4,000 mcg/ml
Solution B	1,000 mcg/ml
Solution C	40 mcg/ml

Agar plate method Melt agar pour tubes, cool to 45°C to 50°C and hold in above rang in a water bath (Temperature above 50°C may deteriorate some antibiotics). Add antibiotic solution to melted agar in tubes as the following concentrations:

Final concentrations	Dilution Volumes
200 mcg/ml of medium	(19.0 ml of medium + 1.0 ml of Solution A)
100 mcg/ml of medium	(19.5 ml of medium + 0.5 ml of Solution A)
50 mcg/ml of medium	(19.75 ml of medium + 0.25 ml of Solution A)
25 mcg/ml of medium	(18.0 ml of medium + 2.0 ml of Solution B)
12.5 mcg/ml of medium	(19.0 ml of medium + 1.0 ml of Solution B)
6.25 mcg/ml of medium	(19.5 ml of medium + 0.5 ml of Solution B)
3.12 mcg/ml of medium	(18.44 ml of medium + 1.56 ml of Solution C)
1.56 mcg/ml of medium	(19.22 ml of medium + 0.78 ml of Solution C)
0.78 mcg/ml of medium	(19.61 ml of medium + 0.39 ml of Solution C)
0.39 mcg/ml of medium	(19.80 ml of medium + 0.20 ml of Solution C)

Mix by inversion two or three times. Pour plates allow plates to solidify and dry before inoculation. A control plate containing no antibiotic was included in each series.

Preparation of inoculum Inoculate the organism to be tested into Triple Sugar Iron agar, incubate 37°C for 18 to 24 hours, and transfer to a 5.0 ml tube of Trypticase Soy broth. Incubate for 5 hours at 37°C. Dilute the 5 hours broth culture in Tryticase Soy broth to contain 10^5 to 10^6 organisms per milliliter. Spot inoculation of the plates was made by using the Steers inocular-replicator. Incubate plates for 18 to 24 hours at 37°C aerobically.

Reading of plates The end point (MIC) is the lowest concentration of antibiotic that completely inhibited growth. Antimicrobial sensitivity was defined as a MIC of 12.5 mcg/ml or less (23).

All experiments were performed duplicately. The results obtained from the average readings. Some doubtful results, the experiments were done repeatedly to obtain the best readings.

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media used in this experiment were dehydrated media of Difco Laboratories Incorporated, Detroit, Michigan U.S.A.