PAKISTAN-SEATO CHOLERA RESEARCH LABORATORY

DACCA-5, EAST PAKISTAN

ANNUAL PROGRESS REPORT

October 1963 - October 1964

PAKISTAN-SEATO CHOLERA RESEARCH LABORATORY

TECHNICAL COMMITTEE MEETING

23 - 25 November 1964

MEMBERS:

U.S.A. Representative :	Dr. Colin M. MacLeod Deputy Director Office of Science & Technology Executive office of the President
PAKISTAN Representative:	Dr. A.K.M. Abdul Wahed Ex-Director of Health Services Govt. of East Pakistan
U.K. Representative :	Dr. Robert Cruickshank Professor of Bacteriology, University of Edinburgh

PANEL OF EXPERT CONSULTANTS:

Dr. Theodore E. Woodward
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OBSERVERS:

Dr. Clifford A. Pease, Jr.
Chairman
NIH Cholera Advisory Committee

Dr. Alexander Langmuir
Chief of Epidemiology Section
Communicable Disease Center

Dr. James L. Goddard
Chief, Communicable Disease Center
U.S. Public Health Service

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CRL Publications

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CRL Publications

1. Greenough, W. B., III, Gordon, R. S., Jr., Rosenberg, I. S., Davies, B. E., & Benenson, A. S. Tetracycline in the Treatment of Cholera (1964) Lancet 1, 355.

2. Gordon, R. S., Jr., Ahmed, J., Akbar, R., Jamiul Alam, A. K. M., Ali, M. M., Barui, R. K., Greenough, W. B., III, Islam, M. R., Islam, M. A., Khan, A. Q., Lindenbaum, J., Rahman, A. S. M. M., Zoha, M. S. Treatment of Cholera in 1964 (1964) East Pakistan Medical Journal 8, 10.

3. Greenough, W. B., III, Cholera: Method of Pakistan-SEATO Cholera Research Laboratory. Current Therapy. (in press).

4. Benenson, A. S., Islam, M. R., Greenough, W. B., III. Rapid Identification of Vibrio Cholera by Darkfield Microscopy. Bulletin of the World Health Organization. (in press).

5. Monsur, K. A., Rizvi, S., Huq, M. I. and Benenson, A. S. Effect of Mukerjee's Group IV Phage on El Tor Vibrios. Bulletin of the World Health Organization. (in press).

6. Pakistan-SEATO Cholera Research Laboratory. Cholera in East Pakistan Families, 1962-63. Bulletin of the World Health Organization. (in press).

7. McIntyre, R., Feeley, J. C., Greenough, W. B., III, Benenson, A. S., Hasan, S. I., Saad, A. Diarrhea Caused by Non-cholera Vibrios. Am. J. Trop. Med. & Hyg. (in press).

8. Rizvi, S., Huq, M. I., Benenson, A. S. Isolation of Hemagglutinative Non El Tor Vibrios. Journal of Bacteriology (submitted for publication).

DIRECTOR'S REPORT

The past year has witnessed the maturation of the CRL into a functioning research laboratory; our level of effort is now limited by our financing and our physical facilities.

Research studies during the past year are best divided into five general functional areas, each of which involves the collaboration and participation of two or more working sections: (1) studies on the patient and the disease; (2) the pathogenesis of cholera; (3) studies of the vibrio; (4) community studies; and (5) last, but far from least, the vaccine study.

(1) The cholera patient and the disease:

The CRL ward facility has provided the opportunity to evaluate therapeutic regimens adapted to the epidemic situation. These procedures are intended for application in endemic areas where qualified physicians and equipped laboratories are few and far between. Our methods received a critical test during communal disturbances in January 1964, when cholera appeared in epidemic fashion among restricted population groups. The ability of our staff to successfully handle a large number of patients encouraged us to approach the Director of Health Services of the Government of East Pakistan with the suggestion that the CRL be designated as the official cholera treatment center for the Dacca area. This was desirable because of the paucity of cholera patients for study between outbreaks of disease. Our proposal was accepted and is the present practice. To move cholera suspect patients to our facility, the CRL maintains an ambulance at the Sir Salimullah Medical College Hospital (formerly called Mitford Hospital), where persons are accustomed to seek treatment. The efficacy of simplified therapeutic regimens is under continual test in the small field hospital in the Matlab vaccine trial area where a Pakistani physician/nurse team operate independently with no laboratory equipment.

The opportunity to observe all suspect cholera patients on admission has emphasised the importance of a group of cases, called for want of a better name, "non-vibrio cholera"; no vibrios are isolated from these cases or no evidence of vibrio infection can be detected, yet the clinical findings on admission are indistinguishable from those seen in the patients with typical cholera infection. It is possible that this dehydrating syndrome, if untreated, can have as high a mortality rate as typical cholera; the syndrome and its etiology require better definition.

Electrocardiographic and circulatory studies suggest interesting possibilities in the pulmonary area for further exploration. With our present therapeutic regimen, we no longer see the pulmonary edema traditionally associated with cholera; extension of studies in this area is projected for early 1965 when a team from the Columbia University College of Physicians and Surgeons will undertake studies on the pulmonary circulation of the cholera patient.

In diagnosis of V. cholerae infection, the use of the darkfield technique has proven a means of almost instantaneous, specific diagnosis on the majority of cholera patients.

Studies of intestinal absorption function in diarrheal states have shown that infection with V. cholerae and with other organisms results in transient, marked

interference with absorption of xylose, vitamin B_{12}, and folic acid. This finding, which suggests that the absorption defect in cholera is much broader than that for sodium, opens new lines for investigation.

(2) The Pathogenesis of Cholera

Studies of possible predisposing factors (potassium depletion, folic acid and B_{12} deficiency, etc.) yielded negative results.

Studies of the host-parasite relationship, designed to elucidate how the cholera vibrio exerts its adverse effect on man, are presented under the pathogenesis of cholera. On a working trip here from the National Institutes of Health, Dr. Leonard showed that cholera stool filtrates may contain a heat-stable factor which has the same effect on the frog heart as digitalis compounds, a phenomenon which is associated with cell wall permeability. Studies carried out by Dr. Craig, who is here on a special NIH Fellowship, have demonstrated the presence of a heat-labile factor in cholera stools which produces capillary permeability and induration twenty-four hours after injection into the skin of a guinea pig or rabbit. This factor can be produced *in vitro* under properly defined conditions. Of great importance is the observation that this toxic effect can be obliterated by the serum of convalescent cholera patients, but not by the serum of rabbits injected with live cholera vibrios or of humans inoculated with commercial vaccine, despite the presence in these sera of high agglutinating antibodies derived from these procedures.

The relation of agglutinating antibodies to the protection of man or animals is still unclear. A micro technique has been developed which gives agglutinating antibody levels indistinguishable from those with standard techniques. This new technique will be of great value in community studies.

Observations on monkeys continue to provide clues for the study of cholera in human beings. Of great importance are two new findings: one, that infection with *V. cholerae* may persist within the upper gut despite consistently negative rectal swabs; and the other, that infection of liver and bile occurs after simple oral challenge. This does not appear to be a consequence of retrograde infection, but probably depends on portal vein bacteremia since infection occurs even after the bile duct has been ligated. No techniques have yet been developed for producing predictable disease in the monkey.

Physiological studies of the upper intestinal tract in human infection with *V. cholerae* have begun. Early findings suggest increased secretory activity of the duodenum, upper jejunum and/or pancreas. The persistent duodenal infection has been confirmed in one human case.

(3) Vibrio Studies

The emphasis in the Bacteriology Laboratories has been primarily directed toward the support of field and clinical studies diagnostically. Towards this end it has been important to critically review the ability of our techniques to detect the presence of vibrios, particularly in the carrier, and to determine any deleterious effect of delay in transit. This has resulted in the isolation of non motile vibrios; a stable non motile variant would provide a valuable tool for serological studies. Considerable emphasis has been placed on methods of recognizing the El Tor type of vibrio since this has been isolated in the periphery of our working area but not within it. Typical El Tor strains have been isolated in the Chittagong district in 1963; in September 1964, classical El Tor vibrios were received from the

Rangpur area in the north west corner of the province. Using chicken cell hemagglutinations for detection has brought to light the presence of cholera vibrios which hemagglutinate chicken cells but behave as classical organisms in all other respects.

Bacteriophage Studies have been conducted largely from the point of view of their value in providing markers for epidemiological purposes. These studies have disclosed that Group IV insusceptibility, the most generally accepted criterion of the "El Tor Vibrio", is a matter of quantitation. Studies have been carried out bearing on the possibility that a prophage may be the diarrhea provoking factor; no promising leads have emerged.

Anti-biograms of locally isolated organisms have been performed to provide guidance to the clinicians in selecting therapeutic agents. They confirm the choice of the tetracyclines for the therapy of the cholera, and suggest that chloromycetin may also prove to be of value.

(4) Community Studies

The aim of community studies is to describe the setting in which cholera occurs. For the past year, Dr. and Mrs. Glasse have studied a rural population providing information on cultural organization and attitudes of people towards disease. Their first hand observations of village life are expected to give insight into the facts involved in the fecal-oral transmission of disease.

To study population and environmental factors involved in the spread of cholera, and to estimate disability associated with infection in a defined population group, families of hospitalized cholera patients have been observed.

A community which had a sharp outbreak of cholera in January, 1964, was selected for a longterm study of cholera edemicity. This provides an opportunity to observe the dynamics of other infections, as well as to search for carriers or other location of cholera vibrios in the interepidemic period.

Studies of ruminants as possible reservoirs of cholera vibrios have been completed with the finding that many ruminants have agglutinins against cholera vibrios but these can be completely absorbed by noncholera vibrios. The rumen frequently contains noncholera vibrios.

Attempts to correlate water with infection in this endemic setting continue to give essentially negative results. Interest continues in the possible role of food, particularly methods of rice cooking which may allow water containing V. cholerae to achieve an infective dose.

(5) Vaccine Study

The vaccine study being carried out in a group of 23 villages in Matlab has involved all sections of the laboratory. Significant protection has been demonstrated during the first 6 months following injection of a commercial vaccine of high potency. The vaccine study is currently being extended in 35 additional villages using three preparations: the same cholera vaccine as was used last year, a purified Ogawa preparation, and an alum-precipitated tetanus toxoid as control. The availability of purified vaccine is an excellent example of international

cooperation. The original material, called a practical antigen, was prepared by Dr. Y. Watanabe at the Kitasato Institute, Tokyo. At our request, this material was flown to Galveston, Texas where Dr. W. F. Verwey extracted a purified fraction which produces a single line on agar diffusion, but retains very high mouse protective potency and relatively low reactivity. It was then taken to West Point, Pennsylvania, where it was lyophilized by Merke, Sharp and Dome. The vaccine was put into the field, using the same general methods as last year, after preliminary testing on laboratory personnel among whom no significant systemic reactions occurred. The same protocol used in the previous trial is being extended. Last year the acceptance rate was 51.7%. However, the response to the present campaign has exceeded 80%, an interesting change in attitude of the population towards our study group.

* * *

The program of work is the reflection of the personnel available to the laboratory. In the past year there have been many changes in staff. In the Clinical Research Section, Dr. Robert S. Gordon, who had been here since October, 1961, functioned as Scientific Director and, for a while, as Acting Director, departed 10 April 1964. This section also lost the services of Dr. Jalaluddin Ahmed on 25 July, 1964. Dr. Shamsuz Zoha, who was one of the first Pakistani physicians deputed by the Government, returned to Government Service in August, 1964, but has been replaced by Dr. Md. Shamsul Islam. The section has also gained the services of Dr. Ally Begum and Dr. Zabedul Huque.

In the Epidemiology Section, Dr. Robert Oseasohn has taken over from Dr. Joe L. Stockard, who departed 24 October, 1963. Dr. A. Q. Khan, who had been with us since August, 1962, left on 24 July, 1964 to become the Director of the Central Malarial Institute. Dr. Md. Fahimuddin, former Director of Public Health, East Pakistan, continues to serve as consultant and active participant in epidemiology projects; data processing has been efficiently performed by Mrs. Johanghir and Messrs. Chowdhury and Hussain.

In the Bacteriology Section, Mr. J. Brennan left on 2 March, 1964 to return to the University of Edinburgh. Dr. R. Akbar, who had previously been in the Clinical Research Section, has been spending the bulk of her time in bacteriology. Dr. John P. Craig, Assistant Professor of Microbiology, Downstate Medical Center, State University of New York, Brooklyn, New York, joined on 9 January, 1964 as a Special Fellow (NIH) and has been working in both the epidemiological and microbiological aspects of cholera.

Mr. Robert Freise joined as Executive Officer on 29 January, 1964.

A notable advance during the year has been the despatch of the first CRL personnel for foreign training. Mr. Imdadul Huq, Assistant Bacteriologist, is currently studying for a Diploma in Bacteriology at the London School of Tropical Medicine and Hygiene; Mr. Sayed Ahmed Khan is a graduate student in Sociology at Western Reserve University. Mr. Syed Iqbalul Hasan is working towards a graduate degree at Jefferson Medical College, Philadelphia. Since August, Mr. M. R. Bashir has been at NIH for on-the-job training, furthering his capabilities in research administration.

SUMMARY OF WARD ACTIVITIES FROM 1 OCTOBER, 1963 to 10 SEPTEMBER, 1964

by
Miss D. Torrance

The activities of the CRL ward have increased notably during the past year. Total admissions this year were double those of the previous year:

	Cholera		Non-cholera	
	Admissions	Deaths	Admissions	Deaths
Children 10 years and under	124	0	107	4
Males over 10 years	83	0	295	2
Females over 10 years	81	1	165	2
TOTAL	288	1	567	8

TOTAL admissions: 1963-64 - 865
1962-63 - 418

In addition to cholera patients, many others with diarrhea not associated with V. cholerae were treated and studied. Of interest among these admissions was the increased number of infants and children with malnutrition, hypovitaminosis and kwashiorkor-like states. Two cases of Kala-azar were documented. The majority of patients with these conditions responded satisfactorily to good nursing care and appropriate medication and dietetic regimes.

Patients with cholera were admitted every month, with the exception of August. The peak months were December, January, April and May.

An epidemic of cholera in January, 1964, put the hospital facilities to full test; 175 patients were admitted in 14 days. All hospital space was utilized; extra beds were placed in other laboratory departments. Three tents were erected to house convalescent patients. A full account of the January epidemic has been recorded separately.

In March, on recommendation of the Director, the Director of Health Services of the Government of East Pakistan officially designated CRL as the cholera treatment center for the Dacca area. Since then, patients thought to have cholera have been admitted to Mitford Hospital only on an emergency basis. The resulting increase in the number of patients and clinical activities at CRL has made it necessary to utilize all available space. Part of the hospital corridor has now been screened off and is used as an examination room for follow-up cases and for employees' medical examinations. A new building has been constructed as a garage which can be converted into two wards for male and female patients.

Medical and Nursing Staff:

Doctors	- 5
Hospital Supervisor	- 1
Clinical Research Nurses	- 2
Senior Staff Nurse and	- 1
Male Aid Nurse (for follow-up)	- 1
Sr. Staff Nurse for medical records and coding	- 1
Sr. Staff Nurse (absorption studies)	- 1

Ward duties:

Sr. Staff Nurses	- 3
Aid Nurses	- 5
Male Staff Nurses	- 2
Male Aid Nurses	- 1

Pharmacy:

Male Nurse	- 1

X-ray & ECG:

Technician	- 1

In addition to all charting, the senior staff nurses on ward duty were responsible for the measurement and recording of intake and output of urine and stool. Meticulous care and a high degree of accuracy were responsible for success in treating patients and conducting research.

Studies:

The double-blind tetracycline study was conducted from November to March and included 45 cases.

The "non-vibrio diarrhea" study began in March 1964, when many patients with watery diarrhea were admitted in a state of severe dehydration, with elevated plasma protein, but were found negative for V. cholerae. These cases closely resembled the disease caused by V. cholerae and provided a unique study opportunity. Thirty-six cases were investigated between March and mid-June.

Intestinal absorption of xylose, folic acid, and vitamin B_{12} was studied. Patients included both those with cholera and with diarrhea attributed to other causes. They tolerated these tests well and were cooperative. One clinical research nurse, assisted by one senior staff nurse, was responsible for performing these tests and ensuring that all samples were collected.

In July, electrocardiographic studies on patients at Mitford Hospital and Dacca Medical College Hospital were started by Dr. K. M. Ally, CRL Physician, assisted by a male staff nurse. They visited the hospitals daily to draw venous blood and to take ECG's on cases selected for the study. Fifty-six patients were observed.

In August, a similar study was started at CRL to obtain further data on factors possibly responsible for the ECG pattern of patients in vascular collapse associated with acute diarrhea.

New Procedures Used During the Year:

The method of intra-peritoneal infusions was used on six children during the January epidemic.

Jejunal intubation by Miller-Abbott tube with suction was performed on patients positive for V. cholerae who were purging severly. The main purpose of this procedure was to see whether suction of duodenal and pancreatic juices diminished the stool volume, and to analyse the composition of these juices.

Jejunal biopsies with the Crosby capsule were done on a number of cases to study the absorbing surface of the mucosa of the jejunum in cholera and other gastroenterological diseases. Patients accepted and tolerated this procedure well; no complications such as haemorrhage or perforation occurred. The Crosby capsule was also used for aspiration of the duodenal juices for culture and biochemical analysis in convalescent cholera patients.

Peritoneal dialysis was done on one patient with renal shutdown and haemolysis probably caused by Primaquin(R). The patient was transferred from another hospital. Fifty-four liters of fluid were exchanged. All fluids were prepared in the CRL pharmacy, and the exchange performed by the CRL nursing staff.

X-Ray Department:

Because of the large load of patients, the work of the X-ray department has increased considerably. From 1 October, 1963 to 30 September, 1964, the following roentgen examinations were made:

Small bowel series	83
Oesophagus	7
Cholecystograms	3
Total opaque media	93
Chest and others	907
Total examinations	1,000

Pharmacy:

During the year, approximately 4,000 liters of intravenous solution for fluid replacement were made in the pharmacy. Approximately 977 liters of solution were used in the January epidemic. On only one occasion when 20 patients were admitted at once, all requiring rehydration, were a few liters of Steriflex(R) isotonic saline used.

The experience of treating a large number of patients during the epidemic, when much time was list in making up a variety of solutions, led to the decision to use a single replacement solution for all uncomplicated cases of cholera or acute diarrhea requiring rehydration. This was called "Cholera Replacement Solution" (CRS) and was made as follows: Potassium chloride - 0.75 gm., Sodium chloride - 5.0 gm., Dextrose - 10.0 g. The ingredients were weighed and stored in clean bottles, when a solution

was required, these salts were dissolved in 1 liter distilled water, one bottle containing 50 mEq sodium lactate was added and mixed well.

In April, CRS was discontinued and replaced by another solution of 6 grams sodium chloride and 4 grams sodium bicarbonate in 1 liter of distilled water. Green coconut water was used as a source of potassium if the patient could tolerate fluids by mouth.

The procedure for filling bottles and making solutions is as follows: One-liter bottles with screw caps are washed in hot water, rinsed twice in distilled water, hung to drain, and then sent to the Bacteriology Department for drying in the oven (170° C dry heat). Bottles are then returned to the pharmacy; when cool, they are filled by direct distillation of water from the Manisty still. Six grams sodium chloride is weighed and added to each bottle of fluid, which is sent to be autoclaved. After autoclaving, caps are screwed on tightly, sealed, labelled and stored. Four grams sodium bicarbonate is weighed out and stored in small clean bottles labelled 6/4. When a bottle of replacement fluid is required, the contents of one of these small bottles is added to the bottle of autoclaved fluid containing 6 gms sodium chloride, making a solution of 6/4. The label from the small bottle is then attached to the liter bottle; date and time of mixing are written on the bottle with a black marking pencil, and the fluid is ready for infusion. This solution is now in use for initial hydration and maintenance of uncomplicated cases.

Recently, Darrow's solution in disposable packs has been used as a replacement fluid for cases low in potassium. The solution contains 4 gms sodium chloride, 2.67 gms potassium chloride, with 53.3 ml sodium lactate in 1 liter of water for injection.

Use of a single intravenous solution for fluid replacement has simplified the work of the pharmacy and of the nurses in charting the intravenous intake. The male nurse in charge of the pharmacy is maintaining the high degree of efficiency.

Cholera patients studied after hospital discharge are given the following tests: rectal swab culture; stool for ova and parasites; urinalysis; body weight; barium meal; and in selected cases, intestinal absorption studies and jejunal biopsies. To date, no cholera patient has been found to have a positive culture after discharge.

Since October 1, 1963, follow-up study has been initiated on 261 cases. Study has been completed on only 190 of 261 patients; others have either refused to return or could not be traced.

One senior staff nurse assisted by a male aid nurse is responsible for the follow-up cases. She accompanies the patients to their homes on discharge, to ensure that she knows where to locate them. Much time is spent in persuading some of these patients to return, as they feel well and are often needed elsewhere. The housewife cannot leave her family,

and the male patient is reluctant to return to the hospital for economic reasons - loss of pay or even job.

Since November, 1963, a small but competent hospital unit has been working at Matlab to support the vaccine trials. From December, 1963 to 25 August, 1964, 602 patients were treated at Matlab:

	Cholera		Non-cholera	
	Admissions	Deaths	Admissions	Deaths
Children 10 years and under	72	1	110	4
Males over 10 years	25	1	182	0
Females over 10 years	35	1	179	2
	132	3	471	6

Approximately 1628 liters of intravenous fluid were used at Matlab, with an average of 4.8 liters per case. Tetracycline was used in all cases.

During the year, only three patients had to be transferred to Dacca from Matlab for more extensive care and investigation. The first, sent in December, was a postcholera patient with acidosis, retention of urine, and heart failure. The second, sent in January, was a young male with a postcholera acidosis. The third, sent in September, was an old man with amoebic dysentery and liver abscess.

Nurses and doctors from the CRL ward have rotated in doing short spells of duty at Matlab, which has proved a most valuable training ground for the staff.

Cholera Outbreak at the Laxmi Narayan Cotton Mill January 1964.

By
Dr. W.B. Greenough III

On Friday, 24 January, 1964, CRL was notified by officials of the GOEP Health Directorate of an outbreak of cholera in a refugee camp at the Laxmi Narayan Cotton Mill. On that evening, the spot was visited by Drs. Gordon and Craig. They found seven to eight patients being attended by a volunteer doctor from the Adamjee Jute Mill. Mr. Alam, the GOEP official in charge of relief work, met the CRL workers there, and arrangements were made for a supply of drugs and intravenous fluids. Dr. Gordon summarized the CRL's therapeutic methods for the Adamjee Mill doctor.

Dr. Greenough went down on 25 January in the morning to survey the situation and Drs. Gordon and Benenson went down in the afternoon. At about 10 A.M. there were about twenty active cholera cases lying in a very small crowded room and in various hallways and porches off this area. It was very congested between patients and almost impossible to move among them to give any sort of treatment. The intravenous solutions which were provided by the Government consisted of 5% dextrose and saline. Intravenous tubing was brought from CRL. The next two hours were spent with the Adamjee Jute Mill doctors giving infusions to patients who appeared to be in the worst circumstances. It was remarkable that many patients who had been treated by dextrose and saline and were well hydrated seemed to be lying in a semi-comatose state despite being out of shock. The situation was extremely chaotic with new patients coming in constantly and old patients and their relatives continually dragging the doctor to their particular family member who might or might not be in a precarious condition. There was no place to give i.v. therapy adequately and a great deal of time was spent finding places to hang up bottles, trying to move people to allow i.v. infusion to be put in place, and re-starting infusions that had infiltrated, etc. Dr. Greenough left at approximately 2 P.M. to return and report to CRL.

That afternoon Drs. Benenson and Gordon found the situation more chaotic with many new admissions and even less space and less ability to render any form of treatment. It was apparent to us at that point that unless further supplies could be obtained and space organized rather little could be accomplished in rendering aid to the cholera patients in this camp.

The following morning Dr. Lindenbaum and one nurse and Dr. Greenough went back to the mill and organized the patients into a larger area where there were places to hang infusion bottles from and arrange patients with space to move between them. This being accomplished, we divided the patient population into those who had apparently good circulation with full radial pulses and good facial appearance and transferred them to another building where they could be observed at longer intervals. The remaining cases who were in circulatory collapse and actively purging were kept in the larger room and spread out in a more orderly fashion. This being done it was possible to set up a large number of infusions and keep them running and keep some track of the number of bottles and state of hydration of each of the more seriously ill cases. During a seven hour period after this separation had been accomplished only three cases had to be brought back from the observation area for emergency treatment with infusions. Antibiotic was administered to all cases both old and freshly admitted in both areas. Due to shortage of time and personnel no records were kept. During a 9 to 10 hour period of treatment of approximately 70 to 100 patients there were two deaths, both in small children. When it was necessary for us to return to our CRL Ward all patients had good circulation and antibiotic had been administered and instructions given for further administration to all cases. The general facilities of this "hospital" and the

infusion treatment therapy we gave were most primitive. Patients all lay on low board benches or on the floor purging into various containers or on to the floor. There were few alcohol sponges, and i.v. tubing and needless had to be re-used from one patient to the next. The mechanism for suspending bottles was not entirely satisfactory and several bottles fell, one causing minor injury to a patient. Lighting was very poor making venepuncture quite difficult. The solutions used were far from ideal in that they were mainly isotonic saline without lactate, bicarbonate or potassium. There was no source of clean drinking water available to either patients or doctors. Administration and organization had to be done by the medical team as there was no local authority in a position to deal with the situation. The experience gained in this situation may be summarized as follows :

1) It is apparent that with less than ideal kinds of i.v. solution, no ability to measure intake or output, no record system, no medical instruments or laboratory, no beds, poor lighting etc., that a large number of active, severely ill cholera cases could be kept alive by a very limited number of medically skilled people.

2) The greatest problem was that of organization and quick evaluation of the status of cases, i.e. "triage". The division of patients into those for observation and those for active therapy removed at least 50% of the cases from the very congested area in which we were initially trying to give therapy, and allowed us to focus on those who were more in need of medical attention.

3) Since many of the patients were small babies and children it was found that scalp vein needles were almost an essential for therapy in this group. It was also found that the jugular venous route seemed to be the only route in many patients where a vein could be found and a venepuncture performed in profoundly collapsed small children. This route was used extensively in both children and adults with great success on these days. The femoral route was found to be somewhat impractical under these circumstances.

4) Division of responsibilities as outlined in the protocol which is appended to this was found to be a great help in keeping infusions running and saving the time necessary for re-starting plugged infusions.

Finally a protocol is appended to this description which is a summary of the ideas for treatment of such a situation in the future and the equipment that would be desirable for accomplishing this.

Protocol for Treating Epidemic Cholera under Field Conditions

EQUIPMENT:

1) A large enough space to organize patients into rows with room for additional admissions to be added as necessary and with room for doctors and assistants to move between patients as necessary to work. Also a separate area for observing patients not receiving intravenous fluids.
2) A rope or wire above the cots from which to hang intravenous solutions.
3) Cholera cots of the simplest type on which to place patients for the collection of urine and stool.
4) Buckets of at least 7 liter capacity calibrated to measure stool and urine volume.
5) Intravenous materials:
   a) Cholera replacement solution of a composition approximating lactate or bicarbonate 50 mEq/l, sodium chloride 5 gms or 85 mEq/l, potassium chloride 10 mEq/l. This should be contained in a bottle or plastic bag with a matching tubing for administration.
   b) Needles: Needles of the scalp vein type would be entirely satisfactory for all purposes; otherwise another kind of matching needle no smaller than 20 gauge that will fit the tubing should be available.
   c) Several syringes for clearing plugged intravenous infusions and starting new infusions.
   d) Adhesive tape, scissors, sponges.
6) A vibriocidal antibiotic (tetracycline is of known potency).
7) There should be a disinfectant and a disposal area for the stools.
8) There should be a clean drinking water source for both patients and doctors.
9) Record forms and clip boards, or other record holders, and pens or pencils to write with. There also should be a marking pen which could write on bottles and patients themselves.
10) One or two firm pillows would be useful in extending the neck of patients for external jugular venepuncture.
11) A flashlight and mantle lantern would be useful if lighting conditions are not good.

PERSONNEL:

With division in responsibility in the following fashion, three skilled workers could handle about 50 active cases. Of course a relief team would have to be available to permit rest for the initial team.

1) An individual working to start intravenous infusions on cases.
2) A person to change bottles and keep intravenouses open and running. This person would also be responsible for marking bottles and keeping a record of what has been given so that the amount of treatment on any single patient would be immediately seen.
3) One person to give antibiotics, observe the condition of patients and point out and give priority to the more severe cases for infusions.

APPROACH TO THE SITUATION:

All cases, old and recent, who are in the area of the epidemic under treatment shall be admitted to the space selected for rendering treatment. This space will be divided into treatment and observation areas. All patients will be marked with a number on a clearly visible place on admission to the areas. Any case

requiring i.v. infusion as judged by general appearance and pulse quality will be placed on the cholera cots in the treatment area. All other cases will be admitted to the observation area, and all cases immediately on admission will be given a priming dose of vibriocidal antibiotic. Infusions will be started according to a priority based on the severity of the patient's condition. Infusions will be maintained until the patient is judged to be in good condition and no longer actively purging. When patients are out of danger and able to be moved from the cholera cot they will then be admitted to the observation area for continuing antibiotic treatment and observation for another 24 hour period. Any new cases admitted to either area shall also be immediately numbered and given a priming dose of antibiotic. All cases in both areas shall be given an antibiotic on a set schedule four times during any 24 hour period. The division of labour between the personnel on the team treating the epidemic is as outlined under the previous section. The buckets under the cots need not be emptied on any set schedule; however, it is important to note the time and the volume whenever a bucket is emptied on the patient's record. It is also mandatory that when a patient is moved off a cholera cot his bucket be removed and emptied and a new one replaced for the next patient. It is important to note that the treatment team need not be made up of fully qualified doctors. Ideally a doctor should be present on each team; however, one doctor could manage several teams, being on call for consulting on particular difficult cases. What is necessary is that the team be made up of people skilled in the organization of handling cholera and skillful in giving i.v. fluid therapy.

MODIFICATIONS TO MEET WORST POSSIBLE FIELD CONDITIONS:

When the situation arises under which only a minimal amount of equipment can be available the following things may be omitted from the above protocol without great detriment to the patients:

1) The patients may be kept on the ground without cholera cots or buckets for collection of stool and urine.
2) Disinfectant or disposal means might not be possible under such circumstances.

3) Charts for individual patients will not be possible however the general organization, space requirement, and needs for i.v. infusions and personnel remain the same. Records of the number of bottles given each patient must be kept by marking directly on the patient himself with a marking pencil in an obvious place.

SUMMARY:

The most important elements of this protocol are as follows:
1) Organization of space, personnel and equipment into a pattern for rendering most efficient treatment.
2) Ability rapidly to evaluate needs for rehydration and to administer required i.v. fluids based on the appearance of a patient and his pulse quality.
3) The prompt administration of antibiotic to all new cases or cases still mild in nature, as well as to the severely effected cases. By doing this it is likely that in an appreciable number of cases the disease may be aborted before needing large volumes of i.v. fluid therapy.

TREATMENT OF EPIDEMIC CHOLERA AT CRL, JANUARY 1964

by

Dr. R. S. Gordon

During the latter half of January, 1964, the Dacca-Narayanganj area experienced a sharp outbreak of cholera. This particularly affected refugees who were congregated under crowded and unsanitary conditions. Food and water had been limited for a few days and in many cases there was inadequate shelter from the cold night weather. Extensive inoculations against cholera and smallpox were undertaken in these refugee camps shortly after they were set up, but cholera developed nethertheless.

From 15 January, 1964 onward, several thousand people were congregated in an area surrounding the Rayer Bazar Pottery Center. Since the CRL had been receiving proven cholera cases from this community, it was no surprise to find the disease spreading in the camp, and to have cholera victims apply to the CRL for treatment. For seven days, this community provided the major part of the clinical load. Then, on 24 January, cholera broke out in a number of much larger refugee camps in the Narayanganj industrial area. CRL clinical personnel gave some emergency aid in this situation, and the ward accepted cases transferred from the area. The outbreak waned as rapidly as it had developed, however, and by the end of the month the number of true cholera cases appearing for treatment was down to a level that had been handled before. This report will give an account of the arrangements that were made to deal with the unusual number of cases, none of whom were turned away.

SPACE: As the CRL ward was planned for 20 beds, the number of admissions far exceeded its capacity. It was necessary to utilize corridors and the Epidemiology apartment to provide room for convalescent patients who were out of danger, but not yet ready for discharge. The GOP Health Services were asked to provide tents, but these were not actually in place and ready for use until 28 January, after the peak load had passed. Three tents, each accommodating up to 14 beds, were set up; they could have solved the space problem entirely if available earlier. The ward itself was reserved for active cases on cholera cots; forty or more of these could be fitted into the wards and the hospital corridor without causing unmanageable congestion.

BEDS: The acute need was for extra cholera cots and rubber drain sheets, of which only 16 were on hand when the emergency developed. Additional cots were made from folding camp cots, which could be procured within a few days, and these proved very satisfactory. About twenty old mattresses which had been replaced by newer ones but not thrown away yet, were got out and placed on the floor for convalescents. Linens, rubber sheets, and stool buckets could be procured locally quickly enough that no critical shortage of these items developed.

STAFF: Fortunately, the CRL had sufficient medical staff that there was no problem in this area. The normal ward staff of three American and five Pakistani physicians,

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one British and 19 Pakistani nurses, was supplemented by three physicians and four nurses loaned from Epidemiology. Two doctors and three nurses from the Peace Corps also gave as much time as they could.

PLAN OF TREATMENT: The objectives of treatment were twofold - to rehydrate the newly admitted patient and keep him in reasonable fluid balance, and to combat the infection with antibiotics. The evidence favoring the use of antibiotics in cholera is currently in press (Greenough, et al. 1964). Patients were allowed to eat as soon as they felt able to, and were discharged when their diarrhea had ceased and they had begun to regain strength. The record of success was excellent; there was no hospital deaths, and no known relapses after discharge. Follow-up is not yet complete, but it is felt that any relapse in Rayer Bazaar would have come to our attention immediately by seeking readmission. The details of the therapy will be taken up serially.

REHYDRATION AND FLUID THERAPY: When the physician had satisfied himself that he was dealing with a case of cholera, or other seriously dehydrating diarrhea, he began intravenous rehydration with normal saline. After the initial liter (or less in children), fluid therapy was restricted almost entirely to "5/4/1", a solution of 5 gm. $NaHCO_3$, 4 gm. NaCl, and 1 gm. KCl per litre. The amount of fluid required was judged on clinical grounds, by noting pulse and blood pressure, skin turgor, appearance of the face, etc. Rehydration was deemed to be adequate when the patient's face filled out, urine was passed, or both. Fluid balance was then maintained by observing the stool output, collected in calibrated buckets, and the patient's vital signs. In no case was a laboratory test utilized to determine the volume of fluid to be given. Rounds were made at least twice a day on active cases, and the senior staff physicians helped to rectify any errors in fluid volume that had been made. Overhydration was common, especially in small children, but appeared to do not serious harm. Pulmonary edema did not develop in any case.

With the exception of a few litres of"Steriflex"saline used when twenty cases were admitted within an hour on the evening of 26 January, all fluids used were prepared by the CRL Pharmacy. Bottles were washed and rinsed with pyrogen-free distilled water in the pharmacy, dried in the ovens in the Bacteriology sections, and filled from the large water still in the Water Laboratory. Water not to be used at once was autoclaved, but most was immediately returned to the ward for administration. Carefully weighed CP reagent salts were added, dissolved, and the fluid administered at once without further sterilization. Pyrogenic reactions did not occur; only one case in whom phlebitis developed around an ankle vein infusion had troublesome fever.

Almost all fluids were given by the classic intravenous route. However, five children received intraperitoneal fluid. This procedure will be the subject of a seperate report. Results were encouraging, and it deserves further study.

ANTIBIOTICS: Because the supply of intravenous tetracycline was quickly exhausted, oral antibiotic therapy was the rule. Adults received tetracycline capsules, and children pediatric suspensions of tetracycline or oxytetracycline, whichever was available. The standard doses are given on the order sheet, which

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is appended. In some cases, the doctor preferred to give a larger initial loading dose. Although the records are not sufficiently detailed to permit careful analysis, the impression was gained that omitting the parenteral antibiotic after the supply ran out did not decrease the effectiveness of treatment.

MEDICAL RECORDS: Our normal medical record procedures proved too cumbersome for use in this emergency; therefore a condensed form was drawn up and used. It permitted all records for one patient for one day to be on a single sheet. Nursing observations, like TPR and stool volume were recorded directly on this sheet, and not transcribed from books onto graphs. Medical notes and orders were put in at the bedside, and the most recent ones were always visible on top. A set of standard orders was drawn up, which could be put into effect on any case by merely writing "Standard orders." Each chart was kept on the bed of the patient to whom it applied. Patients' case numbers were written on their foreheads to avoid confusion. On discharge, each patient was fully identified with the form usually used at CRL. Copies of the condensed clinical record forms are appended to this report.

LABORATORY STUDIES: Except for the admission rectal swab for bacteriologic study, all routine laboratory work was suspended. Hematologic and chemistry laboratory staff could therefore give immediate attention to any urgently required test. This was of great importance when we received cases of cholera complicated by other diseases, or cases that had been under treatment elsewhere with saline alone and were severely acidotic. Likewise, ECG and X-ray examinations were performed only when specifically ordered by the physician. Mr. Sarkar, the nurse specializing in electrocardiography, was freed for ward duty, as Mr. Costa, X-ray technician, could handle both ECG and X-ray requirements.

RESULTS: The most important result of this episode is the demonstration that the CRL can, if necessary, adapt to epidemic conditions successfully and yet maintain its excellent record of minimal mortality. Table I shows the daily census, admissions, and discharges for the period. The Rayer Bazaar cases are tabulated separately, as their numbers represent an almost complete record of the outbreak in that one camp. The number of cases confirmed bacteriologically is also tabulated.

A second conclusion to be drawn from this experience is that well-trained personnel can handle routine acute cases of cholera without laboratory help. Only neglected, complicated, or previously mismanaged cases require individualized hematologic or biochemical study for optimal results. Whereas bacteriologic study is of importance for research and public health reporting, it too is superfluous in the clinical management of the case.

Finally, the epidemic enabled us to make certain observations that might have taken much longer to accomplish under ordinary circumstances. For some reason, an unusual proportion of pregnant females was encountered. Their course and the outcome of the pregnancy will be dealt with separately.

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In addition, the pressure of treating many cases at once, and the impossibility of finding veins in some babies, drove us to try the intraperitoneal route for fluid administration in a few cases. In the first such case, a totally collapsed four-month old infant in whom no veins could be located, the situation was desperate and the result of intraperitoneal fluid administration dramatic. This success has set in motion a study of the intraperitoneal route which may lead to useful practical results.

On the basis of this experience, the Clinical Research Section of the CRL has formulated a plan, which is appended, for the management of future epidemics in which it may be called upon to accept large numbers of patients.

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CRL CASE LOAD DURING EPIDEMIC OF JANUARY 1964. MORTALITY RATE 0%

Table I

Date	Census at 1201 A.M.	Number of Admissions	Number of Discharges	No. of Adm. from Rayer Bazaar	Adm. Bact. Confirmed
1/Jan/64	15	1	1	0	1
2/	15	6	1	0	4
3/	20	2	2	0	1
4/	20	2	4	1	1
5/	18	2	1	1	2
6/	19	2	3	0	1
7/	18	2	1	1	2
8/	19	1	8	1	1
9/	12	3	3	0	2
10/	12	3	1	0	1
11/	14	2	2	•	0
12/	14	1	0	0	0
13/	15	3	2	0	3
14/	16	4	3	3	4
15/	17	0	0	0	0
16/	17	0	3	0	0
17/	14	1	6	0	1
18/	9	4	2	0	1
19/	11	11	1	7	7
20/	21	10	5	10	9
21/	26	14	6	13	13
22/	34	18	5	14	17
23/	47	9	10	8	8
24/	46	13	12	11	9
25/	47	12	18	8	12
26/	41	32	19	7	30
27/	54	16	17	14	16
28/	53	9	26	2	7
29/	36	15	21	3	14
30/	30	6	7	2	4
31/	29	6	9	5	4
1/Feb/64	26	0	12	0	0
2/	14	1	1	0	0
3/	14	1	9	0	0
4/	6	0	1	0	0
5/	5	0	3	0	0

Plan for Epidemic Management at CRL.

Epidemic conditions shall be considered to prevail when the incidence of cholera in the vicinity of the CRL is such that admissions exceed six cases per day, and more may be anticipated. When the decision to initiate emergency procedures is made by the CRL Director or his designee the following steps will be initiated immediately.

1) Set up tents in the field adjacent to the ward, and place all regular beds and mattresses in these tents for accommodation of convalescents. Extra beds and mattresses may be required on loan from Central Medical Stores. Remove existing convalescent cases to the tents.

2) Set up cholera cots in the main wards and hospital corridor. Reserve the small isolation room, if possible, for special problems like deliveries, other contagious diseases, etc. Cots should not be set up on the admitting porch; this area will be needed for sorting new cases in order of severity, weighing etc.

3) Prepare condensed clinical record forms for immediate use. Under this record system, order books, TRP books, etc shall be discontinued, and all nursing and doctors' records made directly on each chart at the bedside. The chart must remain with the patient at all times, and each patient be marked with his identifying number. Stool and urine volumes are to be read from calibrations on the bucket or by a dipstick and recorded at the time the container is first taken out from under the bed. Routine procedures to be discontinued will be blood work, ECG's, X-rays, and daily weights.

4) Alert other sections that their help may be required if the load is large. This includes Epidemiology for extra personnel, Bacteriology for help with cleaning and drying intravenous bottles, and Water laboratory for making extra distilled water. Personnel of these laboratories should be available during off-duty hours on a standby basis. Administrative and General Services sections should be alerted to the possible need for extra drivers, darwans, etc., and extra supplies.

Treatment of Cholera at the Pakistan-SEATO Cholera Research Laboratory

by

W. B. Greenough, III, M.D.

In 1832 correspondents to the Lancet ascribed the collapsed state of the cholera patient to the loss of water, salt, and alkali from the blood into the stools (O'Shaugnessy 1832, Latta 1832). Latta noted that the signs and symptoms of the disease could be relieved by the intravenous administration of saline and sodium bicarbonate solutions. Precise balance studies with detailed analysis of blood and stool by Captain Robert A. Phillips and associates have confirmed these observations and for the first time provided the basic scientific data necessary to construct a fully rational and effective program of therapy. His group has demonstrated repeatedly that the mortality in cholera can be reduced to the vanishing point by the use of a program of fluid therapy based on observations of the blood electrolytes and degree of hemo-concentration as measured by the specific gravity of whole blood and plasma.

When CRL received its first cholera patients in December of 1962 the plan of intravenous fluid therapy was based on considerations outlined by Captain Phillip's group. Blood was taken for sodium, potassium, chloride, carbon dioxide, plasma protein, and hematocrit on admission, after hydration, and usually on the 4th and 7th days. Isotonic saline was infused rapidly until a full radial pulse had returned and the blood pressure was normal. Thereafter a solution approximating the composition of cholera stool excepting potassium was used for further replacement. Precise calculations of losses were not made, however, from the initial values; but initial values and serial values after rehydration were used to check the physician's judgment of the adequacy of treatment. Simple bedside observations, none of which along would suffice as a guide to the progress of therapy, taken together rarely lead to serious errors of either under or over-hydration. Some of these are obvious "foot of the bed" observations - the fullness of the face, restlessness and general appearance. The resilience of the skin, the depth and rate of respirations, the rate and fullness of the radial pulse, the blood pressure, and presence or absence of rales at the lung bases form the other data that may be rapidly collected at the bedside. These coupled with accurate observation of intake, output and, especially, body weight have formed the entire basis for regulation of fluid therapy at CRL. Laboratory measurements have been taken when any question of the adequacy of hydration has arisen and also as a routine. This report will describe the evolution of our approach to fluid therapy and its results.

Phase 1 - December 1962 through April 1963.

Early in this period, Drs. Gordon, Greenough and Rosenberg evaluated and treated all cholera patients. Later this responsibility was gradually shifted to Drs. Akbar, Ali, Islam and Zoha. Isotonic saline was given for initial rehydration and a solution containing 5 grams sodium chloride, 4 grams sodium bicarbonate and 1 gram potassium chloride was given to match stool losses for maintenance of hydration. The value of tetracycline as a supplement to fluid therapy was demonstrated. A total of 155 cholera patients were treated; 64 were admitted directly without prior therapy and 91 were taken in transfer after varying amounts of hypertonic saline and alkali. There were seven deaths during this period. Two small children

expired due to inadequate fluid replacement. There were four deaths attributed to severe acidosis in patients admitted late in the course of disease after 3 or 4 days of diarrhea (3 of whom were transferred from other hospitals). The striking feature common of all the late acidotic patients was the presence of pulmonary edema despite dehydration. Other complications were restricted to the group treated elsewhere before admission. There were several instances of oliguria of more than 3 days duration all with eventual recovery. One of these was treated with peritoneal dialysis. There was one death due to orally administered potassium chloride in a healthy convalescent cholera patient.

Total cholera admissions	-	155	-	7 deaths.
Direct admissions	-	64	-	3 "
Prior hospitalization elsewhere	-	91	-	4 "
CO_2 < 10 mEq/L	-	14	-	3 "

Phase 2 - May 1963 through December 1963.

From the start of this phase onward our trained Pakistani physicians took over full responsibility for the evaluation and treatment of all patients. We saw the patients with them but wrote only orders for particular research studies. The experience during this period was excellent and it was apparent that our colleagues had profited from our earlier observations and errors. Further experience with severe acidosis indicated that isotonic sodium bicarbonate could be given despite pulmonary edema and, in fact, produced a resolution of signs of heart failure when given in quantities sufficient to fully correct the acidosis. Several cases required over 500 mEq to accomplish this goal. The system of therapy during this time remained the same as during phase 1 excepting the treatment of late, severe acidosis. Because of the difficulty and inexperience in prompt recognition of severe acidosis such cases frequently received some isotonic saline initially which increased the pulmonary edema. Because of this it seemed desirable to start hydration of all cases with a solution containing at least some alkali.

Total cholera admissions	-	104	-	0 deaths
Direct admissions	-	79	-	0 "
Prior hospitalization elsewhere	-	25	-	0 "
CO_2 < 10 mEq/L	-	17	-	0 "

Phase 3 - January 1964 through September 1964

The epidemic of January 1964 necessitated simplification of therapy. From this time we have used a single solution for the treatment of each case unless acidosis was very severe or potassium replacement by vein was indicated. Initially we used solution containing: sodium chloride 5 gms., (85 mEq), sodium lactate 50 mEq., potassium chloride 0.75 gm., (10 mEq). After a brief period 4 grams of sodium bicarbonate were substituted for lactate when supplies of lactate were exhausted. During use of this solution there were no instances of potassium intoxication by ECG during the very fast infusions given during the period of rehydration. There were also no cases with long-standing diarrhea that showed serious

signs of potassium depletion. Following this we decided to delete potassium chloride from our intravenous solutions to determine whether intravenous potassium was essential. Since then we have used a solution with 6 grams of sodium chloride and 4 grams of sodium bicarbonate. Potassium replacement by vein has ultimately proved necessary in some cases during this period. In two children serious cardiac arrhythmias associated with hypokalemia occurred despite oral administration of coconut water. In a third child there were several days of stupor refractory to various therapeutic measures associated with hypokalemia and electrocardiographic "u" waves. All three patients responded dramatically to intravenous potassium. There was a single death due to cholera during this period in a patient admitted moribund who expired within a half hour after admission. Another patient admitted with cholera died 3 weeks after admission of amebic colitis with liver abcesses; there was no clinical or bacteriologic evidence of cholera at the time of death.

Total cholera admissions	-	224	-	2 deaths
Direct admissions	-	219	-	2 "
Prior hospitalization elsewhere	-	5	-	0 "
CO_2 < 10 mEq/L	-	24	-	0 "

Matlab - December 1963 through August 1964

Therapy in Matlab was carried out by our Pakistani physicians under primitive conditions, no laboratory support or consultation. Equipment consisted of cholera cots, a scale, a stethoscope and sphygmomanometer. Fluid replacement was carried out with isotonic saline for initial rehydration followed by 1 liter of Darrow's solution for every two liters of saline. Tetracycline was given in all cases. Darrow's solution was the only lactate-containing solution available at that time in plastic bags with attached tubing and needle, the form best suited for use in the field. Darrow's solution contains:

Na	-	121.7 mEq/L
K	-	35.8 "
Cl	-	104.2 "
Lactate	-	53.3 "

This program was unsatisfactory on two accounts:

1) Too little alkali replacement,
2) Excessive potassium concentration in the Darrow's solution with the risk of intoxication by overly rapid infusion.

Tetracycline was used in all cases. Despite the obvious inadequacies of the initial Matlab experience there were only three deaths in the first 132 cases - a mortality of 2.4%. In one of these cases, death was attributed to potassium intoxication from rapid infusion of Darrow's solution.

Currently our Matlab hospital is using a single solution (Serofusine without glucose) which matches the composition of cholera stool within safe limits of rapid intravenous potassium infusion. Serofusine contains:

Na	-	133 mEq/L
K	-	12 "
Ca	-	3 "
Mg	-	4 "
Cl	-	103 "
Lactate	-	49 "

Shaitnal

To illustrate that even a simple step can greatly alter the very high village mortality of cholera, Dr. Glasse, our anthropologist, has instructed local practitioners in venopuncture and the administration of isotonic saline only. A total of 12 confirmed cholera patients have been treated without a single death. Tetracycline was given to most of the cases. This experience shows that it is possible to teach untrained people the simple skills necessary for judging how much of a single solution should be given under the crudest conditions.

Discussion:

Fluid Therapy - The experience at CRL indicates that fluid replacement therapy of cholera patients can be adequately guided by bedside and nursing observations. These guides to therapy are more readily applicable in remote areas, where cholera occurs very frequently, than those which depend on laboratory measurements. Calculation of exact losses from plasma protein levels may be misleading since there is so wide a range of variation in convalescent values.

Convalescent plasma protein values observed in our cholera patients were:

Number of cases	-	324
Range	-	4.5 - 9.4 gm.%
Mean and 2 S.D.	-	6.9 $\pm$ 1.8 gm.%

We favor the use of a single solution for both rehydration and subsequent maintenance of fluid balance. Serofusine without glucose (Vifor Co. Geneva) approximates a composition we have found satisfactory, which is packaged in plastic bags. Calcium and magnesium are not necessary; both are not harmful given in physiologic amounts and may contribute a small extra increment to care of patients with major stool losses. The simpler solution prepared in the laboratory which we have used extensively contains: 5 grams sodium chloride, 4 grams sodium bicarbonate, 1 gram potassium chloride.

Acidosis - The clinical judgment of acidosis has been a more serious problem than that of dehydration. Nearly all our mortality has been in cases who had prolonged diarrhea with inadequate alkali as well as volume replacement. At postmortem these patients have shown marked dilation of pulmonary vessels and hyaline-like membranes present in many alveoli. The clinical events preceding death were pulmonary rales and edema despite significant hypovolemia and shock. When isotonic sodium bicarbonate has been infused in sufficient quantity to overcome the acidosis death has not occurred. Thus this syndrome appears to be a consequence of acidosis and underlines the importance of proper correction of acidosis in all cholera cases.

Potassium - We have seen that both oral and intravenous solutions containing excessive amounts of potassium may result in serious clinical accidents. We have also seen that oral administration of potassium may not be sufficiently reliable to avoid serious consequences due to hypokalemia.

Consequently we have adopted a level of potassium in our intravenous solutions that has proven safe and should avoid the consequences of both potassium intoxication as well as hypokalemia leading to cardiac arrest. In field treatment such as at Shaitnal, Matlab or during the cotton mills' outbreaks oral replacement is often impracticable.

Glucose - Since a simple diet of skim milk, banana and bread has been allowed to all patients at all ages when they are first hungry, we have not seen serious hypoglycemia. Previously several cases of hypoglycemic coma were seen in children. Intravenous solutions of high glucose content have been observed to cause both clinical deterioration and changes in ECG ST segments; for a brief period in January we used a solution containing 1% glucose by vein with no adverse reactions. Feeding of carbohydrates by mouth even during diarrhea avoids hypoglycemia, satisfies hunger which is frequently intense and does not seem to aggravate the existing diarrhea.

Antibiotics - In a study reported to the Technical Advisory Committee last year, tetracycline was shown to shorten the duration of bacterial positivity and of diarrhea in cholera patients. Further experience with tetracycline, especially during the January epidemic has confirmed these findings. The experience of Matlab and Shaitnal, where long term fluid and electrolyte replacement cannot be adequately given, shows that the addition of an antimicrobial agent (tetracycline) saves lives. A double blind tetracycline study was carried out, but due to the inclusion of a group of cholera-like cases from whom V. cholerae was not recovered, it was abandoned before reaching significance. Other antibiotics active in vitro against vibrios are currently under clinical trial.

Summary:

In 362 cholera patients not treated prior to admission to CRL there have been 3 deaths due to the disease. There have been 4 deaths in 121 patients admitted after intravenous therapy at other institutions. Four of the 7 deaths were attributed to prolonged severe acidosis. There has been one death late in an uneventful convalescent cholera case due to excessive intake of potassium by mouth. All except one death occurred before Pakistani physicians took charge of the patient care during the first 102 cases. At the Matlab treatment center, in the absence of laboratory support, 132 cholera cases have been treated with two deaths due to cholera and one probably due to potassium intoxication. In Shaitnal 12 cholera cases have been treated by paramedical personnel with no deaths or complications. The syndrome of pulmonary edema seen in patients with severe acidosis has been treated successfully when enough isotonic sodium bicarbonate was infused to overcome the acidosis. Failure to replace potassium by vein can be potentially lethal in children. The optimal level of potassium in a replacement solution has yet to be determined. Tetracycline has given consistently excellent results in stopping diarrhea; we have not yet seen a case of prolonged diarrhea in a cholera patient adequately treated with this agent. A simple replacement solution in a conveniently packaged unit is a highly desirable simplification of therapy.

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Nonvibrio Cholera

by

J. Lindenbaum, W. B. Greenough, III,
R. O. Oseasohn, S. Rizvi, A. Saad.

In February and March 1964, during a seasonal period usually associated with a fall in the incidence of cholera in Dacca, a number of cases were admitted to the CRL ward in a state of circulatory collapse after an acute illness of less than 48 hours' duration, associated with the passage of copious rice-water stool. In most of these cases, no vibrios were isolated from daily rectal swab cultures. During the period from February 10, 1964 through March 26, 1964, 15 patients were admitted in a completely collapsed state, without pulse or blood pressure, in whom no bacteriologic evidence of cholera infection was found. An additional 29 bacteriologically negative cases were admitted with moderately severe dehydration (plasma protein greater than 9 gm.%) associated with watery diarrhea. During the same period, a total of 14 bacteriologically proven cases of cholera were admitted. It was apparent, therefore, that during this period of time a cholera-like illness without bacteriologic evidence of cholera infection was more than twice as common on our wards as cholera itself. Accordingly, a prospective study was undertaken to obtain more complete clinical, physiologic, bacteriologic, and immunologic data on these cases and to compare them with established cases of cholera.

Plan of Study:

The study was initiated on March 27, 1964, and continued until June 11, 1964. All patients with diarrheal disease admitted to the CRL ward in a state of circulatory collapse (absent pulse or pulse too rapid and faint to be counted) were admitted to the study. On each patient, admission plasma protein, electrolytes, white blood count and differential, serum for agglutinin titers, blood culture, and electrocardiogram were obtained before the initiation of any therapy. All patients were treated with intravenous fluids. Only two patients, both of whom had bacteriologically documented infection with *V. cholerae*, received antibiotics. All of the determinations performed on admission were repeated on the seventh hospital day. Thirty to 100 cc of liquid stool was obtained under oil from the sigmoid colon via a sterile catheter inserted through the rectum within a few hours of admission. On material obtained in this way, stool electrolytes, pH, sugar, gross and microscopic examination, and guiaic test for occult blood were performed, and an aliquot of the stool was employed for bacteriologic studies. The material was streaked on SS, MacConkey's, blood, gelatin, and tellurite-taurocholate-gelatin agars and was placed in bile peptone enrichment fluid. Seven ml aliquots of stool were distributed in 5 to 7 phials as soon after being obtained as possible and stored at minus 45°C. In addition, dark-field examination was performed on fresh stool or rectal catheter specimens according to the method of Benenson, Greenough, and Islam (1964). Throughout the hospital stay of the patient, daily cultures of rectal swab material were placed on gelatin and tellurite-taurocholate-gelatin agar and in bile peptone enrichment fluid. Strict intake and output measurements were made throughout the duration of illness. Stool was collected eight-hourly for description and volume measurement. The end of the last eight-hour period before the passage of non-liquid, semi-formed to formed stool was considered to be "end of diarrhea".

Immunologic Studies:

Paired acute and convalescent sera were available on 33 of the 34 cases, and were tested for agglutinating antibodies against V. cholerae Inaba and Ogawa. Three cases, from whom noncholera vibrios were obtained on stool culture, were also tested for agglutinating antibodies against the homologous organism.

Bacteriophage studies:

Stools from 28 of the patients were examined for the presence of bacteriophage lytic for cholera vibrios as well as bacteriophage which could lysogenize cholera vibrios. Frozen aliquots were tested. Eight classical cholera vibrios, 4 El Tor vibrios, and 2 noncholera vibrios were used as indicator organisms. Stool aliquots were treated with chloroform, heat and ultraviolet light to liberate lysogenic phage. Samples were also enriched with the indicator organisms overnight and then plated to detect the presence of phage.

RESULTS:

Bacteriologic findings - Stool cultures from fourteen patients were positive for V. cholerae (11 Inaba, 3 Ogawa). In each of these patients, repeated stool cultures showed cholera vibrios on 2 to 7 successive days. In 4 other patients, no cholera vibrios were isolated at any time, but noncholera vibrios were demonstrated on 2 to 5 consecutive days on tellurite-taurocholate gelatin agar and/or gelatin agar streaked directly with the rectal swab. No vibrios or other recognized enteric pathogens were isolated from the stools of 15 patients. In a single patient whose admission bacteriologic cultures and dark-field examination were negative, and in whom no serum agglutinating antibodies against V. cholerae were detected in either acute or convalescent sera, V. cholerae Inaba was reported on rectal swab culture from the second hospital day only. Bacteriologic cultures were again negative on days 3 - 8. Although the positive second-day culture appeared to be a sampling error, this patient will be excluded from subsequent analysis of the data. Blood cultures showed no growth in all cases.

Bacteriologically, therefore, the patients in the study fall into three groups: (1) Cholera vibrios - 14 cases, (2) Noncholera vibrios - 4 cases, and (3) No vibrios or pathogens - 15 cases.

Dark-field microscopy - In all of the 14 patients repeatedly positive on bacteriologic culture for V. cholerae, the identical diagnosis, including typing, was made on dark-field examination. In the 15 cases where no pathogen was isolated, no vibrios were noted on dark-field examination. In the 4 cases with noncholera vibrios on culture, noncholera vibrios were identified in the dark-field in two, no vibrios were seen in one, and V. cholerae Ogawa was reported in one. In the last case no cholera vibrios were isolated bacteriologically at any time during the patient's illness, and no antibodies against cholera vibrios were detectable in acute and convalescent sera; this case was considered to be a dark-field false-positive.

Immunologic studies - (Table I) Paired sera were available in 32 cases. In 14 of 14 cases from whom V. cholerae was isolated, a four-fold or greater rise in agglutinin titer occurred between samples taken on admission and those taken after the sixth hospital day. In two of three cases with noncholera vibrios, a four-fold

or greater rise in agglutinin titer against the homologous organism (but not against V. cholerae) was demonstrated. No rise in agglutinating antibody titer against V. cholerae was observed in any of the 15 cases from whom no pathogen was isolated.

Bacteriophage observations - Lytic phage was not isolated from any of the 28 stools examined. . lysogenic phages were isolated, two from stools infected with V. cholerae Ogawa and two with noncholera vibrios (Table II). After overnight incubation of the stools with the indicator strains, lysogenic phage was detected in 4 of 14 stools that were bacteriologically negative, indicating the presence in the stools of phage with a wide host range.

Clinical and biochemical observations - In Tables III through VI, various clinical and biochemical features of the cases are summarized and compared. It is apparent that the clinical symptomatology in the three groups (Table III) was identical, as was the degree of dehydration on admission (plasma protein, Table IV). Most of the patients were young adults, predominantly male. There were two 6-year old children in the group with V. cholerae, while the youngest patient in the group with no pathogens was 12. Leucocytosis was similar in degree and character (increased neutrophils, with a predominance of band forms) in the three groups. Admission blood electrolytes were similar. Similar electrocardiographic abnormalities were common in the 3 groups. Stool was alkaline and electrolyte-rich in all groups (Table V). The patients with V. cholerae tended to have a slightly higher mean pH and bicarbonate content than those with no pathogens. The so-called "typical rice-water stool" of cholera was seen in all groups, though none of the patients with V. cholerae had the reddish-brown stool frequent in the patients with noncholera vibrios and with no pathogens. The most striking differences were in the mean duration and volume of diarrhea (Table VI). The average cholera patient purged for 4.6 days, in comparison to the 1.5 days of those with no pathogens, and passed more than 8 times as much watery stool. Even in these respects, however, there was overlap between the two groups. Thus one patient with V. cholerae passed a total of 5.2 liters of stool over 2.7 days of diarrhea, while a patient from whom no pathogens were isolated had diarrhea for 4.3 days and purged 17.9 liters of stool. In general, however, the duration of illness in the group with no pathogens was short, and most patients stopped purging by the second hospital day. All of the patients in the three groups responded rapidly to intravenous fluid and electrolyte replacement therapy, and there was no mortality in any of the groups.

DISCUSSION:

These findings indicate that a syndrome occurs commonly in East Pakistan which often mimics classical cholera gravis in every respect, but which cannot be attributed to infection with V. cholerae. In none of the 15 patients who presented with watery diarrhea and circulatory collapse with repeated negative bacteriologic cultures were vibrios found on dark-field examination; and in no case was there a rise in serum agglutinating antibody titers against V. cholerae. The striking agreement between three independent techniques (bacteriologic culture, dark-field, and serologic) in failure to demonstrate evidence of vibrio infection in all 15 cases would appear to safely rule out V. cholerae as an etiologic agent. The bacteriologic studies undertaken did not reveal the agent responsible for the watery diarrhea. Virus studies have not yet been completed on the material collected from these cases. In light of our present state of ignorance, and the clinical similarity of the cases to classical cholera, the syndrome may be dubbed "nonvibrio cholera".

The findings further support a view held by many clinicians since the nineteenth century - that the cholera syndrome in all its manifestations, including circulatory collapse, muscle cramps, hoarseness, unconsciousness, and urinary suppression, is not specific for infection with V. cholerae, but is rather the physiologic result of extreme dehydration and electrolyte loss resulting from diarrhea and vomiting.

Each of these 15 cases on admission presented the full-blown clinical cholera syndrome and could not be differentiated from classical cholera. Each responded dramatically to therapy with intravenous electrolyte solutions. Even on retrospective analysis only relative, rather than absolute, differences were noted between the patients with true cholera and those in whom no pathogens were found. In the latter group, the diarrhea usually tended to be shorter in duration and less voluminous, and was sometimes associated with a pinkish, or reddish-brown stool color that is uncommon in patients with V. cholerae infection.

In view of the failure to demonstrate an etiologic agent, it may be asked whether "nonvibrio cholera" as seen in East Pakistan represents a single disease entity, or merely a potpourri of conditions of varying etiology having in common watery diarrhea, severe dehydration, and absence of infection with vibrios. Present information is inadequate to resolve this question. Though our experience with the syndrome is limited at present, certain features suggest that at least some of the patients form a specific disease entity. To date, over the 23-month period since the CRL ward opened, the syndrome has not been seen in children below the age of eight (a group commonly afflicted with V. cholerae). In addition, during the past 12 months, the syndrome was encountered frequently in February through May, with relatively rare sporadic cases in other months. Similar observations, with regard to age-group affected and seasonal incidence, were made in the course of a cholera vaccine field trial among 14,057 persons in a group of 23 villages near Matlab Bazar, 40 miles south of Dacca. The incidence of severe, disabling cholera (i.e., associated with inability to walk or respond) in a control (TAB vaccinated) group during the 9-month interval from December 1963 through August 1964 was 3.3 per 1000. During the same period the incidence of severe, disabling diarrheal illnesses (using the same criterion of severity) from which V. cholerae was not isolated was 15.7 per 1000. More than half of these illnesses unassociated with vibrios occurred during March, April, and May. While most of the severely disabling diarrhea due to V. cholerae occurred in persons below 15 years of age, nonvibrio diarrhea of similar severity was more frequent among those 15 and over.

Information collected both from the Matlab Bazar field trial area and from Shaitnal indicates that untreated or inadequately treated nonvibrio cholera may, like true cholera gravis, result in a fatal outcome.. It must also be emphasized that the present study selected only the severest cases - those with the most profound dehydration; and in the absence of a known etiologic agent, certain identification of milder cases of the syndrome is difficult, and description of its full clinical spectrum is impossible.

Noncholera vibrios were demonstrated on repeated stool culture from four of the patients included in this study. Like those in whom no known pathogens were recovered, these cases mimicked classical acute cholera (Tables III through V), though, as in the group with no pathogens, average duration and volume of diarrhea was less than in the patients with V. cholerae infection (Table VI). In two of the cases with noncholera vibrios, antibody titer rises occurred against homologous noncholera vibrios, but not against V. cholerae. That infection with noncholera

vibrios may occasionally give rise to the full-blown cholera syndrome has been observed repeatedly (Pollitzer, 1959, McIntyre et al, 1964).

Summary and conclusions:

The syndrome in which the passage of copious quantities of rice-watery stool results in extreme dehydration and circulatory collapse is not specific for infection with V. cholerae. Such a syndrome, in which no evidence for V.cholerae infection was demonstrable by repeated bacteriologic culture, dark-field examination and immunologic methods, occurred more frequently in areas of East Pakistan in the spring of 1964 than did true cholera itself. The duration and volume of watery diarrhea was usually less than in cases of true cholera. In a few cases, infection with noncholera vibrios was associated with the syndrome; but in most, no known pathogen was recovered.

Table I

Bacterial isolations and Vibrio Agglutinating Antibody Titer Rises

Bacterial Isolate	Significant** Antibody Rise	No Antibody Rise	Inadequate Sera	TOTAL
V. cholerae Inaba	11	-	-	11
V. cholerae Ogawa	3	-	-	3
Noncholera vibrios	2*	1	1	4
No pathogens	0	15	-	15

* Rise in antibody against homologous organism; none against V.cholerae Inaba or Ogawa.

** Four-fold or greater rise in titer.

Table II

Bacteriophage Isolations

Bacteriological Findings	Lysogenic Phage	No Phage Isolated	TOTAL
V. cholerae Inaba	0	9	9
V. cholerae Ogawa	2	0	2
Noncholera vibrio	2	1	3
No Pathogen isolated	4	10	14
TOTAL	8	20	28

Table III

Clinical Features

	CHOLERA	NO PATHOGENS	NONCHOLERA VIBRIOS
Watery diarrhea	14/14	15/15	4/4
Vomiting	14/14	15/15	4/4
Muscle cramps	13/14	15/15	4/4
Abdominal pain	5/14	11/15	3/4
Hoarseness	14/14	14/14	4/4
Unconsciousness	3/14	4/15	1/4
Oliguria	13/14	13/15	2/4
Documented fever	10/14	11/15	4/4
Duration of symptoms from onset to time of admission:			
Mean	14 hrs.	10 hrs.	11 hrs.
Range	3 - 32 hrs.	5 - 21 hrs.	4 - 18 hrs.

Table IV

Clinical and Biochemical Findings

	No. of Cases	Sex Ratio M : F	Age in Years	Body Weight in Kg.	Plasma Protein in gm.%	
					Admission	Convalescent
V. cholerae	14	11 : 3	28 (6-45)	39 (13-63)	11.7 (8.7-14.2)	7.1 (6.0 - 8.6)
No Pathogens	15	10 : 5	35 (12-60)	38 (34-41)	11.8 (9.8-14.4)	7.2 (6.0 - 8.0)
Noncholera vibrios	4	2 : 2	26 (18-30)	39 (23-54)	11.3 (10 - 13.2)	6.9 (6.5 - 7.4)

	Total White Blood Count	SERUM ELECTROLYTES mEq/l			
		Na	K	Cl.	CO_2
V. cholerae	21,700 (4,000 - 36,000)	140 (139-144)	4.5 (3.5-5.0)	96 (86 - 119)	15.3 (9.1 - 22.0)
No Pathogens	22,300 (6,450 - 38,000)	138 (126-142)	4.1 (3.2-4.7)	96 (82 - 110)	14.5 (9.5 - 20.3)
Noncholera vibrios	21,400 (14,800-32,200)	141 (138-144)	4.2 (4.0-4.6)	96 (90 - 100)	16.0 (14.5 - 18.9)

Comparison of various parameters in the 3 groups of patients included in the study. Mean values are presented. The figures in parentheses following the mean values indicate the range of values recorded.

Table V

Stool Characteristics

	pH*	Na*	K*	Cl*	CO_2*
V. cholerae	7.54 (6.85 - 7.95)	139 (46 - 161)	24.3 (10.2 - 82.0)	106 (63 - 129)	48.5 (26.9 - 66.5)
No Pathogens	7.11 (6.36 - 7.80)	123 (70 - 163)	31.1 (7.7 - 66.5)	99 (89 - 130)	41.5 (18.1 - 68.0)
Noncholera vibrios	7.52 (7.08 - 8.12)	111 (99 - 130)	46.4 (25.6 - 56.4)	91 (82 - 98)	45.3 (39.6 - 55.2)

	Glucose*	Appearance	Mucus	WBC	RBC	Guiaic positive
V. cholerae	0 (0 - 0)	9 "rice-water" 5 yellow	+ - +++	+ - ++	0 - +	3/13
No Pathogens	2.8 (0 - 17)	5 "rice-water" 4 yellow 6 red-brown	+ - +++	+ - ++	0 - +++	5/11
Noncholera vibrios	1.7 (0 - 5)	1 "rice-water" 3 red-brown	+ - +++	+ - ++	0 - +	0/3

* Mean values (range in parenthesis)

Table VI

Duration and Volume of Diarrhea

	No. of Cases	Mean duration, days after admission	Range	Mean Volume, Liters	Range
V. cholerae	12*	4.7	2.7 - 6.3	30.8	5.2 - 69.1
No Pathogens	15	1.5	0 - 4.3	3.7	0 - 17.9
Noncholera vibrios	4	2.1	1 - 3	3.0	2.3 - 3.4

* Two cases of the 14 with *V. cholerae* that received tetracycline therapy are not included.

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Laboratory Diagnosis of Cholera

by

A. S. Benenson, M. D.

The use of the darkfield vibrio immobilization test has provided a method by which vibrio infection can be specifically recognized within minutes. Since preparation of the original report accepted for publication by the Bulletin of The World Health Organization (see CRL Recent Publications), this technique has become a routine carried out by the technicians in the clinical pathology laboratory. Comparison of early and later results reveals that more infections have been missed in the later period. However, in the majority of cases, the procedure has been of great help because the results are available so soon.

Direct Recognition	January Epidemic	Jan. to Sept.
Total examined	62	69
Positives correctly identified	49	24
Vibrios recognized-too few to type	-	11
Missed by darkfield	13	34
False positives	0	0
After Overnight Enrichment		
Total examined	70	93
Positives correctly identified	66	69
Vibrios recognized-too few to type	0	4
Missed by darkfield	2	20
False positives	0	1

In several instances, the darkfield technique has recognized specific infections overlooked on plates containing many non-cholera vibrios. The single false positive instance was one in which a non-cholera vibrio was reported as Ogawa cholera.

It was originally reported that this technique was only positive if there are more than 10^5 vibrios per ml. A review of the degree of culture positivity of stools of all patients from whom vibrios were isolated during their illness shows that 82% have stools which are graded as +++ or higher

on admission. A +++ report is equivalent to 105 organisms and is at the borderline of recognition by this method (see Section III(a)). In the routine CRL culture technique including enrichment, 93% of subsequently confirmed cholera infections are positive on the first day, with a pattern of positivity thus:

	Degree of Positivity on Day of Admission				
Total Cases	Neg.	Enrichment only	+	++	+++ & ++++
144	10	4	3	10	118
	7%	3%	1%	7%	82%

The severity of disease, as judged by admission plasma protein level, had no evident effect on bacteriological positivity.

The likelihood of recognising infection decreases slowly in the patients not treated with antibiotic, as is seen by reviewing the results of daily culturing, with direct plating on TTGA and GA, and enrichment through tellurite bile peptone broth.

	Day of Disease									
	1	2	3	4	5	6	7	8	9	10
	% of cultures positive									
No antibiotic	93	90	89	72	67	54	30	13	9	0

The inclusion of serological studies for agglutinins against living vibrios increases the recognition of specific infection. Results of examination of adequate paired sera (second sample after the 6th day) of cases admitted because of diarrhea (CRL Nos. 258 to 1000) are as follows:

		Vibrio Isolations		
	Total Positive	Ogawa	Inaba	None or NCV
Total No. of cases with adequate sera	178	118	60	85
% serologically positive	86.5	88.1	83.3	5.9
Fourfold or greater agglutinin rise	154	104	50	5
No. with equal Ogawa and Inaba titers		35	22	2
No. with greater Ogawa titer		57	6	2
No. with greater Inaba titer		12	22	1

The 5 cases serologically positive but culturally negative include one with mild gastroenteritis and 4 will illnesses resembling moderately severe cholera. Antibiotic therapy had no demonstrable effect on the antibody responses - the geometric mean titer on days 7-10 was 1:127 among those who did not receive antibiotic, and 1:126 among those who received antibiotic.

In summary, the bacteriological technique using two plates and enrichment broth detects 93% of Vibrio cholerae cases on the first day of illness; testing for agglutinins against living vibrios detects 86.5% of the bacteriologically confirmed infections and 5.9% of those missed bacteriologically. The darkfield vibrio immobilization procedure allows almost immediate recognition of over half the vibrio infections, utilizes minimal equipment, and as an additional diagnostic technique identifies some infections which might otherwise have been missed.

Pulmonary Hypertension. A Consequence of Shock Due to Diarrhea.

by

Drs. K. M. Ally and W. B. Greenough, III.

Pulmonary hypertension is a recognised feature encountered in some congenital heart conditions, chronic lung diseases, and acute pulmonary embolism. To our knowledge, it has not been reported in shock due to severely dehydrating diarrheal disease.

During the winter of 1962-63 three phenomena were observed in cholera patients in circulatory collapse which suggested the presence of pulmonary hypertension. One of these was auscultatory: accentuation and splitting of the pulmonic second heart sound. The other two were electrocardiographic: giant "p" waves and right axis deviation. Further electrocardiographic observations on cholera patients indicated that these changes were always present to some extent during the stage of vascular collapse. Rapid infusion of even relatively small amounts of either saline or alkali-containing solutions resulted in changes toward normal in the "p" waves and shift of the electrical axis to the left.

As part of the prospective study of severely dehydrating diarrheal disease described elsewhere in this report (Lindenbaum et al, "Nonvibrio cholera") serial electrocardiograms were taken on 30 of 34 cases admitted in shock in the spring of 1964. Analysis of these tracings showed that the electrocardiographic findings in cases of vascular collapse due to V. cholerae were identical to those seen in cases from whom no pathogens or from whom noncholera vibrios were isolated. A further study was conducted at Mitford and Dacca Medical College Hospitals to determine whether the changes observed in collapse due to diarrhea would be found in shock due to hemorrhage and other causes.

The results are summarized in the following table:

Table I

The Electrocardiogram in Vascular Collapse

	"p" wave 3mm or more	Axis Shift of 20° L or more	Plasma Protein 10 gm.% or more	CO_2 15 mEq/L or less	Total Cases
True cholera	17/22 (77%)	18/20 (90%)	18/22	11/18	22
Noncholera diarrhea*	10/12 (83%)	7/9 (78%)	11/12	9/12	12
Haemorrhage	2/11 (19%)	- -	0/10	2/10	11
Miscellaneous	4/12 (33%)	- -	0/12	1/11	12

* "Nonvibrio cholera" and cases with noncholera vibrio on stool culture.

The height of "p" waves in electrocardiograms of 171 normal East Pakistani subjects (collected by the Nutrition Survey of East Pakistan) ranged from 1.1 to 1.7 mm. Data on axis shift changes are not available on the cases with shock due to nondiarrheal etiology because serial electrocardiograms were not taken on these subjects. The two patients with haemorrhagic shock with elevated "p" waves had normal serum carbon dioxide contents. Four cases in the "miscellaneous" category had elevated "p" waves. The admitting diagnoses at Mitford Hospital in these 4 cases were: amebic hepatitis with collapse, acute urinary retention, enlarged prostate with uremia, and eclampsia.

Thus we see that cases of shock due to non-diarrheal etiology, in our limited experience, were only occasionally associated with electrocardiographic findings suggestive of pulmonary hypertension. Even in those patients with non-diarrheal shock with "p" wave elevations, the amplitude was not as great as in the typical case with shock due to diarrhea.

Comparison of the height of "p" wave in the diarrheal cases with the degree of acidosis or dehydration did not yield any correlation in our small series. Rehydration, however, rapidly alters the electrocardiographic pattern towards normal as summarized in the following table.

Table II

The Effect of Rapid Intravenous Infusion on the Electrocardiogram of Patients with Shock Due to Diarrhea

Fluid received at time of initial ECG.	Height of p wave 3mm or >			Axis Shift 20° or more		
	Cholera	Non-cholera*	Total	Cholera	Non-cholera*	Total
No infusion	17/22	10/12	27/34 (80%)	18/20	7/10	25/30 (83%)
Less than 500 cc	5/7	3/5	8/12 (67%)	5/7	4/5	9/12 (75%)
More than 500 cc	6/34	1/4	7/38 (18%)	12/26	2/3	14/29 (49%)

* "Nonvibrio cholera" and cases with noncholera vibrios on stool culture.

The accentuation of the pulmonic sound persists for 2 to 5 days, however, even when normal hydration is maintained.

Our current hypothesis is that pulmonary hypertension is consistently present in vascular collapse due to dehydrating diarrhea. We would guess that it may depend on some phenomenon associated with hemoconcentration such as sludging of cells in the pulmonary capillaries, or viscosity changes. Acidosis and decreased blood volume probably play less important roles in the pathogenesis of pulmonary hypertension.

These hypotheses will be subject to direct experimental verification by a team of cardiologists including Drs. R. Harvey, Y. Enson, and M. Lewis, who will be working at the CRL in the winter of 1965. Direct measurements on the pulmonary and systemic circulations by means of cardiac catheterization will be made to determine whether or not pulmonary hypertension is indeed present and to investigate its pathogenesis.

Malabsorption During and After Recovery From Acute Intestinal Infection

by

John Lindenbaum, M.D.

In recent years increasing interest has been shown in a variety of diarrheal diseases associated with small intestinal malabsorption. Though malabsorption, as indicated by a number of tests of absorptive function, has been reported in many chronic diseases involving the gastrointestinal tract, interest has centered on conditions associated with jejunal atrophy - including idiopathic steatorrhea ("gluten-induced enteropathy") and the syndrome of chronic diarrhea associated with malabsorption in persons living in tropical countries ("tropical sprue"). The tropical sprue syndrome is of unknown etiology, though considerable evidence exists that infection may play a role in some of the cases. Symptomatic and biochemical responses to antibiotic therapy have been reported (French, Gaddie, and Smith 1956, Sheehy and Perez Santiago 1961), and the syndrome may occur in epidemic form (Baker, Mathan, and Joseph 1962). Studies from Thailand, South India, and Pakistan indicate that while symptomatic tropical sprue occurs only occasionally in the population, abnormalities of mucosal appearance and function are common in asymptomatic "normal" individuals without diarrhea (Sprinz, Sribhibhadh, Gangarosa, Bonyagati, Kundel, and Halstead 1962, Baker, Ignatius, Mathan, Vaish, and Chacko 1962, Aziz 1964, Lindenbaum 1964a).

The effect on absorptive function of acute intestinal infection, the most common form of gastrointestinal disease the world over, has received little attention. Many cases of acute enteritis are too mild to require hospital admission, or are discharged from hospital soon after symptomatic recovery. Occasional patients have been reported, however, in whom a transient diarrhea associated with malabsorption appeared to follow acute intestinal infection (Achor and Smith 1955, King and Joske 1960). In the past year we have also encountered two cases in which diarrhea and weight loss associated with malabsorption appeared to follow episodes of acute intestinal infection in previously healthy individuals (Lindenbaum 1964b). Interest in these cases, as well as in the possible relationship of previous bouts of acute small bowel infection to the asymptomatic malabsorptive state common in tropical countries, prompted a study of absorption during and after recovery from acute intestinal infections in East Pakistan. Preliminary results will be presented at this time.

Methods and Materials:

The 95 patients included in the study were Pakistanis admitted to the Pakistan-SEATO Cholera Research Laboratory hospital ward for acute diarrheal illness of less than two week's duration. The majority of patients were admitted after only one to two days of acute illness. While most of the patients were young adults of either sex, the entire group ranged in age from 7 to 65 years.

In the majority of cases a bacteriologic diagnosis was established by culture of rectal swabs. In 47, *Vibrio cholerae* was obtained; in 6, *Shigellae* (types A, B, or D); and in 2, *Salmonellae* (one type D, one type B). In four patients who had the classical clinical picture of staphylococcal food poisoning, *Staphylococcus aureus* was isolated from stool culture and from food recently ingested by all of

them. Nine patients, from whom no known pathogens were isolated, had an acute severely dehydrating illness resulting in profound circulatory collapse unassociated with rises in antibody titer against Vibrio cholerae ("nonvibrio cholera"). Clinical features of these cases will be reported elsewhere (Lindenbaum, Greenough, Oseasohn, Rizvi, and Saad 1964). In the remaining 27 cases, all of whom had an acute gastrointestinal illness of one to four day's duration not associated with circulatory collapse, no pathogens were isolated ("acute gastroenteritis").

The five hour urinary excretion of D-xylose after a 25-gram oral dose, as well as plasma xylose levels at two hours, were measured by the method of Roe and Rice (1948). Normal values for 5 hour urinary xylose excretion for this laboratory range from 5.0 to 10.0 grams. Folic acid absorption was studied by microbiologic assay of serum with Streptococcus faecalis after an oral dose of 40 microgm. of folic acid per kg. Values of > 40 millimicrogm. per ml at one or two hours are considered normal (Chanarin, Anderson, and Mollin 1956). The 24 and 48 hour urinary excretion of vitamin B12 was measured after an oral dose of 1 microgm. of Co-58 labelled cyanocobalamin given simultaneously with 50 mg. of intrinsic factor. "Flushing" doses of 1000 mcg. of nonradioactive vitamin B12 were given at 0 and 24 hours (Schilling 1953, Ellenbogen, Williams, Rabiner, and Lichtman 1955). Normal values in this laboratory are 9-25%/24 hours and 13-36%/48 hours.

Since the tests of xylose and vitamin B12 absorption depend on urinary excretion of the absorbed substances, care was taken to exclude patients with impaired renal function. Oliguric patients were not included in the study. Those who had subnormal urinary xylose excretion associated with two-hour plasma xylose levels greater than 36 mg % were considered to have renal impairment and were excluded from the study. Cases whose urinary excretion of Co-58 cyanocobalamin during the second 24 hours after the oral dose exceeded that of the first 24 hours, were also excluded. Patients who vomited within several hours of the administration of the test substances also were not included.

Stool was collected for gross description and volume measurement over eight-hourly periods. The end of the last eight-hour period before the passage of normal appearing, formed stool was defined as "end of diarrhea". In some patients studies of xylose, folic acid, and/or B12 absorption were performed soon after admission (after dehydration and circulatory collapse had been corrected with intravenous fluids) in the presence of continuing diarrhea. Others were first studied during the first week after end of diarrhea. In 54 patients, serial studies of one or more parameters of absorption were performed.

Results:

During diarrhea - The results of absorption studies performed during diarrhea due to a variety of gastrointestinal infections are shown in figure 1. It will be seen that over 90% of patients studied during the acute infection showed impairment of absorptive function, often marked in degree. It is of interest that despite the milder degree of clinical illness and dehydration, and much less voluminous stool output in the patients diagnosed as "acute gastroenteritis", the degree of impairment of xylose absorption (Table I) was greater in this group of patients than in those with acute cholera (11 of the 16 cholera patients were admitted in a state of complete circulatory collapse, without pulse or blood pressure; none of the 15 gastroenteritis cases had circulatory collapse or severe dehydration). The difference between the mean xylose absorptions in the two groups

is statistically significant ($p < 0.01$).

After end of diarrhea - The results of absorption studies carried out during the first week after end of diarrhea are shown in figures 2A,B. The great majority of these patients had undergone complete clinical recovery and were asymptomatic at the time of study; a few complained of weakness or mild abdominal discomfort. Malabsorption of xylose and vitamin B12 was present in the majority of patients in all groups studied, though it appeared to be slightly less common in the cholera group than in the others. While post-diarrhea malabsorption of xylose was common in those patients with gastroenteritis who had minimal clinical symptoms and minimal or no dehydration, it was the rule in those with severe initial dehydration. Thus 15 out of 23 gastroenteritis cases with admission total plasma proteins of less than 9.0 gm.% showed post-diarrhea malabsorption, whereas all of 7 patients with admission plasma protein greater than 9.0 gm.% had subnormal absorption. All of six patients with "nonvibrio cholera", admitted in circulatory collapse with severe dehydration, malabsorbed xylose after end of diarrhea. In the cholera cases, however, severity of illness bore no relation to subsequent malabsorption after clinical recovery. Six of 10 cholera patients with admission protein of less than 10 gm. % showed subsequent xylose malabsorption, as did a similar proportion (13 of 24) with admission protein of greater than 10 gm.%.

Vitamin B12 malabsorption was prevalent after end of diarrhea in all groups, and in the small numbers of patients studied, there was no evident correlation with bacteriologic diagnosis or severity of illness. Absorption of folic acid, however, as measured by S. faecalis serum levels, was normal after end of diarrhea in most cases.

Serial Studies - (Table II) In 43 patients in whom malabsorption of xylose was present when first studied either during diarrhea or during the first week after end of diarrhea, the test was repeated one or more times at varying time intervals after end of diarrhea. In 29 absorption eventually returned to normal; in 22 of these a normal urinary excretion of xylose was achieved within eight days of end of diarrhea. Fourteen patients showed persistent malabsorption of xylose when last studied (11, 12, 15, 18, 26, 31, 34, 43, 52, 180, 200, 338, 371, and 378 days after end of diarrhea). Only 3 of these 14 had abdominal symptoms when last studied and 8 had gained weight; in the remaining 5, body weight was stable. Including those patients whose xylose excretion was normal when first studied after end of diarrhea and who therefore did not undergo serial study, 14 of a total of 70 patients (20%) malabsorbed xylose when last studied.

Persistent malabsorption of vitamin B12 was also common. Of 22 patients with B12 malabsorption during or after end of diarrhea, only 9 had returned to normal, mostly within the first month of end of diarrhea. Thirteen individuals had subnormal cyanocobalamin absorption when last studied (8, 9, 11, 13, 14, 14, 17, 21, 30, 31, 33, 36, and 138 days after end of diarrhea). Only 1 of these 13 had gastrointestinal symptoms and all had maintained or increased body weight. Including those patients who did not undergo serial study because of initially normal B12 absorption the prevalence of persistent B12 malabsorption in the entire group was 43% (13 of 30).

In all of nine patients who had malabsorption of folic acid during acute cholera, repeat studies were within normal limits, seven within the first week after end of diarrhea.

Patterns of recovery of typical cases where serial studies were done are illustrated in figures 3-6.

Antibiotic therapy - A few patients received antibiotic therapy. Of eight patients with cholera treated with tetracycline, four malabsorbed xylose after end of diarrhea. Two patients with Shigellosis treated with antibiotics showed post-diarrhea xylose malabsorption.

Discussion:

These findings indicate that during acute intestinal infection due to a variety of pathogenic organisms, moderate to severe impairment of absorptive function is the rule. In addition, in most patients with acute enteritis of infectious origin, subnormal absorptive capacity is present for variable periods of time after end of diarrhea and apparent clinical recovery. In most cases, return of absorptive function to within normal limits was noted within a week. While an episode of acute infectious enteritis may rarely be followed by a period of transient diarrhea and weight loss (Achor and Smith 1955, King and Joske 1960, Lindenbaum 1964b), nearly all individuals who survive an enteric infection appear to recover completely.

Of the substances studied, xylose and folic acid are believed to be predominantly absorbed from the duodenum and upper jejunum (Fordtran, Soergel, and Ingelfinger 1962, Herbert 1959) while vitamin-B12 absorption has been shown to occur almost exclusively in the ileum (Booth and Mollin 1959). Therefore the absorptive disorder demonstrated in these patients is a widespread one, involving at least carbohydrate and water soluble vitamins, and implicating both the upper and lower portions of the small bowel.

That there is widespread derangement of function during acute intestinal infection is not an unexpected finding, though the severity of absorptive impairment in some of the mild cases of gastroenteritis was indeed surprising. The degree of impairment was often similar to that seen in patients with idiopathic steatorrhea and advanced mucosal atrophy. The mechanism underlying the malabsorption present during acute infectious enteritides is uncertain. Decreased transit time, or other effects on bowel function attributable to the mere presence of diarrhea, cannot account for it. Experimental evidence exists that increased bowel motility may actually enhance absorption (Cummins and Almy 1953). Studies performed in this laboratory on asymptomatic subjects with normal baseline xylose and B12 absorption revealed minimal or no change in absorptive capacity during, or in the week following, several days of copious diarrhea induced by repeated doses of magnesium sulfate (Lindenbaum 1964b).

It is curious that the degree of derangement of intestinal function was greater in patients with "acute gastroenteritis", including rather mild cases, than in those with full-blown severe cholera admitted in circulatory collapse. This finding may be consistent with present concepts of the pathogenesis of cholera. Recent demonstrations of intact intestinal mucosa on jejunal biopsy during acute cholera (Gangarosa, Beisel, Benyajati, Sprinz, and Piyaratn 1960, Fresh, Versage, and Keyes 1964) as well as the absence of increased protein loss into the gut (Weaver, Johnson, and Phillips 1948, Gordon 1960) have refuted the formerly held idea that massive mucosal denudation was responsible for the copious rice-water stool passed during the disease. It is currently

felt that cholera vibrios remain for the most part in the lumen of the intestine and exert their damaging effect by means of a toxin which results in the passage of rice-water stool. A cell-free material that causes inhibition of sodium transport in a number of in vitro systems has been demonstrated in cholera stool (Huber and Phillips 1960) and in culture filtrates (Fuhrman & Fuhrman 1960). According to the hypothesis currently favored by Phillips and associates, the passage of rice-water stool is the result of toxic inhibition of mucosal sodium absorption (Phillips 1963). The data obtained in the present study neither support nor negatethis hypothesis, but indicate that if sodium malabsorption exists during cholera, it may be part of a generalized disorder of absorption involving xylose, folic acid, and cyanocobalamin as well.

Malabsorption of one of the test substances in the present study was not always paralleled by malabsorption of the others when all three tests were performed within a day or two of each other (except during diarrhea when concomitant impairment of absorption of all three substances was almost universal). During the first week after end of diarrhea, 77% of patients studied showed cyanocobalamin malabsorption, 70%, xylose malabsorption, and only 7% folic acid malabsorption. The folic acid absorption test, in which S. faecalis serum levels are assayed after an oral dose, may provide a less quantitative measure of absorption than the tests employed involving xylose and vitamin B12, in which the percentage of the oral test dose excreted in the urine is measured. A test of folic acid absorption, in which the percentage urinary excretion of a test dose of the tritium-labelled vitamin is measured, has recently become available (Anderson, Belcher, Chanarin, and Mollin 1960). This procedure may provide a more quantitative estimate of absorptive capacity. Malabsorption has been demonstrated by means of the latter test in three individuals with chronic small bowel disease who had normal S. faecalis levels after the test dose (Klipstein 1963). On the other hand, the microbiologic folic acid absorption test is felt to be the more sensitive one by others (Anderson, et al, 1960). Future studies are planned in which the sensitivity of the two procedures in detecting inability to absorb folic acid will be compared in patients after recovery from enteric infection.

While the majority of patients who showed post-diarrhea malabsorption eventually regained normal function, a significant number showed persistently subnormal xylose and/or B12 malabsorption, in some cases on repeated study extending several months after end of diarrhea. It is planned to continue these longitudinal studies to determine whether or not full recovery of absorptive function will occur.

The significance of persistent malabsorption in these cases is uncertain. The majority of these individuals were free of gastrointestinal symptoms. Sprinz and co-workers studied 20 cholera patients in Thailand one year after recovery and found malabsorption of xylose in 9; of a control group of 66 "apparently healthy" Thai army recruits, 42 malabsorbed xylose. Nearly all persons in both groups showed slight or moderate chronic duodenitis or jejunitis on mucosal biopsy specimens (Sprinz, Sribhibhadh, Gangarosa, Benyajati, Kundel and Halstead 1962). In a survey of healthy, East Pakistani employees of the Pakistan-SEATO Cholera Research Laboratory, 40% were unable to absorb xylose normally (Table III). Therefore, persistent malabsorption after intestinal infection may merely be a manifestation of an underlying bowel disorder which preceded (or perhaps even predisposed to) the intestinal infection. On the other other hand it cannot be excluded that failure to recover absorptive function after single or repeated

bouts of intestinal infection may be responsible for the widespread abnormalities of intestinal structure and function noted in Pakistani and Thai people.

These preliminary findings will be extended by further serial study of selected patients with acute intestinal infection, in whom xylose, folic acid, vitamin B12, and fat absorption will be correlated with small intestinal appearance on mucosal biopsy and roentgenologic examination.

Summary:

Preliminary results of a study of xylose, folic acid and vitamin B12 absorption in Pakistanis with acute intestinal infections are presented.

During infection with a variety of organisms, widespread, moderate to severe impairment of absorptive function was the rule. Malabsorption was also common during the first week after clinical recovery from infection. In most individuals impairment of absorption was transient. However, some showed continued malabsorption weeks or months after the acute infection. The possible relationship of these findings to the prevalence of subclinical malabsorption in East Pakistan is discussed.

Table I

Xylose Absorption During and After Acute Infectious Diarrheal Disease

	Number of Patients	Mean 5 hr. xylose excretion, grams.	S.D.	Range
During diarrhea:				
Cholera	16	3.7	± 1.5	1.4 - 6.2
Acute gastroenteritis	15	1.8	± 1.1	0.6 - 4.5
All patients	36	2.6	+ 1.6	0.6 - 6.2
First week after end of diarrhea:				
Cholera	34	4.7	± 1.4	2.1 - 7.7
Acute gastroenteritis	30	4.1	± 1.7	1.0 - 7.8
All patients	81	4.3	± 1.7	1.0 - 7.8

Table II

Recovery of Absorptive Function in Patients Abnormal When First Studied

Disease	Xylose Excretion*	B12 Excretion*	Folic Acid Absorption*
Cholera	11/15	6/8	9/9
Acute gastroenteritis	11/18	1/8	
Non-vibrio cholera	3/5	2/3	
Salmonellosis	1/1	0/1	
Staphylococcal food poisoning	2/2	0/2	
Shigellosis	1/2	-	
TOTAL	29/43	9/22	9/9

Results of studies performed serially after end of diarrhea in patients with initial malabsorption.

* Number of patients normal when last studied/ total number serially studied.

Table III

Xylose Absorption in "Healthy, Normal" Subjects Residing in East Pakistan

	No. of Subjects	Mean xylose excretion, gm.	S.D.	Range	No. of subjects < 5.0 gm
Americans and Europeans	19	7.3	± 1.1	5.8 - 10.0	0 (0%)
Pakistanis	73	5.4	± 1.5	2.4 - 9.3	29 (40%)

I(h)

FIGURE 1

Absorption Studies During Diarrhea.

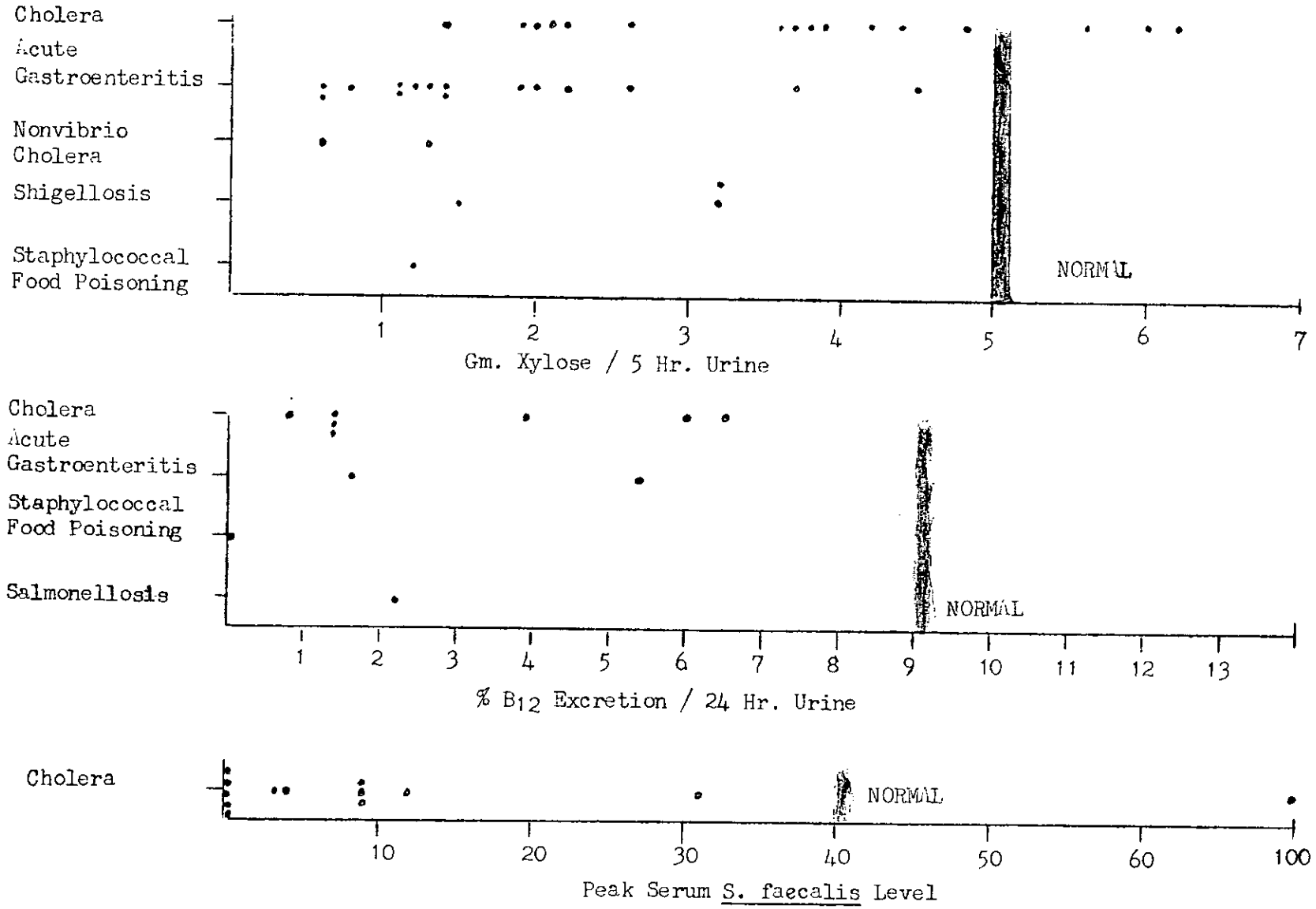
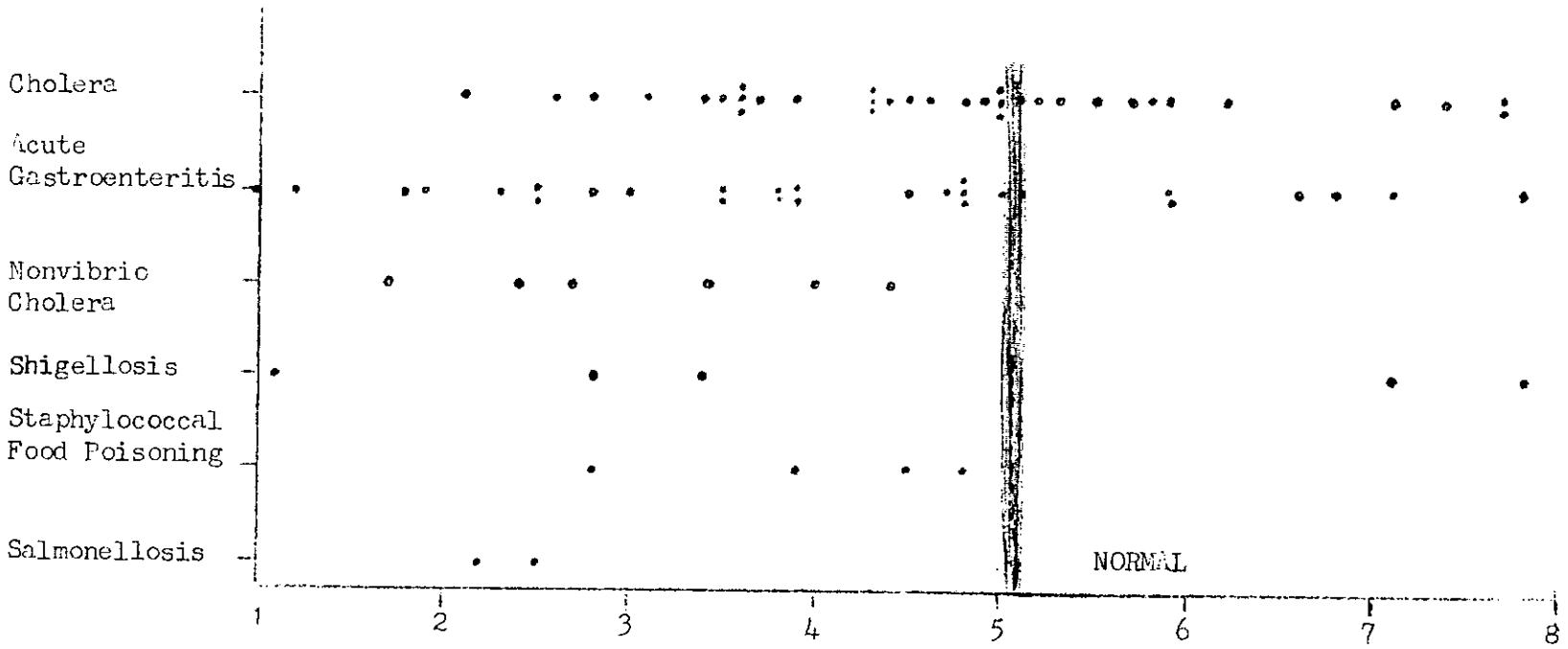


FIGURE 2A

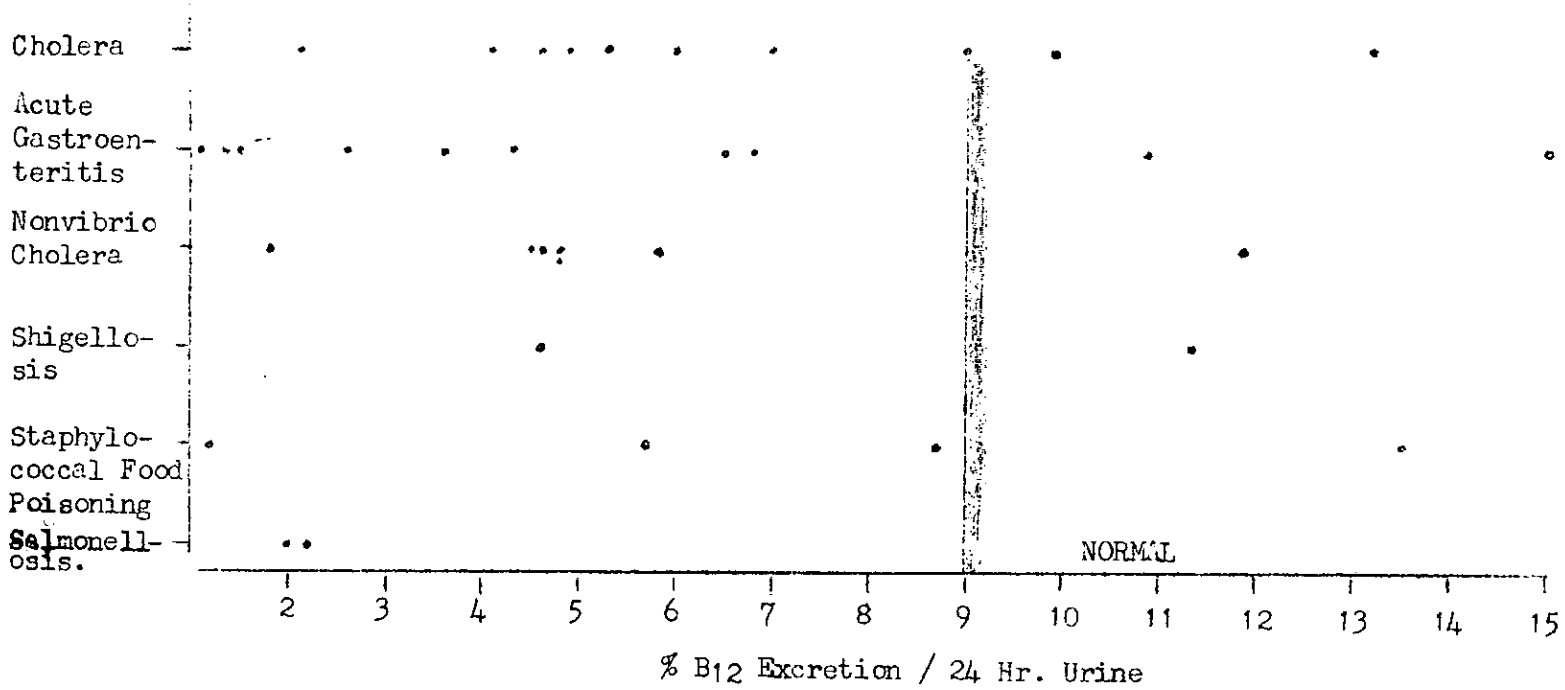
Xylose Absorption Study
During First Week After End of Diarrhea



I(h)

FIGURE 2B

B_{12} and Folic Acid Absorption Studies
During First Week After End of Diarrhea.



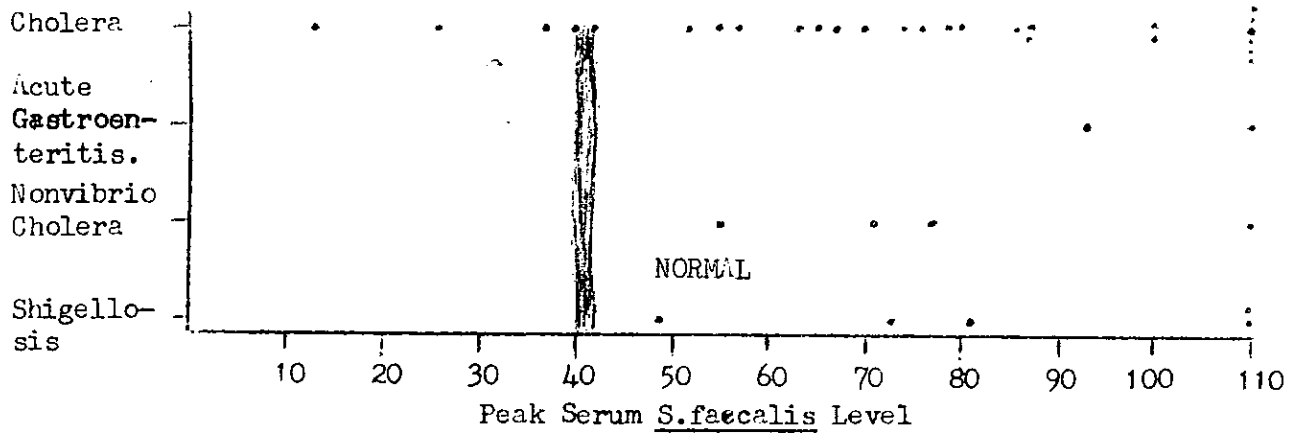


FIGURE 3

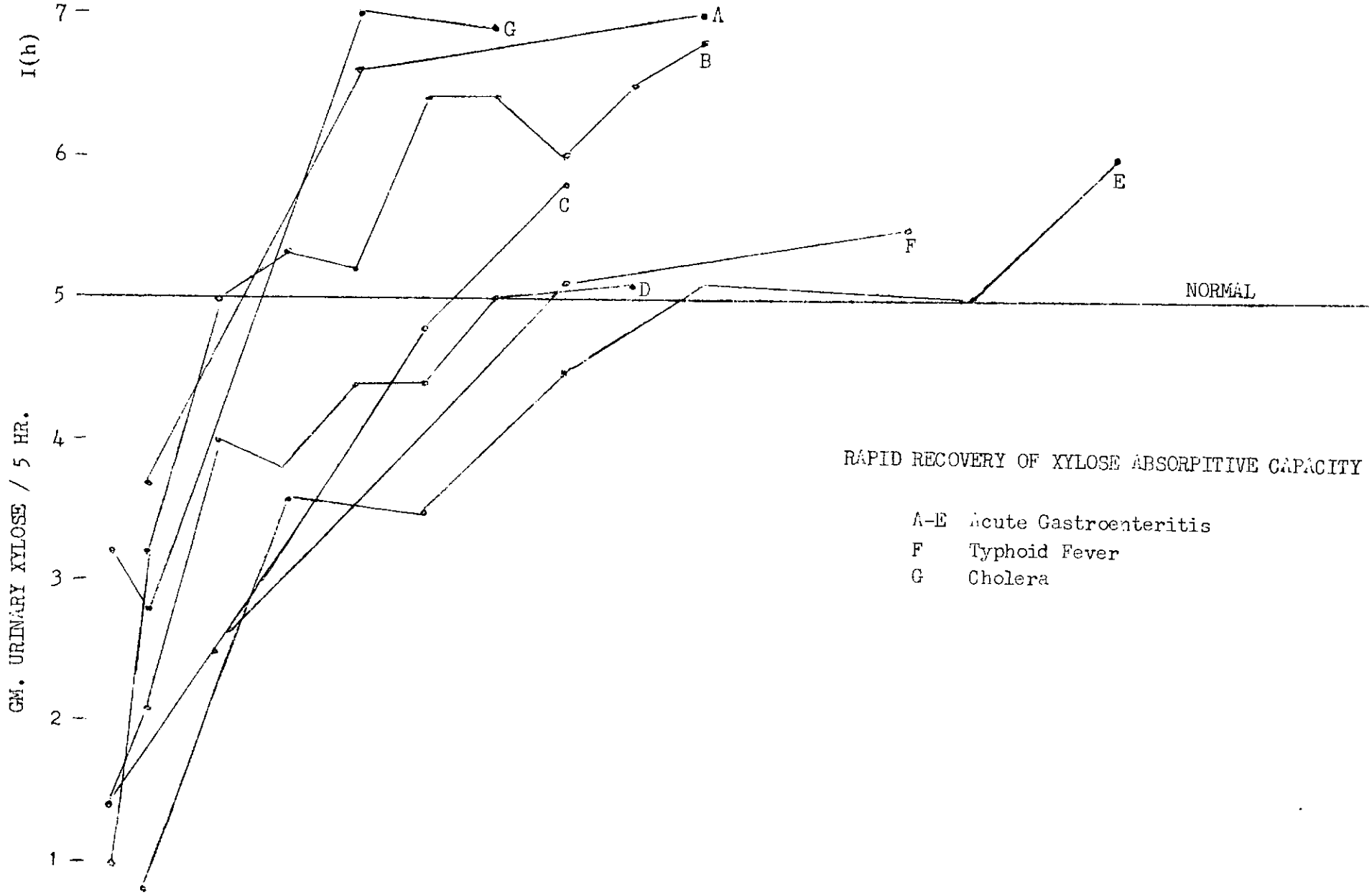

I(h)
GM. URINARY XYLOSE / 5 HR.
7
6
5
4
3
2
1
A
B
C
D
E
F
G
NORMAL
RAPID RECOVERY OF XYLOSE ABSORPITIVE CAPACITY
A-E Acute Gastroenteritis
F Typhoid Fever
G Cholera
During

FIGURE 4

PERSISTENT XYLOSE MALABSORPTION

A-B Acute gastroenteritis
C Nonvibrio cholera

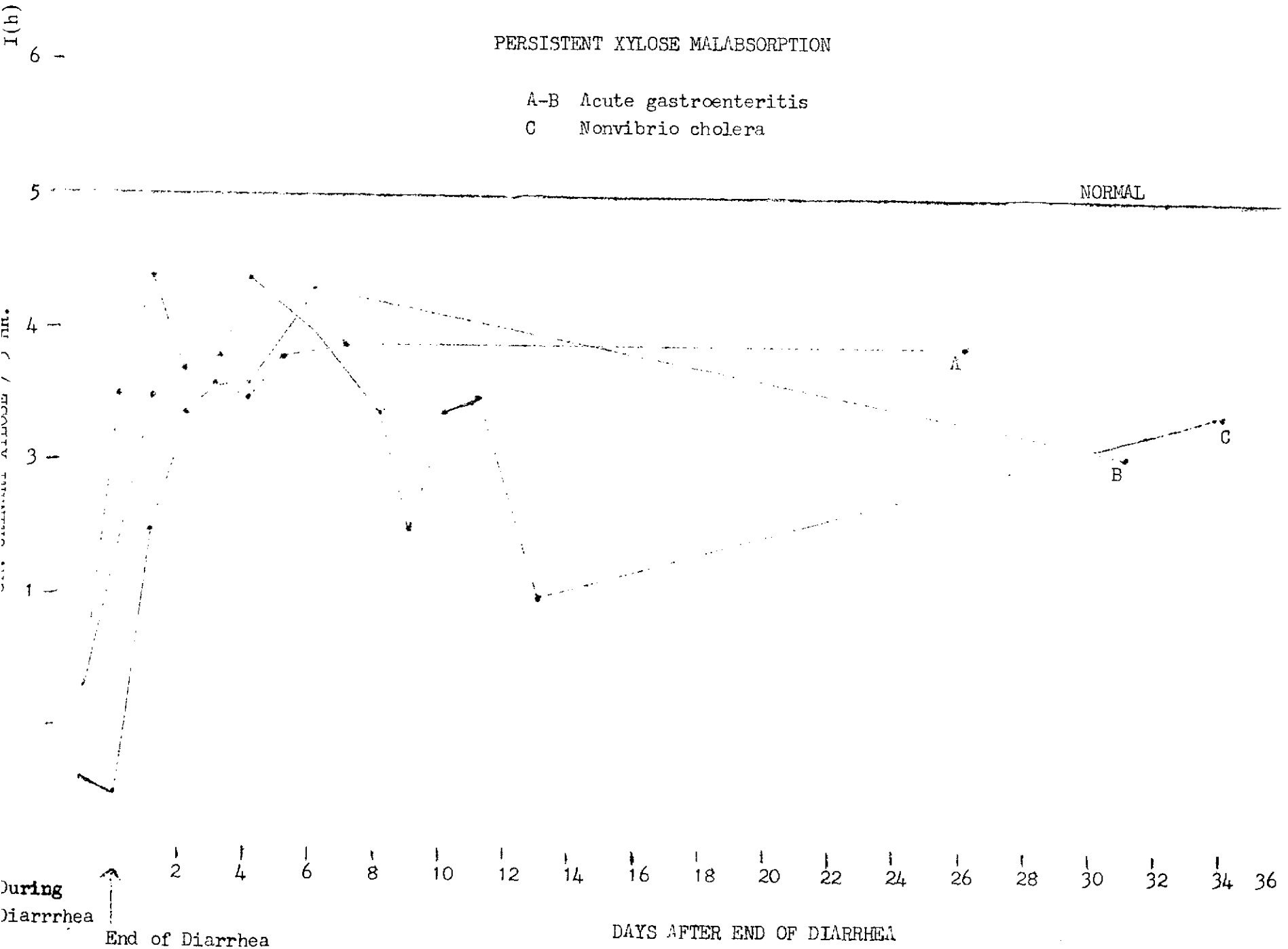


FIGURE 5

B_{12} MALABSORPTION WITH
RAPID RECOVERY

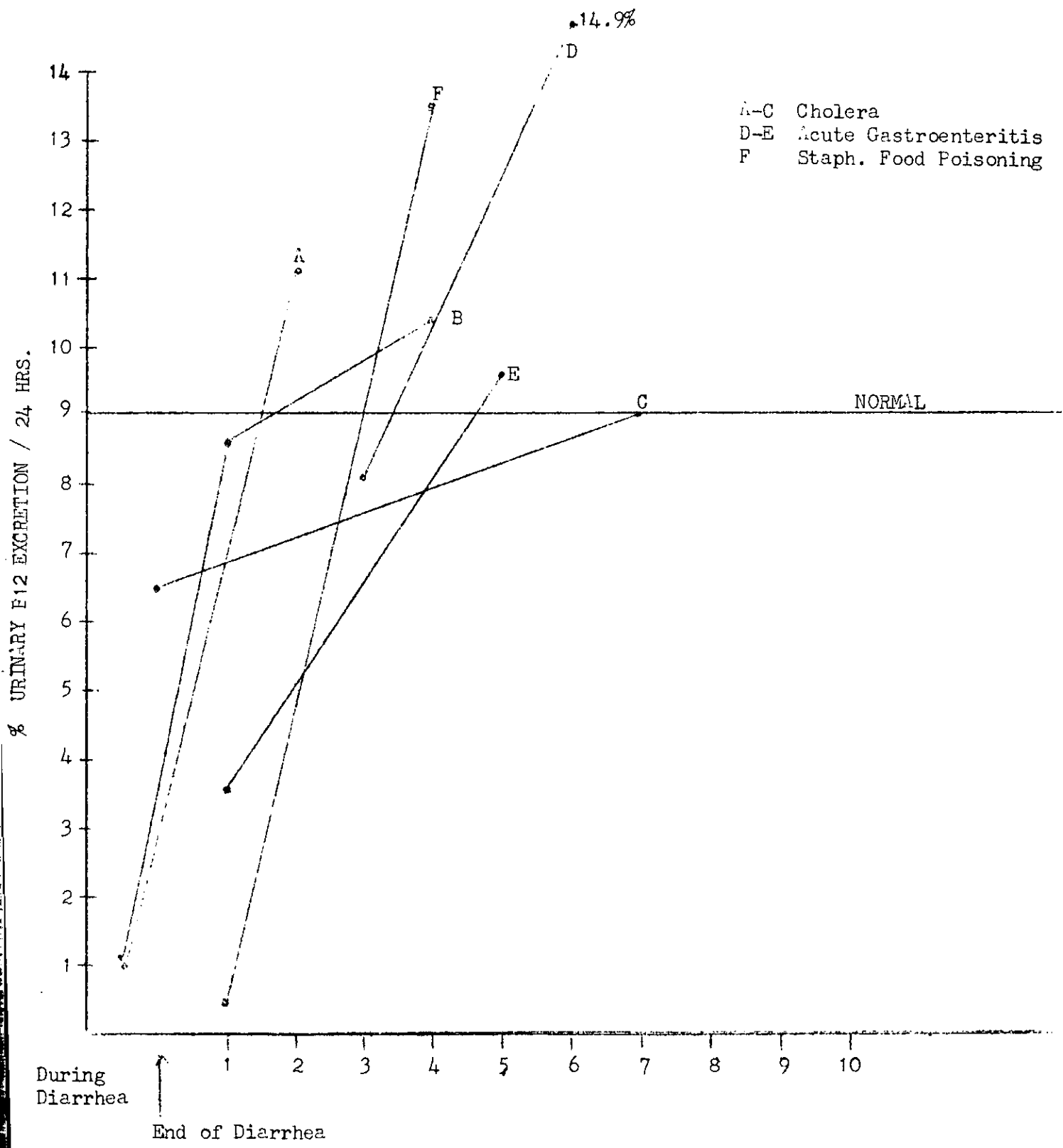
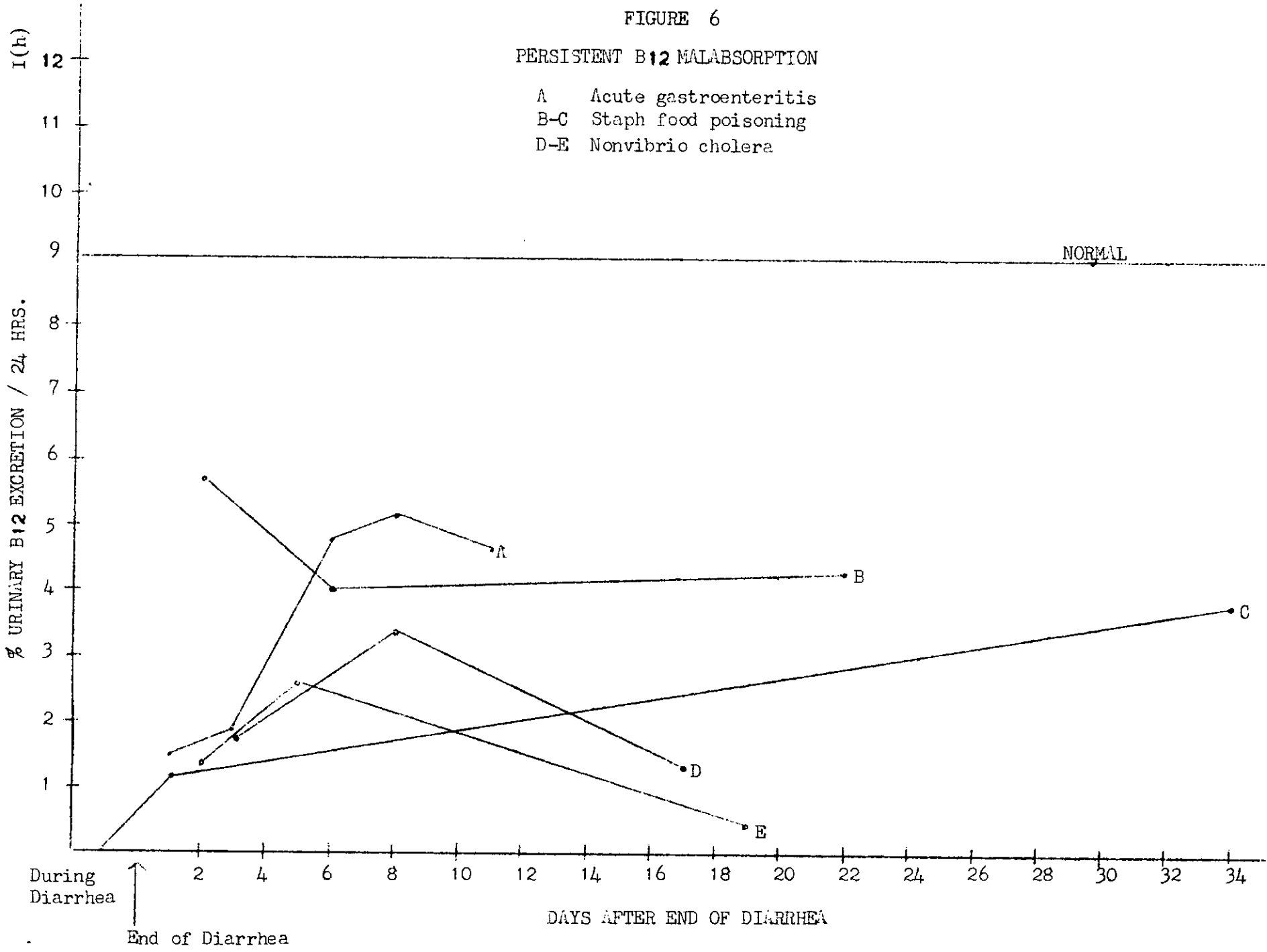


FIGURE 6

PERSISTENT B12 MALABSORPTION

A Acute gastroenteritis
B-C Staph food poisoning
D-E Nonvibrio cholera



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24-HOUR EXCHANGEABLE POTASSIUM CONTENT OF CHOLERA PATIENTS

by

Dr. R. S. Gordon & Mr. M.A. Quddus

In a report presented to the CRL Technical Committee in October, 1963, the hypothesis was advanced that potassium deficiency might exist among the population of East Pakistan, and that this should be investigated as a possible factor predisposing to cholera infection. It was suggested that potassium deficiency could develop as a result of excessive losses of potassium in sweat, and the consumption of a diet with a relatively low potassium content. Rice, the staple diet of East Pakistan, contains very little of this element. In a survey of electrolyte concentrations in urine specimens collected by the Nutrition Survey of Pakistan, it was found that 2½% of specimens showed potassium to creatinine ratios below 15 mEq/gm., suggesting that these individuals might in fact be conserving potassium maximally. The conclusion reached in the above-mentioned study was that potassium deficiency might actually exist in a segment of the population, but of course no relation between potassium deficiency and cholera susceptibility could be demonstrated.

In the present investigation, twenty-four hour exchangeable body potassium contents have been measured in a small group of cholera patients. Results are compared with those obtained in a similar series of patients admitted to the CRL ward with diarrhea, who on bacteriologic examination proved not to have true cholera. It was anticipated that a major potassium deficiency occurring in the cholera patients prior to onset of disease would be reflected in a lower exchangeable potassium content in this group.

Materials and Methods:

The isotope dilution procedure was done with potassium 42, supplied by the Indian Atomic Energy Authority reactor in Bombay. An order was placed for a weekly shipment of 2 millicuries of potassium 42 chloride (calibrated as of the time of arrival in Dacca on the Wednesday), dissolved in sterile isotonic sodium chloride solution. Each patient received a tracer dose of one to two hundred microcuries by either the oral or intravenous route. For the following 24 hours, all excreta were collected and assayed for radio-potassium. By this means it was possible to determine the fraction of the administered dose remaining in the patient at the end of the first 24 hour period. A urine specimen was then collected between the 24th and 28th hour, and the specific activity of potassium in this specimen determined. Assuming that this specimen indicates the specific activity of radio-potassium in the patient's body as a whole, it is then possible to calculate the total 24 hour exchangeable potassium.

The radio-isotope assays were carried out with liquid specimens of from 200 to 900 cc volume. These specimens were placed in screw-capped plastic bottles, and known aliquots of the standard radio-isotope solution were diluted to equal volume in identical bottles. These were then counted inside a lead shield over a three-quarter inch thallium activated sodium iodide crystal scintillation counter (Picker model no. 5817). Corrections for physical half-life were eliminated by concurrent counting of known standards and the unknown specimens. All count rates were high enough to reduce the statistical counting errors to 5% or less.

The patients for the study were selected primarily on the basis of availability. The short half life of potassium 42 made it imperative to perform

the study on patients on the ward on the day that the radio-isotope was received. Young children, and patients who might be uncooperative in the collection of excreta were necessarily excluded. Whenever possible, cholera patients who had been under treatment for only a short time were studied; however, it was never possible to realize the ideal of instituting the study at the time a patient was admitted. Patients being studied for potassium content were at the same time receiving standard CRL treatment for their disease. This treatment consisted of intravenous fluid replacement designed to maintain a physiologic balance between intake and output, and all received replacement fluids which averaged out to contain 10 to 13 milliequivalents of potassium per liter. Some also received tetracycline, but because the double blind tetracycline study is coded, it is not yet possible to specify which individuals received active drug.

Results:

The important data are collected in tables I and II. Table III presents a comparison of mean figures for the various groups. It may be seen that these data do not support the hypothesis that cholera patients are necessarily potassium deficient.

Discussion:

The present study should be considered as an exploratory investigation, and not as a definitive answer to the question. However, the results being negative, one does not feel strongly motivated to undertake the trouble and expense that would be necessary to obtain an unequivocal answer.

A serious drawback in the present investigation was the difficulty of obtaining the radioactive isotope. The K42 has a physical half life of only 12.5 hours, and must be used immediately on its receipt. Thus there was only one day per week in which potassium dilution studies might be initiated, and on many occasions, the shipment failed to arrive on schedule. Since cholera is itself an unpredictable and short lived disease, only coincidence could make a suitable cholera patient and a supply of active potassium 42 available at the same time.

It was never possible to institute the study immediately after the admission of an acute cholera case. The interval between onset of disease and the performance of the isotope study was as long as seven days in one instance, raising the question whether a potassium deficit which had existed previously might have been corrected before the study was done. In most of the cases however, the exchangeable potassium measurement was made on the first to fourth day after onset of disease, and before the patient was back to normal food intake. Therefore it is unlikely that a major potassium deficiency, occurring before onset of cholera in the typical case, would have been missed entirely.

It should be pointed out that there are inherent limitations in the measurement of total body potassium by the 24 hour isotopic exchange method. The results found by this procedure are always lower than total body potassium measured chemically, as a fraction of the body content of potassium is not free to exchange with the isotope in the short mixing period that is allowed (Veall and Vetter, 1958). The rapid decay of the isotope and radiation

safety considerations preclude the use of longer equilibration periods. A recent study (Flear, et al., 1963) indicates that the exchangeable potassium measurement is inherently subject to wide variations. They disapprove of the use of a single urine specimen for the determination of specific activity, but their work did not come to our attention until the present study was almost completed. If it is determined that the present line of investigation should be continued, it would be desirable to obtain several urine specimens on each case, and make repeated determinations of total potassium pool size.

Total exchangeable potassium content is, of course, related to body size, and the results are therefore expressed as a ratio to body weight. It is more difficult to allow for variations in body composition, but this will affect the normal value for exchangeable potassium. Most of the body potassium is in intracellular water, there being very little in fatty tissue, and much in the muscle mass. The differences in normal average potassium content between males and females, and the decline in potassium content with advancing age (Veall and Vetter, 1958), may be explained on this basis. Because the series of cholera patients whose potassium content was determined might well have been leaner, on the average, than the normal individuals on which the data quoted by Veall and Vetter (1958) were obtained, it was considered essential to measure the potassium content of a number of individuals, not affected by cholera, who would be otherwise comparable. Because of the need for quantitative collections of excreta, we chose hospital patients with non-cholera diarrhea rather than healthy controls. The fact that the average value found in this series agrees so closely with that quoted by Veall and Vetter (1958) is probably fortuitous, and a result of mild potassium depletion occurring as a consequence of diarrhea in a population which, by virtue of leanness, has a slightly higher initial normal level of 24 hour exchangeable potassium.

Since large amounts of potassium may be lost from the body in the diarrheal stools of cholera, the question arises whether or not potassium deficiency found in cholera patients might be solely a consequence of the illness, and not reflect an antecedent potassium deficiency. In the present context, this question is not of great importance, as in fact there is no evidence of potassium deficiency in the cholera-affected group. It should be noted, however, that these patients were maintained in approximate potassium equilibrium throughout their course of hospital treatment. Cholera stools in active cases have been found to contain 16-17 milliequivalents of potassium per liter on the average (Watten et al. 1960). Since our replacement solutions contain 10 or 13 milliequivalents of potassium, a cumulative deficit of approximately 5 milliequivalents per liter of fluid turnover might be anticipated. Considering the volumes of replacement fluid which had been administered prior to the potassium 42 study, this might have resulted in a total loss of up to 100 or even 150 milliequivalents of potassium, reducing the final value of potassium content per kilo body weight by at most 2 to 3 milliequivalents per kilogram. This effect would be a relatively small one compared to the variation observed within each series of patients. However, it should be noted that the lowest individual value for total exchangeable potassium was found in a severely ill female cholera patient whose intravenous fluid requirement up to the time of the study had already reached 36 liters. In her case, a potassium deficit incurred during the course of cholera may have been a significant factor. This uncertainty could

be avoided in a future study if careful balance measurements were made from the time of hospital admission until the time of the radio-isotope investigation. Such a procedure should be considered for the future in some centre having a more predictable supply of cholera patients, and readier access to supplies of the radio-isotope.

These data should not be taken to disprove the suggestion that potassium deficiency may occur in the population of East Pakistan. The earlier hypothesis related potassium deficits to losses in sweat, and the present study was carried out during the cool months of the year, when sweating is minimal. It should also be noted that the number of individuals studied was too small to have been expected to demonstrate a deficiency that might affect only a few percent of the total population. The effect of a hot environment on human potassium metabolism remains a subject requiring further research.

Summary and Conclusions:

The 24 hour exchangeable potassium has been measured in a series of 8 cholera cases and 9 patients suffering from non-cholera diarrhea. Potassium 42 was used as the tracer isotope, and the results expressed in milliequivalents of exchangeable potassium per kilogram body weight. The mean value for the series of cholera cases was 44, and for the non-cholera cases 42. These results offer no support for the hypothesis that a pre-existing potassium deficiency predisposes to cholera infection in man.

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14 March, 1964.

TABLE I

24-hr Exchangeable K in Cholera Patients

Case No.	Age	Sex	Weight kg.	Admission Plasma Protein (gm %)	Days Onset to K42 Study	Liters IV Fluid before K42 Study	Total K_E mEq	K_E mEq/kg.
442	30	M	39.9	11.4	3	12	1960	49.0
451	6	M	11.9	10.2	1	3	401	33.8
477	25	M	45.8	12.8	4	21	2200	48.0
480	25	M	51.1	11.6	3	5	2540	49.5
484	13	M	27.0	10.9	7	12	1339	49.5
493	30	M	46.4	13.2	3	11	2195	47.3
699	28	F	31.6	14.6	6	24	1330	42.2
711	25	F	41.8	12.4	2	36	1300	31.0
Averages	22.7		36.9	12.1	3.6	15.5	1658	43.8

TABLE II

24-hr Exchangeable K in Non-cholera Diarrhea

Case No.	Age	Sex	Weight kg.	Admission Plasma Protein (gm %)	Days Onset to K42 Study	Liters IV Fluid before K42 Study	Total K_E mEq.	K_E mEq/kg.
423	65	M	46.5	6.9	4	0	2200	47.3
693	60	M	44.1	13.2	5	6.5	1990	45.0
696	25	M	39.6	7.9	2	0	1500	38.0
706	50	F	36.9	11.0	5	5	1450	39.3
709	40	F	41.3	11.5	3	7	1410	34.2
713	70	M	32.9	8.5	2	10	1300	39.6
730	31	M	55.0	9.6	2	2	2600	47.5
732	45	M	45.4	12.0	2	5	2140	47.3
733	18	M	48.2	8.8	1	0	1900	39.4
Averages	44.9		43.3	9.9	2.8	3.9	1832	42.0

TABLE III

Mean Values of 24-hr. Exchangeable K.

Type of Patient	Mean K_E mEq/kg.	Std. Error of Mean
Cholera (this study)	43.8	2.6
Non-cholera (this study)	42.0	1.6
All males (this study)	44.7	1.4
All females (this study)	36.7	2.2
Normal males (V. & V. 1958)	"about 45"	---
Normal females (V. & V. 1958)	" about 35"	---

FURTHER DATA ON URINARY ELECTROLYTE EXCRETION IN EAST PAKISTAN

by
Dr. R.S. Gordon
&
Mr. M.A. Quddus.

A report on results of sodium and potassium analyses on urine specimens collected by the Nutrition Survey of Pakistan was prepared for the CRL Technical Committee in October, 1963 (q.v.). On the basis of ratios of electrolytes to creatinine in casual urine specimens, it was concluded that at least a fraction of the population might be relatively deficient in potassium, at least deficient enough to be sharply limiting their urinary output of this ion. The probable basis for deficiency was felt to be a diet low in potassium (rice contains very little K), combined with losses of potassium in the sweat. The question whether or not a potassium - deficient individual might be more susceptible to cholera infection was raised.

In the months since, the study has been continued. Four localities have been visited by the field teams of the Nutrition Survey, one of them (Jamalpur) having been studied previously. The results are attached, tabulated in the same form that was used previously, along with the earlier data for comparison purposes. In addition, a few more specimens were obtained from follow-up visits of former cholera patients, and are included.

The present results round out a full year of this study, and are the only ones to represent the cooler season of the year. It is interesting to note that the mean values of electrolyte excretion (both Na and K) found in these last four locations are higher than those recorded earlier. The frequency of potassium: creatinine ratios equal to or less than 30 is lower, and is markedly lower if the data from Khulna are omitted. As Khulna was studied in November, at the very beginning of the cooler season, it may be that these results still reflect the influence of heat. Jamalpur, a village which had been surveyed in August, 1963, was revisited in early March, 1964. There is a marked difference in electrolyte excretion values, with higher mean values of sodium and potassium excretion in March, and many fewer individuals showing low potassium output. These differences are statistically significant. The comparison is particularly useful, as the location was the same, and regional differences, as opposed to seasonal differences, are thus excluded.

The present data do not give cause for any important alteration in the interpretation of the earlier results. It still appears that potassium deficiency may exist, especially in the hot months. Further study of this possibility appears indicated, quite apart from its possible relationship with the etiology of cholera. This might best be implemented by a search for electrocardiographic abnormalities indicative of hypokalemia during the hot season, and serum potassium analyses on suitably selected and collected samples. If hypokalemic individuals are found, they should be checked medically for chronic diseases that might cause the condition and their response to dietary potassium supplements documented.

Whether or not potassium deficiency predisposes to cholera infection in man is still an open question. In a small series of patients tested during the cool months of 1963-1964, we could find no marked abnormality of 24-hour changeable potassium content in either cholera or non-cholera diarrhea patients. The study did make two things clear - that a flagrant potassium deficiency demonstrable by that method is not a necessary prerequisite for cholera infection, and that K

is difficult to procure and use in Dacca. Further study of this important question of the relationship of potassium nutriture to susceptibility to cholera (and perhaps to other diarrheal diseases of similar nature) is in order.

2 April, 1964.

R.S. Gordon, Jr., M.D.
M.A. Quddus, M. Sc.

Electrolyte excretion (mEq. per gm. creatinine) in East Pakistan, 1963-64

Number of Samples	Location	Dates of Survey	POTASSIUM				SODIUM			
			Mean	Std.Error of Mean.	% Samples 30 or less	% Samples 15 or less	Mean	Std.Error of Mean	% Samples 20 or less	% Samples 300 or more
18	Dinajpur	16-23 April	58.4	15.5	28	6	241	32	0	28
82	Comilla	16-22 May	72.5	4.1	10	1	185	13	0	10
53	Faridpur	10-15 June	89.1	8.2	4	0	263	19	0	30
64	Bogra	26-30 June	66.0	5.4	14	0	239	17	0	30
85	Pabna	3-8 July	55.5	4.5	25	4	158	9.5	1	9
78	Jessore	25-31 July	63.0	3.9	9	1	210	17	0	13
77	Jamalpur	23-29 Aug.	44.1*	3.7	38 **	5	222	25	0	26
72	Kushtia	19-25 Sept.	56.9	4.3	22	6	244	30	1	32
79	Khulna	16-20 Nov.	47.9	3.4	28	4	235	17	0	24
49	Rangamati	19-23 Dec,	97.7	14.3	8	0	273	22	0	37
58	Pabna	11-16 Jan.	87.2	6.6	7	0	583	24	0	43
45	Jamalpur	3-6 Mar.	72.8*	5.2	7 **	0	318	27	0	47
82	Parpara	1-4 Mar.'64	65.8	7.2	10	0	219	24	1	24
134	Azampur	10-13 Mar.	54.9	5.4	16	1	215	19	0	20
104	Fulbaria	18-21 Mar.	52.7	6.5	20	0	208	23	0	14
235	Sylhet	24-28 March	57.3	2.1	21	2	358	21	0	49
236	Comilla	4-7 April	43.0	2.7	37	9	231	17	0	24
321	Chittagong	10-13 April	40.7	1.4	36	7	186	13	0	13
529	All Locations	Apr. - Sept. 1963	62.5***	1.9	18	2.4	214	5.7	0.4	21
231	All Locations	Nov.- March. 1964	73.2***	1.3	14	1	279	3.5	0	36
1129	All Locations	March-April.	52.4	4.2	23	3.1	235	18	0.3	24
37	Post-cholera	July-Sept.	53.2	7.9	35	8	268	58	0	32
14	Post-cholera	Sept.-Nov.	61.6	15	29	7	370	59	0	57

* These values differ significantly ($t = 4.5$; $p < .001$).

** These values differ significantly ($\chi = 14$; $p < .001$).

*** These

SERUM VITAMIN B12 AND FOLATE LEVELS IN PATIENTS WITH CHOLERA

Previous studies at the Cholera Research Laboratory (cf. "Nutritional status of cholera patients", Report to the Technical Advisory Committee, 1963) failed to reveal nutritional deficiency of thiamine, vitamin C, or riboflavin in the majority of patients with infection with V. cholerae. In order to obtain further evidence of the presence or absence of nutritional deficiency as a possible predisposing factor to cholera, it was decided to evaluate vitamin B12 and folic acid nutriture in a group of patients admitted to the C.R.L. Deficiency of one or the other of these factors is commonly associated with gastrointestinal disturbances. In addition, it has been reported that monkeys fed a folic-acid deficient diet have a greater susceptibility to bacillary dysentery than control animals (Janota and Dack, 1939).

Vitamin B12 - Serum vitamin B12 levels were determined by microbiological assay with Lactobacillus leichmanii, according to the method of Spray (Spray, 1955) in 46 patients with acute diarrheal disease due to V. cholerae. Serum was obtained during the first week after admission, usually on the second hospital day, after rehydration had been accomplished, but before vitamin B12 containing foods had been added to the diet. The normal range in this laboratory, based on healthy American and Pakistani staff personnel, is 180 to 900 micromicrogm. per milliliter. Serum levels of this vitamin have been shown to correlate well with overt deficiency in a variety of clinical situations, and to become abnormal before tissue stores are exhausted or anemia and symptoms appear (Spray, 1962).

Two out of the 46 patients had subnormal levels. One of these was a woman in the third trimester of pregnancy; the other, a 20 year old male with a subclinical chronic malabsorption syndrome. Of the remaining 44 patients, B12 levels were normal in 41 and elevated in 3.

The reason for the elevated B12 levels in the latter 3 patients, all of whom were well hydrated at the time serum was obtained, was not readily apparent. Pre-existing liver disease was not suspected in any. However, since the patients were admitted in circulatory collapse, B12 levels may have become elevated due to hepatic necrosis (as is the rule in patients with infectious hepatitis).

In 15 patients admitted with acute diarrheal disease in whom no pathogen was isolated from the stools, the B12 levels were normal in all.

It would appear, therefore, that vitamin B12 deficiency is uncommon in patients with diarrheal disease in Dacca. This is in accord with the province-wide findings of the Nutrition Survey of East Pakistan, where B12 deficiency was an uncommon finding (Reiner, et al, 1964). It is unlikely that either dietary lack of B12, or disease of the ileum interfering with its absorption, is a factor in the development of cholera in a significant number of cases.

Folic acid - Serum folate levels were measured by microbiologic assay with Lactobacillus casei, according to a modification (Waters and Mollin, 1961)

of the method of Baker and his colleagues (Baker, et al, 1959). Serum folate levels have been shown to correlate well with clinical folic acid deficiency (Herbert, et al, 1960; Waters and Mollin, 1961; Cooper and Lowenstein, 1961; Klipstein, 1963). In normal volunteers on diets deficient in folic acid, serum folate levels become subnormal before hematologic abnormalities make their appearance (Herbert, 1962a and 1962b). In this laboratory, the assay has proved reliable and reproducible, though a wider range of normal (3.5 to 25 millimicrogm. per ml.) has been found than in other laboratories. Patients with megaloblastic anemias due to folic acid deficiency (associated with normal serum B12 levels) were found to have folate levels below 3 millimicrogm. per ml.

Sera from 73 patients with acute diarrheal disease due to V. cholerae and 43 patients with acute diarrhea from whom pathogenic organisms were not isolated, were obtained during the first hospital week, after rehydration had been accomplished, and were assayed for folate activity. The groups were comparable as to age, sex, body weight, convalescent hemoglobin and convalescent total plasma protein.

Twelve out of 73 cholera patients (16%) and 17 out of 43 patients with non-cholera diarrhea (40%) had subnormal folate levels. The mean folate level for the cholera group was 7.8 millimicrogm. per cent, and for the non-cholera group 9.8 millimicrogm. per cent. When the patients with low folates were compared with those with normal levels, there were no significant differences in age, body weight, convalescent hemoglobin and convalescent plasma protein levels in either the cholera or non-cholera groups. Peripheral blood smears from 25 of the 29 patients with low folate levels were available for review (by an observer unaware of the folate levels) for evidence of folate deficiency (hypersegmented neutrophils with or without macrocytes). In 11 patients these abnormalities were found, though usually of mild degree.

It would appear then that in a minority of patients with diarrheal disease in our population, folic acid deficiency is present. The incidence of deficiency appeared to be somewhat higher in the non-cholera than in the cholera group; the difference was statistically significant ($p < 0.01$). These findings parallel those found with regard to ascorbic acid deficiency in our patient population (cf. Report to the Technical Advisory Committee, 1963). This is not surprising, as dietary deficiency of folic acid is frequently associated with deficiency of Vitamin C. As with Vitamin C, it does not appear that folate deficiency is a predisposing factor to cholera, at least in the majority of cases.

Whether or not serum folate levels may fall during the course of acute intestinal infections (as, for example, serum iron levels drop during a variety of infectious diseases during which body iron stores are not depleted) is unknown. To resolve this question, serial studies of folate levels in selected patients with various acute enteritides are planned.

Summary - Subnormal serum vitamin B12 levels were found in 2 (4%) of 43 patients with cholera; subnormal serum folate levels were present in

12 (16%) of 73 cholera patients. These findings indicate that deficiency of neither of these vitamins is a major predisposing factor to cholera in East Pakistan.

A higher incidence of subnormal serum folate levels was found in patients with diarrhea due to no known pathogens than in those with cholera.

* * *

The technical assistance of Mrs. Shiela Wickenden in performing the microbiologic assays is gratefully acknowledged.

John Lindenbaum, M.D.
7 September, 1964.

JL/raf

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Digitalis-like Activity in the Stool of Patients and Experimental Animals Infected with Vibrio cholerae.

by
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and
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Introduction:

Over the years various investigators have published reports concerning extracts of cholera vibrios which have been toxic when injected into experimental animals. Recently, interest in this field has been stimulated by two kinds of results in which the reported "toxic" activity appeared to be possibly related to the pathogenesis of the profuse diarrhea which is characteristic of the human disease. Oza and Dutta (1963) showed that the oral administration to suckling rabbits of a toxin prepared by ultrasonic disintegration of cholera vibrios produced a syndrome similar to clinical cholera, characterized by diarrhea, hypothermia and death. The syndrome was also produced by vibrio-free Seitz-filtered stools from 7 out of 12 patients with cholera (Panse & Dutta, 1961). Huber and Phillips (1962) reported that stools of patients with cholera were capable of markedly decreasing the short circuit current of isolated frog skin, and concluded from these results that there was a material in cholera stool that could inhibit the active transport of sodium by the skin. Fuhrman et al. (1962) found that extracts of some, but by no means all, preparations of dried cholera culture filtrate inhibited short circuit current of frog skin and toad bladder. Both sets of investigators working on the frog skin suggested that the inhibition of sodium transport might be the cause of the diarrhea of cholera. Reports on the chemical nature of the toxin have been fragmentary and in certain respects conflicting. It is of interest that the choleragenic material studied by Dutta is inactivated by pancreatin, phosphorylase and lipase, but not by trypsin or pepsin (Dutta & Oza 1963).

Further studies on cholera toxin appeared worthwhile for several reasons. First, the nature of the action of the toxin on in vitro systems requires extension: how fast does the toxin act, is the effect reversible, have the observed results been a specific inhibition of sodium transport or a more general tissue damage? Second, what is the range of tissue specificity? Can cholera toxin be absorbed and affect heart, skeletal muscle or red cells, or is its action limited to the intestinal mucosa? Third, what bioassay system would be most suitable for purifying the toxin and determining its chemical nature?

Methods:

A number of assay systems were considered before the trip to the CRL in Dacca was undertaken. The isolated toad bladder was tried in a number of experiments, but has the disadvantage that stability of short circuit current is not characteristic of the preparation. Frog skin is more stable, but the same general problem exists. It was decided to set up the isolated frog heart system of Hajdu (1957) in Dacca, with the intention of checking any material that had digitalis-like activity on the frog heart (and therefore presumably sodium-transport inhibiting activity) for its capacity to produce cholera in the suckling rabbit or inhibition of salt and water transport in an isolated

intestinal loop. In Hajdu's assay procedure a distinction between digitalis-like activity and positive inotropic effects caused by a large variety of compounds like adrenalin and bile salts is made on the basis of the staircase phenomenon demonstrated by frog heart. The only substances hitherto known that cause the heart to maintain maximal tension at frequencies of contraction below 3 per minute are the cardiac glycosides, the lysolecithin isolated by Hajdu et al, (1957), and the recently described plasma protein system called cardioglobulin (Hajdu and Leonard, 1958).

Stools were collected and prepared for assay as outlined in the tables. Because the potassium concentration in cholera stools is generally considerably higher than that in Ringer-Conway solution, the stool was either diluted with potassium-free Ringer or dialyzed for thirty minutes against cold Ringer solution. This time was sufficient for the potassium concentration to approach an acceptable level for the assay. Whether a part of the digitalis-like material diffused across the cellophane during this period of time is not known.

Results:

Table I is a summary of experiments with human cholera stool. The small number of stools tested was due to the fact that only three patients with cholera were admitted to the CRL hospital during the period covered by this report. Two out of five cholera stools had digitalis-like activity. Since activity probably often declines rapidly on standing, it is likely that 3 out of the 5 stools assayed were not collected under optimal conditions (standing overnight in collection bucket, or slow freezing of a large volume).

Table II shows the results with experimental cholera. In two attempts with adult rats no sign of intestinal fluid accumulation was noted at the end of 6 hours after inoculation with cholera vibrios. Accumulation of voluminous amounts of fluid in small intestine, cecum or colon was observed in all trials with rabbits. Digitalis-like activity was detected in three out of five rabbits under 19 days of age, and in one out of 4 rabbits aged 25 days. Fluid from a 10 day old rabbit still showed activity after 1.5 hours standing in the cold. Activity in the fluid collected from animal number 9 was shown to survive quick freezing followed by thawing after one or two days at minus 20 degrees. It also survived immersion in a boiling water bath for a period of 8 minutes, after which the activity did not disappear during a period of 3.5 hours at room temperature. Table III is a summary of the characteristics of the digitalis-like activity, based on a limited experience with material from both experimental animal and human sources.

No such activity could be detected in cholera cultures grown in T_1N_1 media, nor was any found in a preparation enriched by addition of suckling rabbit small intestine. One confusing experiment with egg-yolk enriched T_1N_1 gave digitalis-like activity both with and without cholera vibrios, possibly due to lecithin or lysolecithin content of the large amount of egg yolk in the medium (Hajdu et al, 1957).

Discussion:

Because of the paucity of human material during the period covered by this report, attempts were made to produce cholera in experimental animals.

Intraintestinal inoculation of vibrios into suckling rabbits according to the method described by Dutta and Habbu (1955) invariably produced a cholera-like syndrome. Similar results were obtained with 4 older rabbits (25 days) in which fasting and ligation of lower duodenum or upper jejunum were added in order to prevent biliary and pancreatic secretions from reaching the inoculation site. No cholera-like syndrome was produced in two rats within 6 hours of inoculation. It is of interest that the rat is relatively insensitive to the action of cardiac glycosides. Fully adult rabbits and other experimental animals will be studied in the future.

The results show that digitalis-like activity could be demonstrated in fewer than half of the cholera stools examined. It should be remembered that Panse and Dutta (1961) found choleragenic activity for suckling rabbits in only 7 out of 12 stools from patients with cholera. If one assumes that the digitalis-like activity is related to pathogenesis of cholera, why is it not invariably present? It appears that the toxin is readily destroyed, possibly by intestinal enzymes. Support for this view is provided by experiments with purified intestinal enzymes as well as extracts from the intestines of adult rabbits (Dutta and Oza 1963). It is also consistent with our findings of lability at room temperature, but survival (one experiment) at 100 degrees, conditions that might be expected to inactivate many intestinal enzymes. Experiments with frog heart suggest that the toxin becomes bound rather strongly to tissue. If the same phenomenon occurs at the surface of the intestinal mucosa, it is not hard to imagine the toxin being produced by the vibrios at the mucosal surface, followed by binding which would allow the effect of the toxin to persist in the presence of a voluminous diarrhea that might otherwise be expected to wash out the pathogenic material. According to this view, the demonstration of toxin in the stool might well be fortuitous, depending on the following factors: rate of production by organism, fraction bound to intestinal surface, rate of destruction by intestinal enzymes, degree of dilution by intestinal fluids.

Finally, one should raise the question whether the digitalis-like activity is produced de novo by the organism, or whether the organism releases an enzyme which in turn acts on a host substrate to produce the toxin. The cholera vibrio is a rich source of many enzymes, including ones that can attack lecithin at several sites (Felsenfeld 1944). In general, cholera cultures grown in simple media have not contained toxins that inhibit active transport of sodium in an in vitro system. Although the work of Fuhrman et al. (1962) with the commercially available Phillips-Duphar vibrio extract is an exception to this rule, those investigators found many Phillips-Duphar extracts without activity and they list in addition nothing but negative results obtained with extracts of other cholera cultures from various sources. On the other hand, the fact that we have been able to produce a cholera-like picture and demonstrate toxin in a 25 day old rabbit fasted for three days, with upper jejunum ligated, suggests that an exogenous source of substrate is not required. The choice, then, lies between pre-formed cholera vibrio toxin or a vibrio enzyme capable of acting on a substrate that is normally present in the intestine.

Recommendations for Future Research:

1. At CRL: assay of cholera and non-cholera stools from human patients. Attempts to stabilize activity for assay purposes by a simple, preferably one step procedure. This can perhaps be done by heating, possibly

by solvent extraction. Preparation of large amounts of crude material may be a later requirement.

2. At NIH: experimental production of the digitalis-like material and investigations of its chemical nature and mechanism of action.

Edward J. Leonard, M.D.
J. R. Shaha, M.Sc.
20 October - 7 December, 1963.

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Table I

Effect of Human Stool on Frog Heart

Exp. No.	Stool Sample from Case No.	Fresh or Frozen	Diluted or Dialyzed	Other Conditions	Digitalis-like Activity	Comment
a		frozen	2x dil.	-	+	-
b	295* (stored frozen)	frozen	2x dil.	70 min room temp.	0	Same day as 1a.
c		frozen 2x	2x dil.	3 hr 4°C.	0	Same material as 1a, kept cold, refrozen, re-thawed.
d		frozen 2x	30 min dial.	" "	0	
	313* (stored frozen)	frozen	2x dil.	-	0	-
a		fresh	2x dil.	-	●	-
b	418*	fresh	dial.	-	+	-
c		frozen	dial.	-	+	less active than in 3b.
	422	fresh	dial.	-	0	non-cholera diarrhea.
	Mitford*	fresh	dial.	-	0	Stool collected in bucket, had stood at room temp up to 10 hrs
	429*	fresh	dial.	-	0	-

* Confirmed cholera.

Summary:

5 cholera stools, two positive (one fresh, one stored frozen).
1 non-cholera stool, negative.

Experiments 1 (b, c, and d) and 3c suggest activity may decline rapidly, therefore experiment 5 may not be valid.

Table II

Experimental Cholera

Exp.	Animal	Age	Fasting	Other Cond.	Time of Sacrifice	Digitalis-like Activity	Comment
1	rat	adult	2 days	duod. tie	6 hrs.	0	no fluid accum.
2	rat	adult	3 days	duod. tie	6 hrs.	0	" " "
3	rabbit	18 d.	0	-	6 hrs.	+	
4	rabbit	16 d.	0	-	7 hrs.	0	
5	rabbit	11 d.	0	duod. tie	about 6 hrs.	0	
6	rabbit	25 d.	2 days	duod. tie	6 hrs.	+	
7	rabbit	25 d.	2 days	duod. tie	6 hrs.	0	
8	rabbit	10 d.	0	-	4.5 hrs.	+	still active after 1.5 hrs. in cold.
9	rabbit	13 d.	0	-	5 hrs.	+	
10	rabbit	25 d.	3 days	duod. tie	6 hrs.	0	
11	rabbit	25 d.	3 days	duod. tie	6 hrs.	0	

Summary:

2 rats: cholera 0, toxin 0

5 rabbits under 19 days: cholera 5, toxin 3

4 rabbits 25 days, duodenum tied: cholera 4, toxin 1

Table III

Characteristics of Toxin (Preliminary - some results based on one experiment)

1. Acts rapidly (within one or two minutes) on addition to frog heart.
2. Washes out slowly; therefore probably becomes fixed to tissue.
3. Unstable on standing in cholera stool - loss of activity within an hour at room temperature.
4. Survives quick freezing and thawing.
5. Detectable activity after 30 minutes dialysis in cold.
6. Survived 8 minutes at 100 degrees centigrade under carbon dioxide.

Table IV

Digitalis-like Activity of Cholera Cultures

1. T_1N_1 broth, overnight growth of cholera vibrios: no activity.
2. " " " " " " " : no activity.
3. T_1N_1 broth, overnight growth of cholera vibrios, followed by incubation with longitudinally cut suckling rabbit small intestine: no activity.
4. 50 ml. T_1N_1 broth plus 1 egg yolk, overnight growth: digitalis-like activity which washed out rapidly, and similar activity with egg yolk plus T_1N_1 alone.

Summary:

No digitalis-like activity related to growth of cholera vibrios under the above conditions.

The Effect of Cholera Stool and Culture Filtrates on the Skin of Guinea Pigs and Rabbits

by

John P. Craig, M. D.
with the technical assistance of
Russell Faust

The initial aim of the present study was to determine whether the intracutaneous injection of sterile filtrates of stools from bacteriologically-confirmed cholera cases would evoke a skin response distinguishable from that produced by non-cholera stools. The absence of skin lesions in clinical cholera certainly does not preclude the possibility that the noxa responsible for gut damage could also have a deleterious effect upon skin provided it is applied to skin in sufficient concentration. Many examples of this kind exist among the bacterial toxins. The alpha-toxins of Clostridium oedematiens, and Cl. septicum, and the alpha and iota-toxins of Cl. welchii cause an increase in skin capillary permeability. (Elder and Miles, 1957; Craig and Miles, 1961). Diphtheria toxin and the alpha, beta, epsilon and iota-toxins of Cl. welchii all produce erythema and necrosis following intracutaneous injection. (Oakley and Warrack, 1953). Yet in natural infection with these organisms, skin damage is never primary, and usually absent, while the major lesions are found in various viscera. Indeed, infection with the strains of Cl. welchii which produce beta and epsilon-toxins are often associated with diarrhea. (Wilson and Miles, 5th Ed.). Although skin reactions to various components and products of cholera vibrios grown in vitro have been described, little has been reported concerning the skin response to cholera stools themselves. Basu Mallik and Ganguli (1964) have shown that intracutaneous injection of cholera stool filtrates evokes an immediate increase in permeability of skin capillaries in the rabbit, as demonstrated by intravenous dye technique. Similar activity was demonstrated to a lesser extent, however, in non-cholera stools.

If cholera diarrhea is associated with a soluble substance elaborated or evoked in the host by cholera vibrios, it is likely to be detectable in rice-water stools. Moreover, if such a substance exerts any effect on any of the skin elements, it should first be sought in the stools themselves. Search for substances with similar activity derived from vibrios in culture would logically follow.

METHODS

Stools from cases admitted to the Pakistan-SEATO Cholera Research Laboratory were refrigerated immediately after collection and processed as follows: liquid stools were centrifuged at 10,000 rpm at 5° C for 30 minutes and the supernatant fluids were passed through 45 mμ millipore filters in Swinney syringe adapters. Formed stools were diluted 2-fold to 10-fold in sterile saline before centrifugation. Most filtrates were frozen and stored at -20° C, but after it was found that skin-reactive

factor was equally stable in the thawed state, some filtrates were stored in an ordinary refrigerator at 5-8° C. For all specimens the following data were obtained: age and sex of patient; interval between onset of diarrhoa and collection of specimen ; bacteriologic results of initial culture on SS and MacConkey agar plates and daily cultures on gelatin-agar and bile-tellurite-gelatin agar plates (Monsur) and in bile-peptone enrichment broth; severity of disease as measured by presence or absence of pulse on admission, total diarrheal stool volume and admission plasma protein content.

Materials were injected intracutaneously in 0.1 ml volumes in partially-randomized sites on the backs of albino guinea pigs or rabbits. Guinea pigs were prepared by clipping and depilating with barium sulfide paste, while rabbits were clipped only. Guinea pigs were given 24 injections in 6 rows of 4 each; rabbits usually accommodated 60-70 injections. Sites were coded so that readings were made without knowledge of what test material had been injected at any given location. For initial screening, specimens were diluted 1:20 in sterile saline and, if no reaction ensued, were subsequently injected at 1:5 dilution.

RESULTS

It was noted early that stools from some, but not all, bacteriologically confirmed cholera cases produced marked induration and erythema beginning 6 to 8 hrs after injection, reaching a peak at 18-24 hrs, receding somewhat by 48 hrs, but persisting for 4 to 5 days, in both guinea pigs and rabbits. Transient erythema without induration was noted with some non-cholera stools. By replicate titration of "positive" stools in both guinea pigs and rabbits, it was found that the diameter of induration and erythema bore a linear relationship to log dose of filtrate, over a range of 6 to 15 mm. The amount of filtrate in a 0.1 ml volume capable of producing induration of 8 mm in diameter was established as an arbitrary "induration dose" in order roughly to quantitate the activity of the other stool samples to be tested. Induration was selected because it was subject to less variation than erythema. Intensity of induration was estimated from + to ++++ but, since it appeared to be directly related to lesion diameter, it has not been incorporated in the measurements of activity reported here.

The results of the first 56 stools tested in guinea pigs are shown in Table 1. All specimens were tested in at least two animals simultaneously and most were repeated in other sets of animals on different days. The active cholera stools were employed repeatedly for investigation of the properties of the active substance described below. Most of the same stools were also tested in duplicate in rabbits and results were essentially identical. It is of special interest that no activity was present in any of the stools from the 16 patients with the clinical syndrome of "non-vibrio cholera", i.e., watery diarrhea associated with dehydration and collapse, from which V. cholerae was not isolated (Lindenbaum et al). In the cases of early cholera thus far tested, there is no detectable correlation between skin activity titer and severity of illness or time of stool

collection. From three patients with confirmed cholera, stools were collected in the acute stage and again after recovery. In one case, both early and late stools were negative. In the other two, stools collected on the first day were strongly positive (>100 induration doses/ml), whereas stools collected 57 and 68 days after onset were negative (< 5 induration doses/ml). Serial daily stools are now being collected from both cholera and non-cholera diarrhea cases in order to determine the time of appearance and disappearance of skin activity during the course of illness, and to ascertain whether or not similar activity can be found in stools from cases in which vibrios were not isolated.

The effect of stool filtrates on skin capillary permeability was investigated by intravenous injection of Pontamine Sky Blue 6XB, 0.12 ml of a 5% solution/100 g., at varying times after the intracutaneous injection of test materials. Preliminary experiments showed that filtrates capable of evoking induration also caused an increase in skin capillary permeability of at least 24 hours duration, and that the diameter of the blue lesion also bore a linear relationship to log dose of filtrate, within the range of 5-12 mm. The time-course of increased permeability was determined by injecting filtrates at various intervals followed by intravenous injection of dye when the skin was bearing lesions of different ages. A final injection of filtrate and of saline was given immediately after the dye to determine the immediate permeability effect as well as the effect of trauma. Readings were made 1 hr after the injection of dye. Physiologic saline injected into a guinea pig with dye in the circulation evokes a 2-4 mm lesion of increased permeability due to trauma alone. Within 30 minutes after injection of saline, permeability has returned to normal. A graph of the time-course of both permeability-effect and induration evoked by 0.1 ml of a 1:20 dilution of an Ogawa rice-water stool is shown in the upper two curves of Figure 1. The graph represents a composite of six experiments in sets of 3 to 6 guinea pigs each. Although there was minor inter-animal variation in the diameter and intensity of the mature 18-24 hour lesions the chronology of permeability events was consistent. Immediately after injection there was a marked increase in permeability of skin capillaries followed by recovery within one hour. During the next few hours the permeability gradually increased reaching the maximum diameter, but not maximum intensity, by 8 hours. The blueing became more intense between 8 and 18 hours and was maximum at 18 to 24 hours. Between 24 and 48 hours intensity faded. Induration developed more gradually during the first 24 hours, but the peak was the same as the peak of blueing. Lesions older than 48 hours have not been carefully studied, but permeability appeared to return to normal by 72 to 96 hours, whereas induration gradually receded over a 4 to 5 day period. Residual induration was palpable at 6 to 7 days.

The time-course of permeability and induration in rabbit skin was essentially the same as that in guinea pigs, but because of the irregularity of hair growth and difficulty of depilation of rabbits, lesions proved to be less reproducible than in guinea pigs. On the basis of time-course studies, 18 hour lesions were selected for the study of other properties of the stool permeability factor (PF). In all stool filtrates

thus far studied, increased capillary permeability at 18 hours has paralleled the induration response.

IN VITRO PRODUCTION OF SKIN-REACTIVE SUBSTANCE

Preliminary attempts to demonstrate skin activity in culture filtrates of V. cholerae grown in tubes of brain-heart infusion, tryptose, trypticase soy, and thioglycollate broth met with failure. Likewise, suspensions of living vibrios injected intracutaneously yielded no induration, erythema or increased capillary permeability greater than that evoked by medium controls. On the other hand, filtrates of V. cholerae cultures grown in 5% Difco Bacto-Peptone®, pH 7.3, as employed by De et al for the production of cholera enterotoxin (1960, 1962) evoked skin lesions indistinguishable from those produced by the skin-factor found in cholera stools.

De et al (1962) showed that the in vitro production of cholera enterotoxin active in the ligated rabbit-gut segment were related to surface/volume ratio of the culture medium. Maximum titers were obtained with a free surface of 1.25 to 2.50 sq cm/ml of medium. This association appears to hold true also in the case of the skin-reactive factor. Figure 2 shows the relative content of guinea pig PF in cultures grown in the same batch of medium under three conditions of surface/volume ratio in Erlenmeyer flasks (20 ml, 2.5:1) Kolle flasks (85 ml, 1.9:1) and test tubes (20 ml, 0.2:1). Shallow cultures yielded approximately 30 times as much active material as did tube cultures.

The influence of time of incubation on skin-factor production was investigated by titrating PF content of filtrates of two strains of V. cholerae grown in shallow cultures in Erlenmeyer flasks (surface/volume ratio of 2.5) for different periods of time at 37° C. The time-course of PF production is shown in Figure 3. Titers did not reach their maximum until 24-48 hours. According to De et al (1962) the titer of cholera enterotoxin, prepared under similar conditions, reached its maximum at 6-12 hours. Parallel titrations of the same preparation in the guinea pig skin and ligated-gut segments are obviously indicated.

The skin reactions evoked by stool and culture filtrates were compared by determining both dosage-response and time-course of induration and increased capillary permeability in parallel tests on the same sets of guinea pigs. For these determinations an active stool filtrate (CRL hospital patient 531) was compared with a filtrate of a 24 hour 5% Bacto-Peptone culture of the Ogawa strain (B 1307) isolated from the same case. Dosage-response curves of induration, erythema and PF of the two preparations were identical. Time-course of PF activity of both stool and culture filtrates in the same animals displayed the same sequence of events as shown for CRL 531 stool filtrate in Figure 1. Thus, the skin responses evoked by stool and culture filtrates were indistinguishable qualitatively and quantitatively, suggesting that the two materials are identical.

In order to determine whether substances with similar effect on skin were elaborated by other enteric organisms, the following strains were

grown simultaneously for 24 hours in 20 ml of 5% Bacto-Peptone, pH 7.3, in 250 ml Erlenmeyer flasks; Shigella B (B37893); Enteropathogenic E. coli serotype 026 (B12282); E. coli, non-typable; non-agglutinable vibrio, Heiberg Group II, (B14051) isolated from a case of diarrhea; and V.cholerae Ogawa (B1307). Although medium control as well as filtrates produced a transient erythema and edema during the first few hours, reading at the routine interval of 18 hours after injection revealed that no induration or change in capillary permeability was evoked by intracutaneous injection of undiluted filtrates of cultures of the first four organisms, whereas the cholera filtrate gave rise to typical lesions at 1 : 10 to 1 : 40 dilutions.

PHYSICAL PROPERTIES

The effects of heat and dialysis upon the skin reactivity of culture filtrates and active cholera stool filtrates have been compared in a series of preliminary experiments conducted in parallel. Both the induration-evoking factor and PF of both materials are completely destroyed at 56° C for 30 minutes. The result of a time-course study with heated stool is shown in the lower curve in Figure 1. Neither induration nor delayed permeability effect was seen throughout a 48 hour period. Studies are in progress to determine more precisely the temperature coefficient of both stool and culture filtrates. Dialysis of both stool and culture filtrates for 24 hours at 5° C against 0.85% NaCl has resulted in 25-50% loss of skin reactivity. Whether this represents slow or partial dializability or the loss of a dialyzable co-factor is not known and requires further investigation. Both materials are inactivated by addition of 0.4% formalin. Stool filtrate retained its full activity after exposure to trypsin, 100 µg/ml for 15 minutes at 37° C at pH 7.0. Preliminary observations were made on the effect of pH by exposing stool filtrates to pH 4.0 and 9.0 for 15 minutes at 37° C and comparing their skin activity with a control kept at pH 7.0. Roughly 50% of activity was lost at pH 4.0, but only very slight loss occurred at pH 9.0.

NEUTRALIZING CAPACITY OF CONVALESCENT CHOLERA SERA

Acute and convalescent sera from cholera cases were inactivated at 56° C for 30 minutes. Three-fold serial dilutions of serum were mixed with constant amounts of stool filtrate or culture filtrate, and the mixtures were incubated at 37° C for 30 minutes. The mixtures were injected intracutaneously in guinea pigs, and intravenous dye was injected 18 hours later. Convalescent serum from confirmed cholera cases was capable of preventing both the induration and PF effects of both stool filtrate and culture filtrate at a higher dilution than was acute phase serum. A graph of typical titration is shown in Figure 4 in which culture filtrate was employed as PF. The results of titrations of a number of sera against culture filtrate are set out in Table II. The values shown in Figure 4 are tabulated under Case 944 in this table. Titer rises were demonstrable in all cases of confirmed cholera, but not in human beings given commercial cholera vaccine nor in rabbits given living vibrios grown on 1% trytose slants. Substantial agglutinin titer rises were demonstrated in both the cholera cases and in persons and animals vaccinated with whole organisms.

Normal persons and some acute phase sera contained low titers of neutralizing substance. The lowest titers were found in normal rabbits and in the acute phase sera of those cholera patients who showed the most marked titer rises during convalescence.

DISCUSSION

Stools from certain cholera patients, and cultures of V. cholerae grown in 5% Bacto-Peptone® contain filtrable, heat-labile substances which exert identical deleterious effects following intracutaneous injection in guinea pigs and rabbits: namely, erythema, induration, and a prolonged increase in capillary permeability. Both substances are neutralized to the same degree by a heat-stable component of convalescent cholera serum. These findings suggest that the two materials are identical. Moreover, their properties are those of a typical bacterial exotoxin. Hence, the term "skin-toxin" will be applied to this material to simplify discussion.

For the preparation of skin-toxin in vitro, the medium and growth conditions employed were those used by De et al (ibid) for the production of cholera enterotoxin. Skin-toxin and enterotoxin are both produced in 5% Bacto-Peptone® with a large surface/volume ratio and both materials appear in relatively young cultures. Neither are produced in poorly aerated cultures nor in any of the other media tested. Both are destroyed by heating at 56° C for 30 minutes. The preliminary findings suggest the possibility that the skin-toxin and the enterotoxin are identical.

At present there is no evidence that the skin-toxin plays a role in the morbid sequence of cholera in man. However, skin-toxin could possibly exert a deleterious effect upon gut tissues, perhaps by altering permeability of mucosal cells or of capillaries in the villi.

Evidence indicates that the skin-toxin is antigenic in man, that it reaches antibody-producing elements during the course of clinical cholera, and that the antibody elaborated behaves like an antitoxin. Moreover, antibody against the skin-toxin does not appear to be evoked by vibrio antigens capable of evoking agglutinins.

Work is now in progress to determine the antigenicity of skin-toxin derived from both stool and culture, and to determine whether antigenicity is retained after treatment with formalin. Agar-gel diffusion studies have been started. The relationship between skin-toxin and the enterotoxin of De et al (ibid) will be investigated by comparing the effects of both stool and culture filtrates in both skin and gut.

SUMMARY

Filtrates of stools from cholera patients and from young V. cholerae cultures grown in 5% Difco Bacto-Peptone® contain a heat-labile skin-toxin which gives rise to induration and increased capillary permeability following intracutaneous injection in rabbits and guinea pigs. Toxic activity has not been detected in stools from other kinds of diarrhea, nor

in filtrates of cultures of either enteric pathogens. The skin-toxin is neutralized by convalescent sera from cholera patients but not by sera containing agglutinins evoked by vaccination with living or killed vibrios.

TABLE I

24 Hours Induration in Guinea Pigs Following Intradermal Injection of Stool Filtrates

Source of Stool Specimen	Induration Doses/ml Stool Filtrate				Positive / Total
	<5	5-10	10-100	100-1000	
ACUTE CHOLERA*					
Ogawa	4	-	3	1	
Inaba	3	2	2	5	
Total Acute Cholera	7	2	5	6	13/20
CONVALESCENT CHOLERA	3	-	-	-	0/3
NON-CHOLERA DIARRHEA*					
Non-cholera Vibrio, Heiberg II	4	-	-	-	
No Pathogens					
Pulseless	16	-	-	-	
With Pulse	13	-	-	-	
Total Non-cholera Diarrhea	33	-	-	-	0/33

* Within 48 hrs of onset

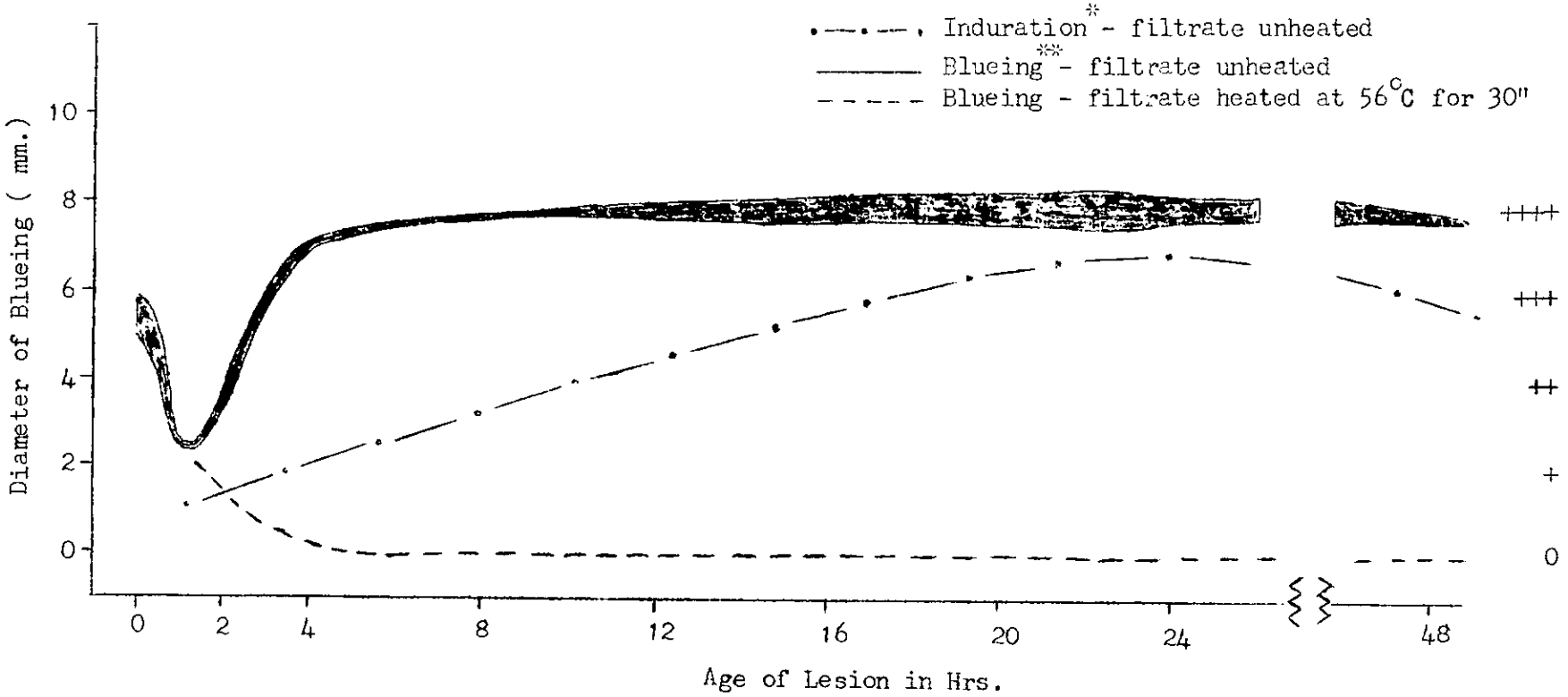
TABLE II

Toxin-Neutralizing Titers of Human and Animal Sera
(Toxin from Ogawa B1307)

Source of Sera	Day of Disease or Vaccination	Neutralization Titer	Agglutination Titer Ogawa	Agglutination Titer Inaba
I. Confirmed Cholera Cases				
Case No. Organism				
977 Inaba	1	10	< 40	< 40
	10	30	1280	1280
1021 Inaba	0	10	< 40	< 40
	10	270	160	160
	30	90	40	80
978 Inaba	1	10	< 40	< 40
	12	30	640	640
1188 Inaba	1	< 10	< 40	< 40
	10	> 270	320	320
944 Ogawa	0	< 10	< 40	< 40
	16	90	160	40
890 Ogawa	0	5	< 40	< 40
	9	640	1280	640
II. Non-Vibrio Cholera				
1011 No pathogen	0	30	< 40	<40
	13	-	< 40	<40
	59	30	-	-
III. Normal Human Controls (East Pakistan Residents)				
44-I -	-	10	< 40	<40
37-I -	-	10	< 40	<40
A-684 -	-	10	< 40	< 40
IV. Pakistani Personnel Vaccinated with killed Ogawa+ Inaba Vaccine				
1 -	0	30	< 20	<20
	28	30	160	320
19 -	0	30	40	40
	28	30	640	640
V. Rabbits Vaccinated with living V.cholerae Ogawa				
RJ-6	0	< 10	< 40	<40
	36	< 10	1280	320
RJ-7	0	< 10	< 40	<40
	36	< 10	10,240	10,240

FIGURE 1 II(e)

Time-course of Increased Capillary Permeability and Induration following Intracutaneous Injection of 0.1 ml. of 1:20 Dilution of Cholera Stool Filtrate (CRL 531 Ogawa) in Guinea Pigs.



* Intensity of induration is indicated by arbitrary scale at right.

** Intensity of blue is indicated by width of solid line.

FIGURE 2

Titers of PF of Cholera Filtrates of Bacto-Peptone Cultures Grown under Different Condition of Aeration.

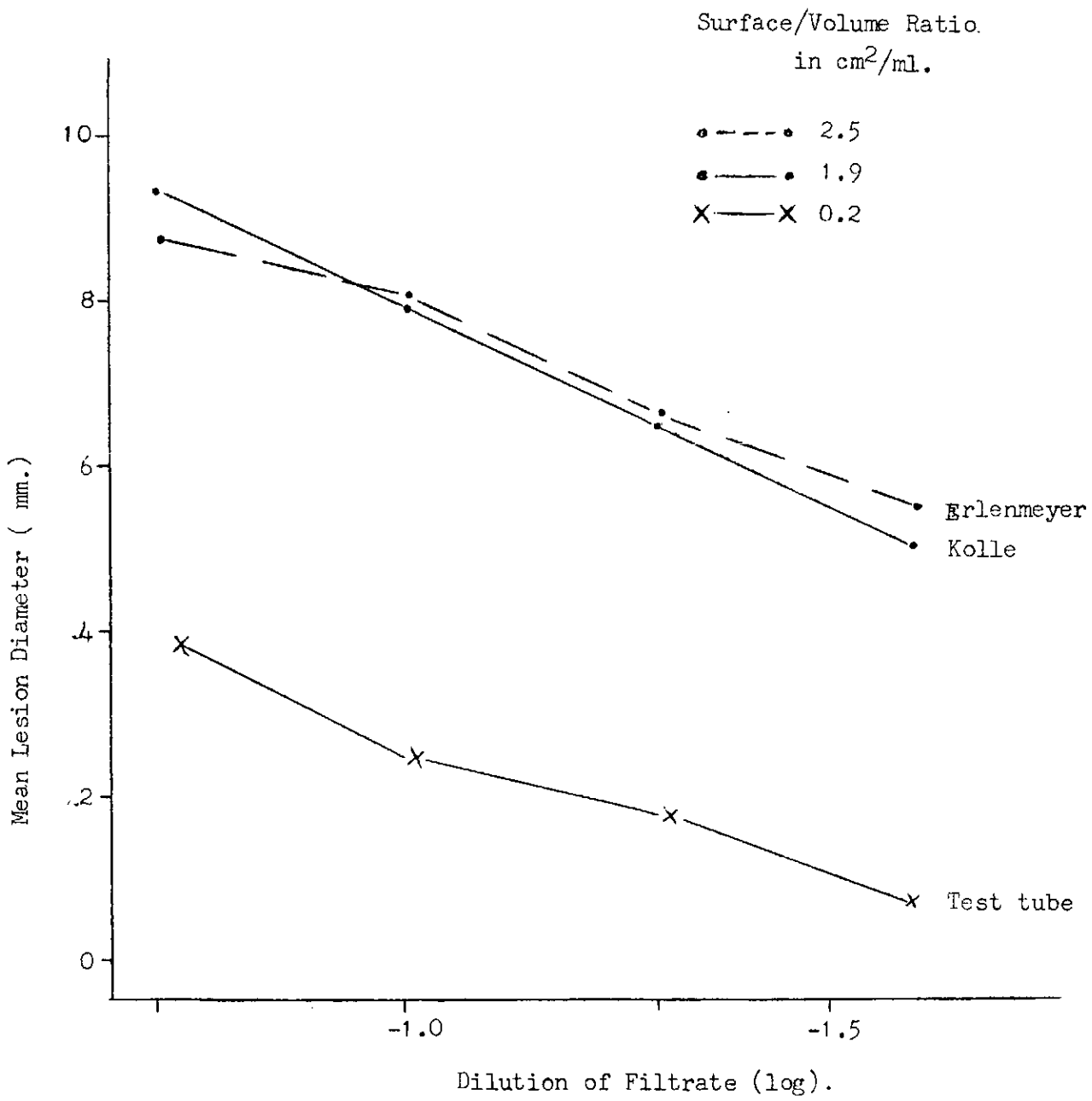


FIGURE 3

Time-Course of Production of Capillary Permeability Factor in 5% Bacto-Peptone, pH 7.3 by two strains of Vibrio cholerae.

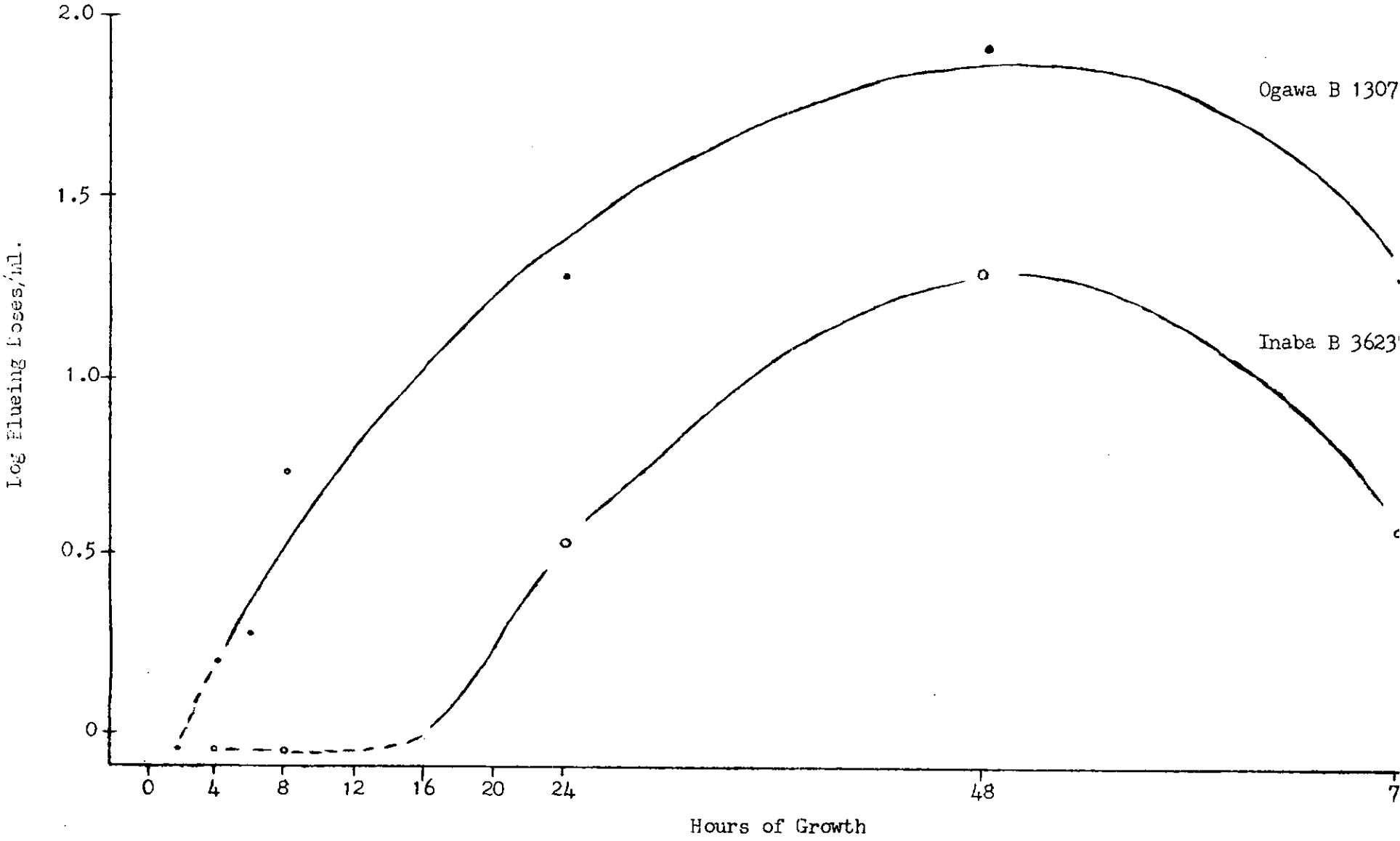
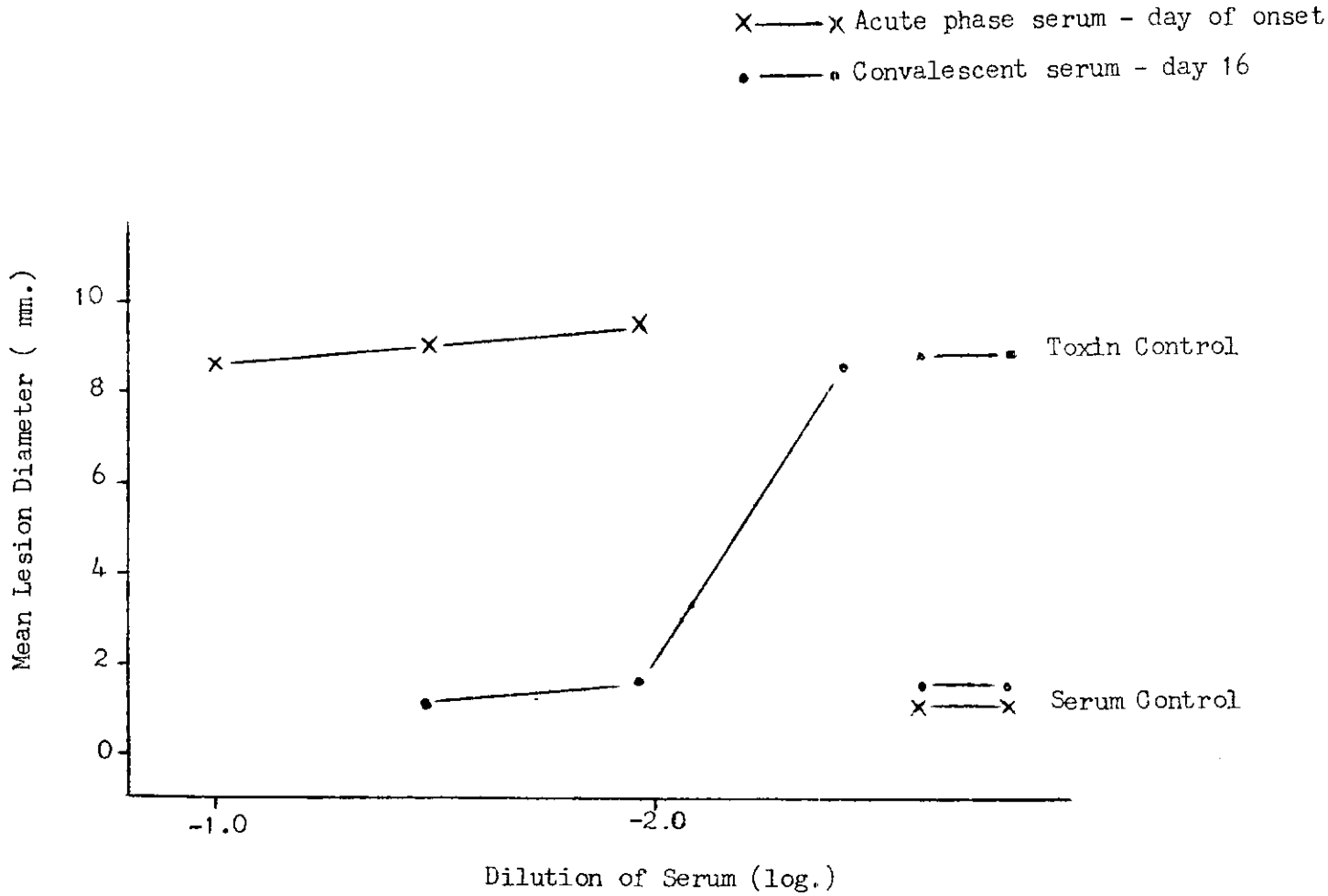


FIGURE 4 II(e)

Neutralization of Permeability Effect of Cholera Toxin by Convalescent Serum from Cholera Case CRL 944



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IMMUNITY IN CHOLERA PATIENTS

by

Dr. A.S. Benenson & Miss Saad

The place of immunity in the epidemiological and clinical pattern of disease can be assess in vivo by studying the effects of an aministered vaccine (See section V), and by examining the sera from cholera and non-cholera patients for the presence of antibodies directed against the vibrio. The technique used at CRL to date for in vitro antibody studies has been that of agglutination of living vibrios, using both the Ogawa and Inaba serotypes. Agglutinins which are demonstrated in this system may be specific, i.e. directed against the somatic substance, or may be directed against flagellar antigens, which might be shared with other organisms.

Comparison of the vibrio positive and vibrio negative diarrhea cases admitted to the CRL ward reveals that an appreciable number of patients in both groups are admitted with agglutinins against one or both cholera serotypes. Antibodies, when present have usually been both anti Ogawa and anti Inaba. The latter are, however, slightly more frequent and at slightly higher titer. Anti Inaba agglutinins are present in 22.2% of CRL cases with vibrio infections and 35.7% of those negative for vibrios; this is based on all cases on whom serum was available in the first three days of disease. The distribution of admission antibody values among CRL cases No. 258 to 1000 was:

	Vibrio Positive Diarrhea Cases	Vibrio Negative Diarrhea Cases
1280	-	1
640	-	2
320	1	4
160	5	22
80	22	38
40	21	42
<40	172	196
Total	221	305

The geometric mean agglutinin titers are significantly higher, ($p<.001$) in the non-vibrio group. These antibodies cannot be attributed entirely to prior vaccination. By history there is no difference in the number of "properly" vaccinated individuals in the two groups, i.e. within 10 days to six months, but the non-vibrio group is, in general, better vaccinated; thus:

	Vibrio Diarrhea	Non-Vibrio Diarrhea
Total Number with Vaccination Data	253	310
No history of vaccination	62%	58%
Vaccine within 10 days	12%	3%
10 days - 6 months	9%	11%
6 months - 2 years	13%	22%
Over 2 years	4%	7%

While the pre-existing antibodies in the non-vibrio group are in some cases clearly a response to vaccine, this is not evident in analysis in pre-existing antibodies (anti Inaba) in the two groups:

DISTRIBUTION OF ANTIBODY TITERS FOUND IN FIRST THREE DAYS OF DISEASE

Tubes Positive	Titer	Vibrio Diarrhea		Non-Vibrio Diarrhea	
		Vaccine	No Vaccine	Vaccine	No Vaccine
6	1280	-	-	1	-
5	640	-	-	2	-
4	320	-	1	1	2
3	160	1	3	11	10
2	80	9	13	17	21
1	40	10	11	20	20
0	<40	62	110	71	122
	Total	82	138	123	175
Geometric mean (tubes)		0.38	0.36	0.87	0.57

Analysis of 104 cases for the time course of the agglutinin response was carried out. Only bacteriologically positive cases were included who had no demonstrable agglutinin to antigen of either serotype in the first serum, and did develop a four-fold or greater rise in titer in later sera. The pattern of titers against the infecting serotype was: (See Table I).

* In this series no antibody response occurred before the fourth day and in only three instances was it not present by the seventh day. Of particular interest is the fact that the antibodies fall so soon after infection, in contrast to the persistence of the "pre-existing antibodies".

Possible relation of cholera susceptibility to other serological findings was explored. Blood type distribution in the two groups was not significantly different.

BLOOD TYPE OF CRL DIARRHEAL CASES

Blood Type	Vibrio Positive Group		Vibrio Negative Group		All	
	No.	%	No.	%	No.	%
O	63	29	24	42	87	31
A	67	30	13	23	80	29
B	71	32	16	28	87	31
AB	19	9	4	7	23	8

6.7% of the vibrio positive and 7.6% of the vibrio negative groups were positive in the cardiolipin microflocculation test.

TABLE 1

	Titer															
7	2560								1							
6	1280								4	2	1					
5	640						1	6	10	0	1					
4	320					3	3	9	12	5	2	2				
3	160				1	1	3	11	10	4	1	3	3			
2	80				4	2	8	9	11	0	0	2	-			
1	40				3	0	4	3	1	1	1	3	1			1
0	240	47	56	26	26	12	2	2	1	0	0	7	9	5	-	1
					14	19	46	120	182	45	23	24	10			
					34	18	21	40	50	12	6	17	13			
Geometric Area		0	0	0	0.41	1.06	2.19	3.0	3.64	3.75	3.83	1.41	0.76	-	-	-
		1	2	3	4	5	6	7	8-10	11-15	15-30	1	2	3	4	5
		Days										Months				

Time after onset of disease

Development of a Microserological Method for Vibrio Agglutinins

by

Miss Anisa Saad and
A. S. Benenson, M. D.

A simple, economical, and convenient serologic technique utilizing capillary blood samples would be desirable in a community study in cholera. Towards this end, a study was undertaken of the microtiter system which had been used for hemagglutination and complement fixation procedures, to determine what changes in the immunological system would be necessary for effective consistent results.

Materials and Methods

The Microtiter Kit (Cooke Engineering Company) was used. This kit includes microtiter plates with conical ("V") or radial-bottomed ("U") cups, pipette droppers calibrated to drop 0.025 ml saline, and calibrated loops which deliver 0.025 ml. A tetrazolium salt kit was procured from the Nutritional Biochemical Corporation, Cleveland, Ohio.

The standard V. cholerae strains previously used for agglutination at CRL were used; these included strain 465 V. cholerae, Ogawa and 466 V. cholerae Inaba, both isolated in this laboratory from cholera patients in Khulna in 1962; strain J89 V. cholerae Inaba was sent by Dr. Goodner.

These organisms were maintained on T_1N_1 (trypticase 1% NaCl 1%) agar slants, and for use were incubated overnight in T_1N_1 broth (pH 7.3).

Agglutination Tests

The agglutination tests were performed according to the procedure outlined by Goodner (SEATO Conference on Cholera, 1960) using living organisms.

Results

Examination of the bottoms of agglutination tubes in the standard tube agglutination test with living vibrios revealed that vibrio agglutination reactions can be read by observing the bacterial pattern of sedimented organisms; the agglutinated vibrios form a shield over the bottom of a tube while non-agglutinated vibrios form a button in the bottom of the cup. With the small volume (0.025 ml of antigen) in the microtiter test, it was thought that the buttons would be easier to recognize if the bacteria were colored and the plate examined against a white background.

The various salts included in the tetrazolium salt kit were dissolved in 0.01% concentration in water (tetrazolium red, triphenyl tetrazolium chloride, and nitro blue tetrazolium), or in 1% alcohol (neotetrazolium chloride diformazan, tetrazolium violet, tetrazolium violet diformazan,

neotetrazolium choloride, and tetrazolium blue), or 2% alcohol (triphenyl tetrazolium chloride formazan, tetrazolium blue diformazan). These stock solutions were added to T_1N_1 cultures of strain 465 to make a final concentration of 0.001%, 0.0005%, 0.00001% and 0.000005%. After overnight incubation, color visible at the 0.00001% concentration had developed in the tubes containing triphenyl tetrazolium chloride, tetraxolium violet, tetrazolium blue, and neotetrazolium chloride. At these levels the dye was not toxic to the vibrios. When these salts were added to an 18-20 hour culture of vibrios, tetrazolium violet at a final concentration of 0.001% or 0.0025% produced a pink to violet color within 15 minutes. A color also appeared in this time with tetrazolium blue, but since this was not as sharp as that of tetrazolium violet, the latter was chosen for further work.

In preliminary studies, doubling dilutions of standard rabbit antiserum in 0.025 ml volume were prepared in the microtiter plates. The calibrated pipette dropper was used to put 0.025 ml of saline in each cup. The microtiter loop was used to make the serial dilution starting with 1:40. 0.025 ml of the dye-stained antigen was then deposited in each cup. The holes were covered with sealing tape and the plates floated in a water bath at 40° - 42° C for one hour and then kept in the refrigerater overnight. Gross readings of the plates were then easily made; when held against a brightly lit white background, the colored buttons of unagglutinated organisms stood out on the plates. Alternatively, the reaction could be read under the stereoscopic microscope which clearly revealed agglutinated shields or unagglutinated buttons.

Later, a simple system was developed using the microtiter test reading mirror. By judicious arrangement of light, the magnified buttons were made to appear white against a black background; with this arrangement, tetrazolium salts were unnecessary for the white buttons of undyed organisms were easily visible.

Initially, sera were tested in the plates with round bottom (U-shaped) as well as conical cups (V) in addition to the standard test tube technique. Because the titer readings in U-cups more closely matched those of the test tube, these cups were selected for subsequent use.

Microtiter Agglutination Method

The following technique was evolved:

Antigen suspensions were diluted to approximate the MacFarland No. 1 standard opacity; in the Coleman Junior Spectrophotometer at a wave-length of 515 satisfactory antigens had an optical transmission of 74% to 80%. When tetrazolium violet was used, a 0.01% solution was added to make a final concentration of 0.001%. After 15 minutes, the dye was reduced and the antigen was ready for use.

Serum dilution

One-to-20 dulutions were prepared by mixing one microtiter loopful of

serum or blood or contents of a 0.025 ml capillary pipette* with 0.475 ml of normal saline.

The Test

0.025 ml of saline was delivered into each cup of the plastic plate by means of a microtiter calibrated pipette. 0.025 ml of the 1-20 serum (or blood) dilution was transferred to the final cup with the microtiter loop; doubling dilutions were made with the loops. They were rotated 4 or 5 times in each cup and then passed to the next row. Four or more loops could be handled at one time, providing simultaneous, multiple serum dilutions. 0.025 ml of the Inaba and Ogawa antigens were then dropped into appropriate rows with calibrated pipette droppers.

Reading of Tests

Absence of agglutination was indicated by a sharply marked button, while agglutinated cells formed a shield over the bottom of the cups. The serum dilution in which no button was present was taken as the end point, since this was an objective observation on which all observers would agree. The presence of partial agglutination at higher serum dilutions was noted, but did not influence the titer.

* * * * * * *

Evaluation of the Method

Comparison of tube and microtiter results. A series of routine clinical specimens was run in parallel using the same antigen in both the microtiter and the tube test (Table I). In 70%, values were in complete agreement; in 18% microtiter results were one tube higher than tube test findings; microtiter readings were one tube lower in 11%. There was a two-tube difference in 2% of samples. In 53 paired samples a rise in titer was detected with both methods (Table 2). The titers were identical in 72% of the pairs and, in the remaining 28%, differed by one tube.

The consistency of the method was studied by comparing the titer of agglutinins in the plasma and serum derived from the same person. The serum was obtained by venipuncture, while the plasma was derived from finger tip blood using lectins as anticoagulant. Results are presented in Table 3.

A series of tests performed with the same lot of reference calf serum, provided the opportunity to measure the inter-test variations of the tube and microtiter systems. In 39 tests there was a slightly wider spread of titers with the microtiter method (Table 4). The lower microtiter values are due to the fact that complete rather than partial agglutination was arbitrarily selected as an end point.

* Drummond Scientific Co., Broomall, Pennsylvania

Development of capillary fingertip blood technique. Venous and capillary blood was simultaneously obtained from a series of patients. Fingertip blood was drawn, added to 0.475 ml distilled water with immediate hemolysis or 0.475 ml of saline to a 1:20 dilution, using either the 0.025 ml capillary pipette or the microtiter loop. Vibrio agglutinin titers were obtained by the microtiter method of the serum, hemolysed blood, and the plasma (after centrifuging out the RBC). Agglutinin titers of the serum tended to be higher than those with fingertip blood; however, 55% of the tests gave identical values. The difference is consistent with the volume of the whole blood sample occupied by the red blood cells. Values obtained when the fingertip droplet was placed in distilled water were similar to those obtained with saline. From a technical point of view, the specimens collected in saline and then centrifuged gave sparkling clean results and made the use of tetrazolium salts unnecessary. On the other hand, hemolysed blood could be tested without centrifugation, but tetrazolium violet was required to differentiate the button of red cell stroma and debris from that of non-agglutination.

Concurrent studies were carried out to determine whether the antibody content in a 1-to-20 dilution is stable under refrigeration or freezing conditions. No significant difference in titer was observed as the result of freezing, thawing, or storage for one week at 4° - 6° C. For routine use, specimens were kept frozen when not in use.

Conclusion

Simplicity, economy, and the ease of reading the microtiter method justified its adoption for routine use. The necessity for thorough cleaning of the plates between use and the complete removal of detergent afterwards is a limitation in the microtiter system; this can be obviated by the use of disposable plates. The use of filter paper would further simplify collection of blood in community surveys and eliminate need for refrigeration.

TABLE 1

Comparison of Tube Agglutination & Microtiter Results of Routine Clinical Specimens

Tubes Positive	0	1	2	3	4	5	6
6	-	-	-	-	-	6	8
5	-	-	-	2	7	20	2
4	-	-	-	14	31	4	-
3	-	-	7	18	3	-	-
2	1	8	19	4	2	-	-
1	7	34	4	-	-	-	-
0	47	8	-	-	-	-	-

Microtiter Cups Positive
(1 = 1:40)

TABLE 2

Comparison of Rises in Titer in Clinical Serum Pairs by Tube Agglutination and Microtiter

Tube Rise in Titer	0	1	2	3	4	5	6
6	-	-	-	-	-	1	2
5	-	-	-	-	2	4	-
4	-	-	-	2	17	1	-
3	-	-	5	6	2	-	-
2	-	1	7	1	-	-	-
1	-	2	-	-	-	-	-
0	-	-	-	-	-	-	-

Rise in Titer by "Tubes" in Microtiter

TABLE 3

Reproducibility of Agglutination Titers with Microtiter

Serum & plasma of an individual in same test

Cups Positive with Serum (from Venous blood)	Cups Positive with Plasma from fingertip blood (1 = 1:40 dilution)						
	0	1	2	3	4	5	6
6	-	-	-	-	-	-	3
5	-	-	-	-	1	2	-
4	-	-	-	-	8	-	-
3	-	-	1	9	-	-	-
2	-	-	4	-	-	-	-
1	-	4	-	-	-	-	-
0	10	-	-	-	-	-	-

TABLE 4

Inter-Test Consistency Using Reference Calf Serum in Different Tests

Positive Tubes or Cups (1=1:40 dilution)	Ogawa Antigen		Inaba Antigen	
	Tube Test	Microtiter Test	Tube Test	Microtiter Test
9	-	2	-	1
8	21	11	4	5
7	17	15	29	13
6	1	11	5	16
5	-	-	1	4
Mean	7.5	7.1	6.9	6.6
95% Confidence Limits	7.4 - 7.6	7.0 - 7.2	6.8 - 7.0	6.4 - 6.8

TABLE 5

Comparison of Titers Obtained with Serum and Whole Blood Diluted 1:20 in Distilled Water

Cups Positive with Serum	Cups positive with hemolysed whole blood (1 = 1:40 dilution)						
	0	1	2	3	4	5	6
6	-	-	-	-	-	3	1
5	-	-	-	-	2	-	-
4	-	-	-	4	4	-	-
3	-	-	7	7	-	-	-
2	1	4	7	-	-	-	-
1	1	5	-	-	-	-	-
0	3	1	-	-	-	-	-

The Rhesus Monkey as an Experimental Cholera Model

by

Drs. W. B. Greenough III, and A. S. Benenson

Since the last meeting of the Technical Committee, rhesus monkeys have been prepared and challenged with V. cholerae in a variety of ways. Initial experiments were carried out to test the possibility of duplicating the results of A. Mendoza, and H. Pottevin and H. Violle (see last year's Report to the CRL Technical Committee). Subsequently, attempts were made to interrupt the flow of bile and pancreatic enzymes by ligation of the common bile and pancreatic ducts. This work was based on a report by Dutta, suggesting that absence of pancreatic lipase and phosphorylase might explain why the infant rabbit is susceptible to cholera. To date a successful reproduceable cholera model has not been achieved in our monkeys; however, several interesting observations have been made and ideas generated from them are being investigated in man.

METHODS:

Rhesus monkeys weighing 1½ to 4 kilograms were used. They were obtained from dealers who had caught them in the Sunderbans. Unless on special diet they received fruit, rice, dhal, fish, and salt. During experiments they were kept in separate cages. Prior to experiments, a rectal swab, body weight, temperature, and general appearance were noted, and blood taken. After challenge, daily rectal swabs were taken. Liver biopsies were performed with the Menghini needle after an iodine-alcohol skin preparation. In case of death or sacrifice an autopsy was performed. At autopsy the abdomen was opened and the intestines clamped at different levels before further manipulation. For sacrifice, animals were anesthetised, the clamps placed and then the animal was killed by an overdose of ether. Cultures of organs for V. cholerae were done by searing the site for aspiration by syringe and needle or excision of small pieces of tissue. The gross appearance of organs was noted and sections placed in 10% formalin or Zenkers solution. The bacteriologic methods for vibrio isolations are those in standard use in the laboratory - direct plating on TTGA and GA and enrichment through bile peptone.

Pretreatment:-

Monkeys were pretreated for challenge in the following ways:-

1) Deficient diets were given consisting of either vitamin free casein or rice and salt alone. After varying intervals on these diets a challenge of V. cholerae was given.

2) 10% sodium sulfate was given by stomach tube sufficient to induce diarrhea. Immediately after diarrhea began, a challenge of V. cholerae was given.

3) A modification of the sodium sulfate preparation was used, giving enough potassium sulfate to prevent excessive potassium depletion before challenge.

4) 3% sodium bicarbonate solution was substituted for the drinking water for 1-2 days before challenge and continued after challenge.

5) In monkeys prepared by 1-2 days of 3% sodium bicarbonate, 10% sodium sulfate was given to the point of diarrhea prior to challenge.

6) Animals were given injections of DOCA 0.1 mg for 3 to 10 days prior to challenge, to induce potassium depletion.

7) The animals were operated and the common bile duct or common bile duct and pancreatic ducts were either tied and cut. Challenge with V. cholerae was given 1-4 weeks after surgery.

Challenge:-

Several methods were used for challenging with V. cholerae. The usual procedure was to give 25 ml of an overnight broth culture of V. cholerae grown in T_1N_1 broth. All monkeys received between 10^6 to 10^{11} organisms by stomach tube. Vibrios were also grown in rice and ricewater and fed either once or repeatedly. Ricewater stool taken directly from patients was used in several cases. One group of monkeys received accidental challenges, presumably through contamination of their food.

Results:-

None of the procedures tried to date has established a method whereby the monkey will predictably develop the clinical or autopsy findings of cholera. An acute cholera syndrome has been seen sporadically in all groups challenged with the gross autopsy findings analogous to those seen in the autopsy of human cholera. On the other hand, infection with vibrios, as evidenced by positive rectal swabs persisting for several days, occurred quite regularly. Five monkeys who had not been challenged deliberately but were held in the infected animal room, developed positive cultures.

Vibrios were recovered at the autopsies of 39 of 69 monkeys dying or sacrificed as much as 61 days after exposure to cholera vibrios. Organisms were more consistently isolated from the small intestine, and particularly from the duodenum, than from the large bowel. This was not the consequence of infection of the gall bladder, as was shown by the analysis of 33 instances in which culture data are available on both organs:

Duodenum Positive Bile Negative	Duodenum Positive Bile Positive	Duodenum Negative Bile Positive	Total
18	13	2	33

The finding of more intense positivity of the small intestine was observed in animals found dead and then autopsied as well as those in whom the infection was more sharply circumscribed by killing the animal after the intestinal loops had been isolated as shown:

Vibrio Isolation

	Liver & Bile		Stomach		Small Intestine		Large Intestine	
	No.	%	No.	%	No.	%	No.	%
Monkeys (dying)	11/28*	39	18/20	90	57/62	92	48/56	85
Sacrificed monkeys	9/21	43	7/17	41	36/51	70	20/41	49
All	20/49	41	25/37	68	93/113	83	68/97	70

* Number positive over number examined.

This phenomenon is seen in its extreme in 12 bacteriologically positive animals whose rectal swabs had been negative for vibrios for 1 to 15 days before autopsy. Three of these had never become positive after the challenge given 2, 5 and 6 days earlier.

The liver parenchyma was cultured nineteen times either at autopsy or by biopsy. In five instances V. cholerae was recovered; in 4 of these the bile duct had been ligated to prevent retrograde infection of the biliary tract. Liver positivity was only observed during the first 8 days as is shown below. Bile, on the other hand, was found positive in 17 of 67 monkeys examined. Common bile duct ligation performed several weeks before challenge, which caused acholic stools, did not prevent the development of positive bile.

Time after Challenge when Positive Cultures of V.cholerae Were Found in Liver and Bile

		8 days or less	more than 8 days
Liver	(Positive	5	0
	(Negative	4	10
Bile	(Positive	7	10
	(Negative	12	38

There is a suggestion in the following table that there is an association between a cholera-like syndrome and an infected liver, while this does not follow with infected bile.

Positive Cultures for V.cholerae in Monkeys Dying of a Cholera-like Illness

		Cholera-like	No Cholera
Liver	(Positive	3	2
	(Negative	2	13
Bile	(Positive	3	14
	(Negative	8	42

Increasing experience summarized below has fortified the earlier impression that the first exposure to vibrios provides statistically demonstrable immunity, although an individual monkey may still proceed to acquire a fatal infection.

Effect of Prior Challenge on Duration of Positive Culture and Incidence of "Cholera-like" Illness

	First Challenge	Subsequent Challenge
* 3 days or more (+) R/S	59	14
Less than 3 days (+) R/S	32	32
"Cholera-like" illness	9	3
No "cholera"	82	43

* Chi square distributions show p values of $< .001$

Discussion:-

Although it has not been possible to define the conditions under which a monkey exposed to vibrios will consistently develop disease, these studies have opened at least two suggestive leads.

The first of these is the observation that the vibrio infection is more intense in the upper than the lower intestinal tract, and that an animal can be consistently negative on rectal swab while harbouring infection within his body. This means that the small bowel, particularly the upper small bowel, may be the reservoir in which the organism survives. The bile was infected in all monkeys positive more than 1 month after challenge, but the duodenal and jejunal infection in infections of shorter duration can occur with negative bile. These observations suggest the need for appropriate studies of man.

Bile and liver positivity, which can occur even after the bile duct is severed, suggests that in the monkey the cholera vibrio is not necessarily confined within the lumen of the intestine. These observations suggest that in this animal a portal bacteremia can occur early in the disease, and raises the question whether this might occur in man.

Histopathological studies are contemplated to see whether tissue changes similar to those seen in human autopsies occur in the monkey; the results will have great weight in determining the validity of the monkey infection as a model of human disease.

VIBRIO STUDIES

by

Dr. S.H. Rizvi, Mr. Imdadul Huq & Mr. C.R. Dey

(A) DIAGNOSTIC BACTERIOLOGY:

Work Load:

The clinical and epidemiological activities of the Cholera Research Laboratory depend on demonstration of the presence or absence of vibrios in clinical or environmental material. The recognition of pathogens other than V.cholerae which might be responsible for a clinical picture resembling that of cholera infection is equally important; therefore, the bulk of bacteriological effort of the laboratory has been diagnostic.

General Report:

From October 1, 1963, to September 30, 1964, a total of 42,120 specimens was examined bacteriologically: 41,478 (98.2%) rectal swabs, 30 bacterial isolates, 170 blood cultures, 75 throat swabs, 51 urine, 32 sputum, 34 worms, 197 water, 7 vomitus, 9 pus, and 37 miscellaneous - spinal fluids, eye swabs, autopsy specimens, etc.

The sources of these specimens were as follows:

1.	Mitford and Victoria Hospitals	650
2.	CRL Hospital	4,584
3.	Rayer Bazar Study	18,490
4.	Matlab Studies	17,844
5.	Shaitnal	309
6.	Other places in E. Pakistan	33
7.	Outside East Pakistan	210

These biological specimens were received in the laboratory in one or more of the following media:

a.	Bile-peptone tellurite enrichment broth	41,051
b.	Selenite broth	1,010
*c.	T_1N_1 Broth	504
*d.	Alkaline peptone water	294
e.	Direct TTGA plates (Monsur)	15,731
f.	Direct GA plates	6,381
g.	Direct SS plates	7,560
h.	Direct MacConkey plates	5,258
i.	Direct blood agar	3

* These enrichment media were used when sodium taurocholate was unavailable.

The following pathogenic organisms were isolated:

Vibrio cholerae	Inaba	789
	Ogawa	499
Vibrio El Tor	Inaba	1
	Ogawa	60
Non-cholera vibrios		192
Other pathogens		
Shigella A (dysenteriae)		9
" B (flexneri)		133
" C (boydii)		4
" D (sonnei)		35
Salmonella paratyphi A		4
B		2
C_1		3
C_2		4
Salmonella typhi		4
Klebsiella		3

All vibrio isolates are routinely subjected to chicken cell agglutination as a screening test for El Tor. During the period covered by this report, eight hemagglutinating strains were obtained which, by all other tests were classical cholera vibrios. A paper has been submitted for publication on the properties of these strains (See 'Isolation of Hemagglutinative Non El Tor Vibrios).

Routine Vibrio Diagnosis for Field Studies:

Based on the results reported last year, tellurite bile peptone broth is being used for all CRL vibrio field studies. Accordingly, studies to determine the efficacy of this medium and of isolating Vibrio cholerae assume importance, particularly because getting specimens from the field to the laboratory sometimes takes several days.

The general procedure for recognition of vibrios in the field is as follows: The rectal swab is obtained with a tellurite impregnated cotton swab which is then immersed in 10 ml of alkaline bile peptone broth; after overnight incubation (or as soon thereafter as possible) it is streaked in a standard manner on gelatin agar (GA) and tellurite taurocholate gelatin agar (TTGA) plates (Monsur).

After overnight incubation, vibrio-like colonies are examined by slide agglutination with specific anti-vibrio serum and subjected to biochemical testing. Degree of positivity based on the number of positive colonies on the surface of the plate is reported arbitrarily in a + to ++++ scale. In order to determine whether there was numerical significance in these degrees of positivity, a series of log dilutions of Vibrio cholerae were prepared, and 12 plates were streaked in the standard fashion for each of 8 log dilutions.

Streaking was done by the laboratory technicians who normally streak the plates, then the plates were coded and after overnight incubation were read by three other technicians who normally read the plates. The relation of plus values to the vibrio count shows an appreciable consistency (Table I).

Stool samples were received in the laboratory both on plates as well as in the enrichment broth from 1108 vibrio positive cases, affording an opportunity to evaluate the consistency and reliability of the enrichment procedure; 239 samples were negative on the direct TTGA plating; thus 21.6% of positive samples were isolated by enrichment only (Table II). Surprisingly, 40 samples positive on the direct plate were negative after enrichment. These are analysed in Table III, which shows that in 13 instances only non-cholera vibrios were isolated after enrichment, which could be explained by more rapid growth or bacterial antagonism of non-cholera vibrios; in the 27 other samples another explanation is necessary. Lytic phages were not found in any of the enrichments.

TABLE I

Vibrio Count per ml	"Degree of Positivity"					
	++++	+++	++	+	Neg.	
10^8	11*	24	1	-	-	-
10^7	7	24	5	-	-	-
10^6	-	9	26	1	-	-
10^5	-	-	21	15	-	-
10^4	-	-	2	33	1	-
10^3	-	-	-	-	36	-
10^2	-	-	-	-	36	-
10^1	-	-	-	-	-	36

* Number of readings

Relation of Plus Values of Vibrio Isolation Plates to Vibrio Count and Inoculation.

TABLE II

Comparison of the Degree of Positivity of Direct TTGA Plate with Positivity on TTGA Plates after Enrichment

Vibrio	Direct TTGA Reading		Specimens after enrichment showing degree of positivity						
	Degree of Direct Positivity	No. of Samples	-	0	+	++	+++	++++	+++++
Ogawa	-	79	0	0	6	26	29	16	2
	0	91	0	0	11	19	38	23	0
	+	33	3	1	4	9	7	9	0
	++	51	6	2	2	9	25	5	2
	+++	101	8	1	5	17	50	18	2
	++++	131	2	6	3	26	41	51	2
	+++++	13	1	1	1	3	4	3	0
Inaba	-	162	0	0	14	22	62	57	7
	0	124	2	0	15	23	62	21	1
	+	47	2	5	6	8	16	10	0
	++	67	3	6	9	16	18	14	1
	+++	158	6	11	13	24	66	36	2
	++++	212	1	7	19	39	68	73	5
	+++++	19	0	0	0	6	5	8	0
NCV	-	127	0	0	13	20	41	50	3
	0	24	0	0	3	2	10	9	0
	+	10	2	0	0	0	3	4	1
	++	11	2	1	1	2	5	0	0
	+++	15	0	1	2	2	3	4	3
	++++	5	0	0	1	0	1	3	0
	+++++	0	0	0	0	0	0	0	0

TABLE III

Enrichment isolations in 40 rectal swabs positive for V. cholerae on direct plating on TTGA and negative after enrichment in alkaline tellurite bile peptone.

Positivity on Direct TTGA plates	'Enrichment Isolation' Non-cholera Vibrios		Other Bacteria		Total
	Ogawa*	Inaba*	Ogawa*	Inaba*	
++++	1	-	-	-	1
+++	3	3	3	4	13
++	-	4	1	7	12
+	1	1	1	5	8
	-	-	1	5	6
Total	13		27		40

* Serotype of V. cholerae isolate.

Dynamics of Vibrio Growth:

To test the effect of non-cholera vibrios on Vibrio cholerae in enrichment media, growth curves were obtained for mixtures of non-cholera vibrio and V. cholerae isolated from the same stool specimen (Fig. 1). In a series of experiments, the NCV's were found to be about 10-100 times as abundant as V. cholerae, but the V. cholerae could be detected on streaked TTGA plates. Thus, the presence of NCV does not cause the disappearance of V. cholerae from bile-peptone enrichment but makes it difficult to recognize the few agglutinable vibrios among the multitude of non-cholera vibrios. Obviously, plating from enrichment broth after 4-6 hours will provide a more favorable ratio.

Occasionally, vibrio negative gelatin agar plates showed profuse growth of Pseudomonas pyocyanea, indicated by green or blue pigmentation of the medium. As many of the Pseudomonas group produce antibiotics with vibriocidal action, the effect of these bacteria on growth of V. cholerae in T_1N_1 and bile-peptone broths was investigated. When Ps. pyocyanea and V. cholerae were mixed in culture, the vibrios disappeared after 24 to 48 hours of growth, depending on the medium (Fig. 2). At first the vibrios multiplied at a greater rate than Pseudomonas, but after 12 to 24 hours of growth, the Vibrio counts levelled off or began to fall, while those of Pseudomonas continued to rise. After 36-48 hours, viable vibrios had disappeared. Pseudomonas pyocyanea grew faster and reached a higher concentration in T_1N_1 broth than in bile-peptone broth.

In stools, however, the number of Ps. pyocyanea may already be large with respect to the vibrio count. When the stool is put in enrichment broth, enough Pseudomonas may be present to kill a large proportion of the vibrios in a short period. In conjunction with other bacteria or with some other component of the stool, this vibriocidal action may be enhanced.

It is evident that the V. cholerae growth is more rapid in the initial few hours than that of at least these two interfering organisms. Accordingly, the standard practice has been changed so that enrichment broths are streaked on plates after 4-6 hours of enrichment, as well as after overnight incubations.

Nonmotile Vibrios:

Several vibrios isolated on gelatin agar plates on which Pseudomonas pyocyanea were also present in large numbers were found to be nonmotile under darkfield microscopy and in the motility tube. When broth cultures of the nonmotile forms are kept at room temperature, a few motile forms begin to appear after 3-5 days. Five colonies from each of 7 nonmotile strains were carried through seven serial broth passages; some strains remained nonmotile, while in others motile forms reappeared with passage (see Table below).

Ps. pyocyanea was suspected as the cause of mutation. When three known motile strains of V. cholerae were grown in bile peptone broth in mixed culture along with Pseudomonas, nonmotile forms appeared; the behaviour of these forms was similar to that of the nonmotile vibrios isolated initially. A number of these nonmotile vibrios have been stocked for further study.

Maintenance of Nonmotility of Vibrio Strains on Repeated Passage in T_1N_1 Broth.

Vibrio Strain	PROPORTION OF ISOLATES REMAINING NONMOTILE						
	Passage Number						
	1st	2nd	3rd	4th	5th	6th	7th
37951A	5/5	4/5	3/5	1/5	1/5	1/5	1/5
37480A	5/5	5/5	5/5	0/5	0/5	0/5	0/5
37815	5/5	5/5	5/5	5/5	5/5	5/5	5/5
38928A	5/5	5/5	5/5	5/5	5/5	5/5	3/5
38428B	5/5	5/5	5/5	5/5	5/5	5/5	4/5
37947A	0/5	0/5	0/5	0/5	0/5	0/5	0/5
37947B	5/5	5/5	5/5	5/5	5/5	5/5	5/5

FIGURE 1

Growth of NCV and V.cholerae Mixture
in T_1N_1 and Bile Peptone Broths

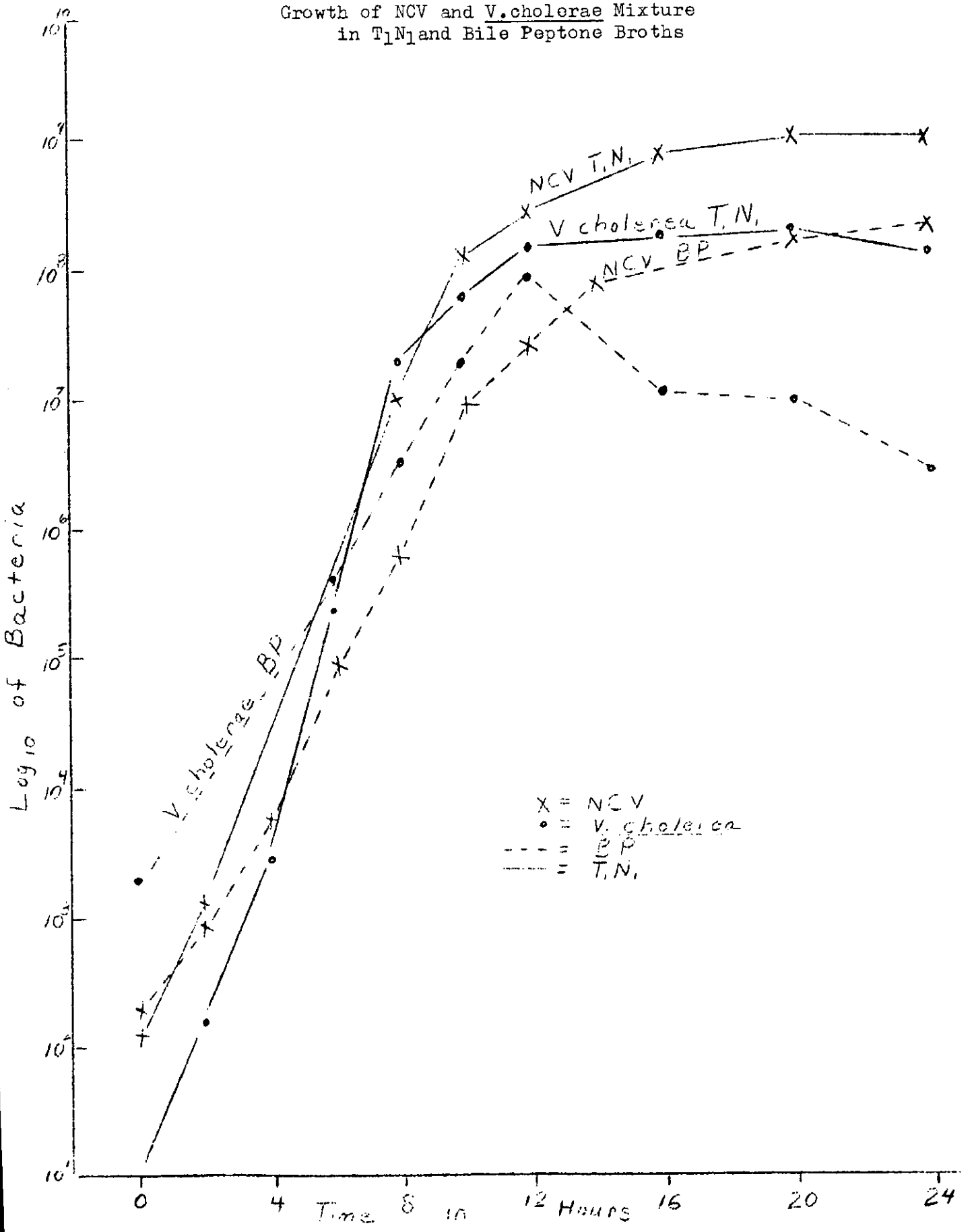
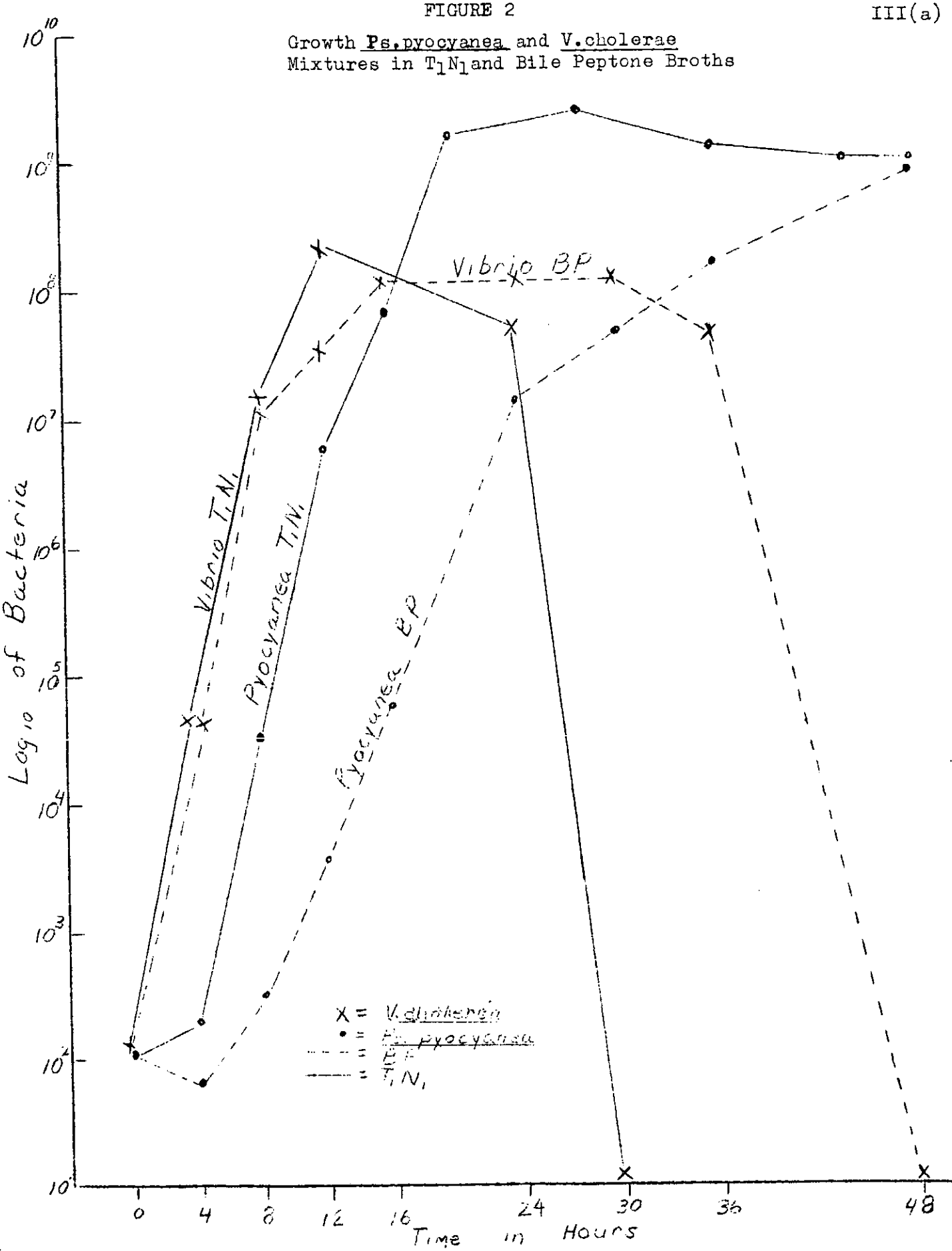


FIGURE 2

Growth Ps. pyocyanea and V. cholerae
Mixtures in T_1N_1 and Bile Peptone Broths



(B) BACTERIOPHAGE STUDIES:

In order to prepare high titer cholera bacteriophage, growth curves of V. cholerae, host strain M-154, were compared under different conditions of aeration. One ml of overnight culture of the bacteria in T_1N_1 broth was put in 50 ml flasks; one flask was left standing in the incubator, the second flask was put on a shaker, and the third was attached to a vacuum pump for aeration with filtered air. Maximum growth took place with aeration (Fig. 1).

Bacteriophages (Mukerjee's typing phages and a local isolate) grown in aerated host cultures achieved the highest titers when the host bacteria were grown for five hours and then enough phage added to infect all the bacteria. In following experiment, 5-hour V. cholerae M-154 cultures were seeded with 4.0×10^{11} phage particles per 50 ml of culture.

YIELD OF BACTERIOPHAGE IN AERATED HOST CULTURES

Phage Type	Total Phage Yield in PFU per 50 ml
Phage I	3.0×10^{12}
Phage II	3.3×10^{12}
Phage III	4.5×10^{12}
Phage IV	9.0×10^{12}
Phage 268	4.0×10^{12}

Bacteriophage Burst Time

The burst time of several bacteriophages was determined at 37° C by mixing phage and the host bacteria, allowing ten minutes for attachment, then diluting the mixture 100-fold. One-tenth ml samples of this diluted mixture were plated at various intervals to determine the time at which increase in phage titer became evident. The results are:

Phage Type	Burst Time (minutes)
Phage I	47
Phage II	47
Phage III	73
Phage IV	41
Phage 268	31

All these cholera phages have longer burst times than generally seen with other phages. Therefore, if phage is contemplated as a therapeutic agent, a cycle of infection and liberation of phage could not be expected to kill all the bacteria. In severe diarrhea, the passage time through the gut is so fast that the infected bacteria may pass from the gut before having time to liberate much phage. However, as treatment with about 10^{12} phage could infect all the bacteria, this might be an effective method for the treatment of cholera.

Factors Involved in Maintenance of Bacteriophages:

If bacteriophage therapy for cholera patients is found to be effective, availability of a stock of high-titer phage would be essential. Therefore, stability of the bacteriophages in relation to pH and other storage treatments was determined. Phage preparations were prepared by chloroform treatment of a phage lysate of the host strain in trypticase broth, followed by low speed centrifugation. Chloroform was removed by aeration before testing.

As shown in Fig. 2, the phages are sensitive to pH levels below 6.5 and over 7.5. The effects of freezing, freeze-drying from trypticase broth and storage at 5-10° C are shown below. Refrigeration and freezing appear to be satisfactory for maintenance of viability for short periods of time.

Treatment	Phage Type	Initial	PFU/ml After 3 weeks
FROZEN ONLY	Phage I	1.7×10^{10}	6.5×10^{9}
	Phage II	3.4×10^{10}	1.43×10^{10}
	Phage III	6.8×10^{9}	6.4×10^{8}
	Phage IV	3.3×10^{10}	1.15×10^{10}
	Phage 268	1.42×10^{10}	7.5×10^{9}
FREEZE - DRIED IN TRYPTICASE	Phage I	1.7×10^{10}	2.8×10^{9}
	Phage II	3.4×10^{10}	2.2×10^{9}
	Phage III	6.8×10^{9}	6.0×10^{7}
	Phage IV	3.3×10^{10}	4.3×10^{9}
	Phage 268	1.42×10^{10}	3.2×10^{9}
REFRIGERATED	Phage I	1.7×10^{10}	4.3×10^{9}
	Phage II	3.4×10^{10}	1.8×10^{10}
	Phage III	6.8×10^{9}	2.2×10^{9}
	Phage IV	3.3×10^{10}	1.7×10^{10}
	Phage 268	1.42×10^{10}	8.2×10^{9}

Sensitivity of El Tor Vibrio to Cholera Phages

Nonsensitivity of El Tor strains to Mukerjee Group IV phage has been utilized to differentiate El Tor from classical cholera strains. When El Tor was tested for susceptibility to Mukerjee phages, by both Mukerjee's method and the soft-agar pour-plate method, all strains were killed if phages I, III, and IV were used at a titer about 10^8 per ml. At routine test dilutions of the phage, the strains were not sensitive. With the pour-plate method, however, phage III formed minute plaques on some of the strains at low concentrations. A report from this laboratory on the effect of group IV phage on El Tor vibrios will be published in The Bulletin of the World Health Organization (Enclosure).

Minute Plaque Formation by Phage III on El Tor Strains

Origin of Strain	Number Tested	Number Attacked by Phage III
East Pakistan	10	4
Malaysia	17	10
Water El Tors	4	3
Indonesia	7	6
Phillipines	3	3
Thailand	8	6
Hong Kong	7	3
Others *	59	12
Total	115	47

These El Tors were not attacked by Phage I, II, and IV at low concentrations.

* After infection with Group I-Group IV phages, some of these strains gave rise to turbid plaque-forming phages that did not attack the universal host for cholera phages.

Phage Types of V. cholerae Isolated in East Pakistan in 1963-64

The phage type of the V. cholerae strains isolated in our laboratory was determined according to the method of Mukerjee. Most of the strains belong to type III, but variations from the classical type were noted. The table below shows the distribution of cultures on which records were kept.

Group	Number of Strains	Effect of Lower Phage I Dilutions	Effect of Lower Phage III Dilutions
a	268	Clearing	Clearing
b	95	No clearing	Clearing
c	15	Clearing	No clearing
d	23	No clearing	No clearing
e	22	Clearing	Very minute plaques only

Study of Non-Vibrio cholera Stools for Lysogenic Phage

The following procedure was used for the detection of lysogenic phage in 44 diarrhea cases admitted to CRL. 28 of these are included in the separate report on "non-vibrio cholera". Stool material had been stored in the deep freeze immediately after collection. At the time of the experiment, a vial was taken out and quickly thawed by immersing in a water bath at 37°. Induction of the production of lysogenic phage was attempted as follows:

1) 1.5 ml of the stool was treated with chloroform for 1 hour and centrifuged to remove bacterial debris. The dissolved chloroform was removed by suction. Drops of the chloroform-free supernates were then spotted on lawns of the indicator strains.

2) 1.5 ml of the stool was heated for 1 hour at 55-56° and centrifuged. Drops of the supernates were spotted on lawns of the indicator strains.

3) 1.5 ml of the stool was treated with ultraviolet light for 3 minutes and allowed to grow for an additional three hours. The sample was then centrifuged to remove bacteria, and drops were put on lawns of indicator strains as before.

4) 0.2 ml samples of the stool were enriched overnight in 4-hour cultures (5.0 cc) of selected indicator strains. The culture was then divided into 3 equal parts and treated as described under (1), (2) and (3).

The following indicator strains were used for detecting the lysogenic phage:

Mukerjee Type Strain	V 154	Ogawa	True cholera
" " "	Pr 508	Ogawa	True cholera
" " "	Pr 528	Ogawa	True cholera
" " "	Pr 542	Ogawa	True cholera
" " "	Og 29	Ogawa	True cholera
East Pakistan CRL	S 10	Nikojima (?)	True cholera
" " "	S 1187	Inaba	True cholera
" " "	S 1197	Inaba	True cholera
" " "	5/64	Water NC vibrio Heiburg group I	
" " "	688/64	Water NC vibrio Heiburg group II	
Indonesia	Ind-9	Ogawa	El Tor
Malacca	3734	Ogawa	El Tor
Bangkok	SE 52	Inaba	El Tor
Bangkok	SE 47	Inaba	El Tor

Five of 44 (11%) of the stools tested directly for lysogenic phage showed phage activity (Table I). With enrichment, phage was detected from 11 of the 44 (25%); it is pertinent that two stools, positive for phage on direct examination, were negative after enrichment. In toto, 13 of the 44 stools (30%) were lysogenic. The phage of five of these specimens was detected by Malacca 3734; the other phages varied in host organism.

Phage activity was detected by chloroform, heat and/or ultraviolet light treatment of the stool or stool enrichments. Ultra-violet treatment appeared to give the most frequently positive results (Table II).

TABLE I

Bacteriology	Results Tested	Lysogenic Before Enrichment.	Phage after Enrichment	Total Positive Stools
Ogawa	2	2	1	2
Inaba	10	0	1	1
NCV	3	2	1	2
No Pathogen	29	1	8	8
Total	44	5	11	13

TABLE II

Effect of Various Inductive Treatments on Lysogenic Phage Activity of Stools and Stool Enrichments.

	Number of Stools showing phage activity after various treatments.						
Stools	Chloroform	Heat	Ultra-violet	Chloroform + heat	Heat + Ultra-violet	Chloroform + Ultra-violet	Chloroform + heat + Ultra-violet.
Before enrich-ment	0	0	2	1	0	1	0
After enrich-ment.	0	2	0	0	0	1	3

FIGURE 1

Growth Curve of V. cholerae (M-154)
under Various Conditions.

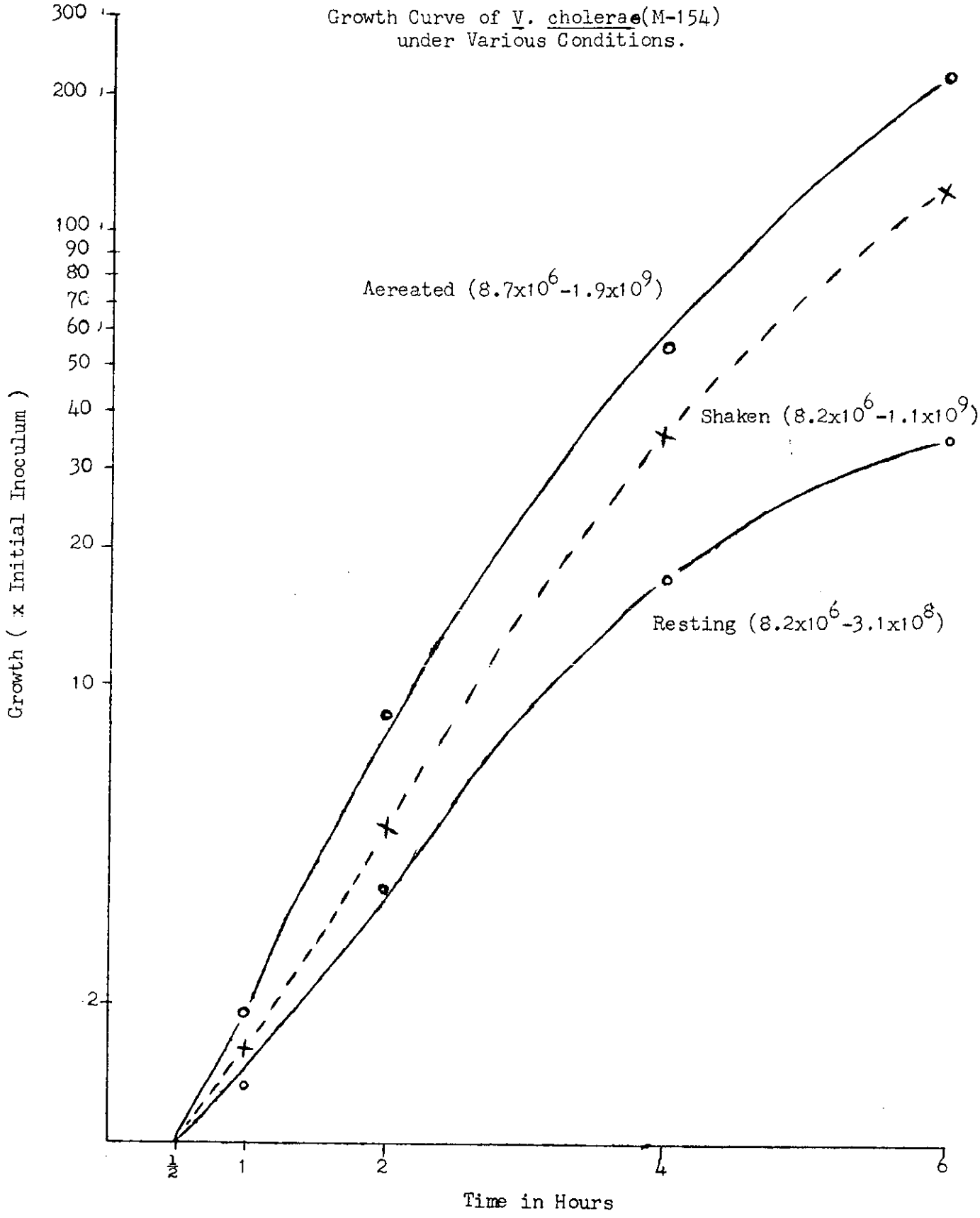
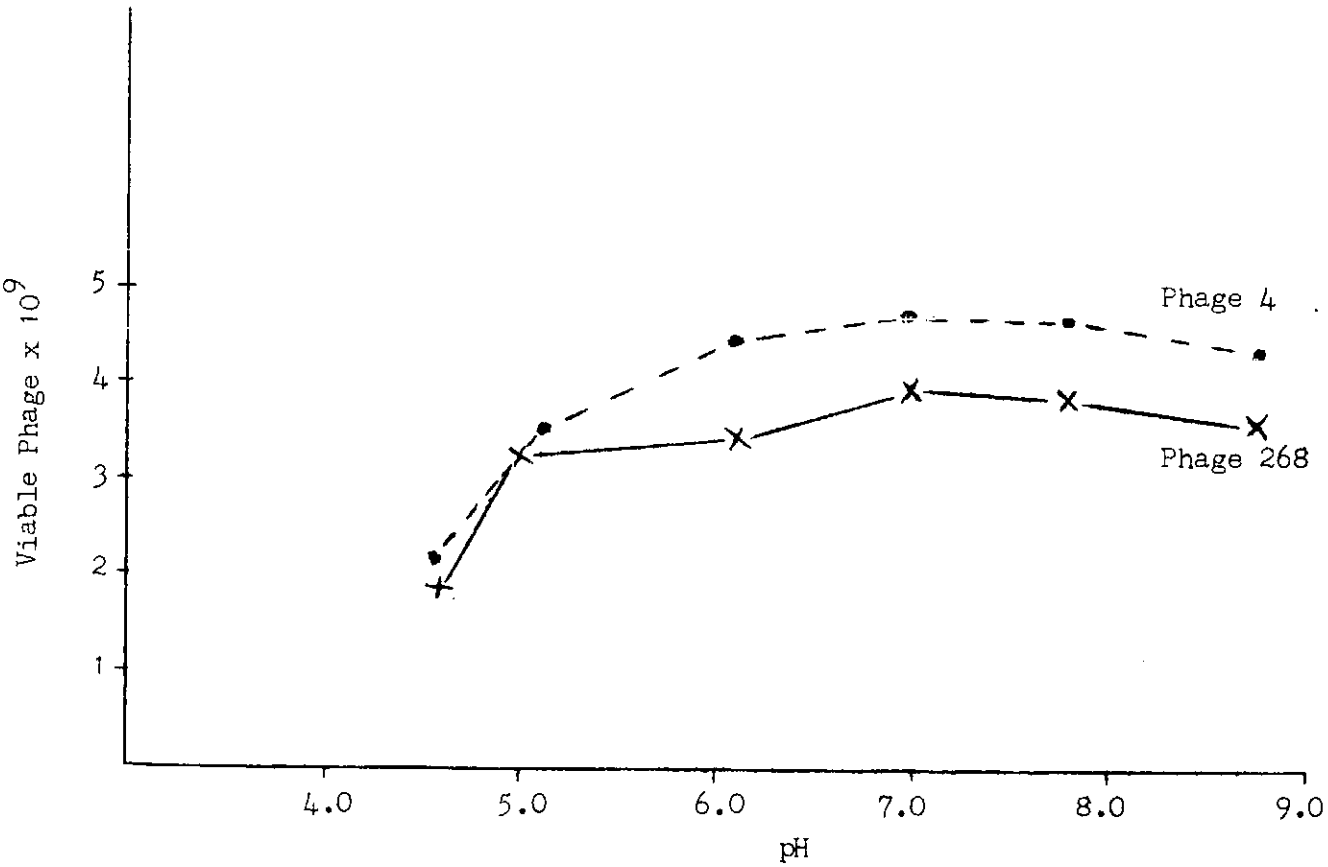


FIGURE 2

Effect of pH for 24 hrs on
Bacteriophage Titer



(C) DRUG SENSITIVITY OF VIBRIOS AND OTHER BACTERIA ISOLATED FROM DIARRHEAL PATIENTS:

The antibiotic and sulfa drug sensitivity of recent isolates from diarrhea patients was determined in order to provide guidance for therapy.

The tests were performed by the tube dilution method. The antibiotics were dissolved in trypticase soy broth while the sulfonamides were dissolved in 25 ml of 0.1 normal NaOH , pH was adjusted to 8.5 and then added in the necessary amount to tubes containing 5.0 ml alkaline peptone water, preparing serial two-fold dilutions. Approximately 10^4 bacteria, grown overnight in T_1N_1 broth, were added to each tube, the samples incubated overnight and observed for bacterial growth or inhibition.

Thus far, this method has been used to study the following: 45 strains of Vibrio cholerae; 30 strains of El Tor; 1 strain of non-cholera vibrio; 4 strains of unidentified organisms isolated from an outbreak of probable food-borne diarrhea in a group of rickshaw pullers; 4 strains of E. coli from "non-vibrio cholera"(1) patients; and 16 pairs of vibrio strains isolated from patients at admission and at discharge. The results are given in Table I.

The antibiotics were effective at 0.625-1.25 µg/ml concentration except for streptomycin which was effective only at concentrations greater than 20 µg/ml. These concentrations can easily be attained in a patient. Only one patient showed a significant difference in sensitivity between strains isolated on admission and at discharge; this was a woman whose first isolate was inhibited by 1.25 µg/ml terramycin and 0.625 µg/ml tetracycline. Her last isolate, 5 days later, was inhibited by 5.0 and 2.5 µg/ml respectively; she had been treated with fluids only and had not received any antibiotics!

Most of the sulfa drugs are effective only at a concentration of 5 mg/ml or more, except for El Tor vibrios in which the effective concentration is 0.625 mg/ml or less. Concentrations of 5 mg/ml [illegible] in stool would be difficult to obtain in a patient, necessitating a daily dose of 50 grams or more if there is no absorption.

(1) See Lindenbaum et al, 'Non-vibrio Cholera' this report.

TABLE I

Susceptibility of Bacteria to Various Drugs

Minimum Inhibitory Drug Concentration	NUMBER OF STRAINS						
	V. cholerae	El Tor	NCV	Food Outbreak	E.coli	Vibrio Pairs from Patients	
						First	Last
Tetracycline:							
5.0 mg/ml or more	0	0	0	0	0	0	0
2.5 µg/ml	9	0	0	2	4	0	10
.625-1.25 µg/ml	36	29	1	8	0	16	15
.31 µg/ml or less	0	1	0	0	0	0	0
Terramycin:							
2.5 µg/ml or more	0	0	0	0	4	0	1
.625-1.25 µg/ml	43	27	1	8	0	15	15
.31 µg/ml or less	2	3	0	2	0	1	0
Chloromycetin:							
5.0 µg/ml or more	0	0	0	0	0	0	0
2.5 µg/ml	4	0	0	0	4	0	0
.625-1.25 µg/ml	41	8	1	8	0	16	16
.31 µg/ml or less	0	22	0	2	0	0	0
Streptomycin:							
40 µg/ml	15	17	0	5	0	7	5
20-30 µg/ml	26	11	1	3	0	7	7
10 µg/ml or less	4	2	0	2	4	2	4

Social Groupings in Rural East Pakistan

by

Dr. R. Glasse

Introduction:

In September, 1962, Dr. Robert H. Black suggested to the Technical Committee of Pakistan-SEATO Cholera Research Laboratory (CRL) that anthropologists be employed to investigate social and environmental factors that might be important in the spread of cholera. He pointed out that little is known of social life in rural East Pakistan and that this knowledge might lead to a better understanding of the epidemiology of cholera. The Committee accepted this recommendation, and asked Dr. Black to recruit a husband and wife team. The Australian government generously undertook support of the work This paper presents some of the findings of the investigation.

Shaitnal Union, the community under study, comprises 11 villages in the north-western corner of Matlab Thana in Comilla District. Shaitnal lies on the Tropic of Cancer, 23 miles south-southeast of Dacca on the eastern bank of the Meghna River. My wife and I carried out a year of field work in this area, mainly in Shotiki and Segundi villages. During most of the year, I had the valuable assistance of Mr. A.K. Mozibul Haque, a local resident from Senerchar; my wife profited from the help of Mrs. Stella Das, a Bengali Christian from Dacca, who lived with us for several months. Our base was at Shotiki Bazar, first in a houseboat (which threatened to sink), then in a tin shed belonging to Sharifullah School, and finally in a brick house built by CRL.

The social groupings in Shaitnal are representative of those found in south-central East Pakistan. As several studies of Bengal Hindu society have been published (see bibliography), I shall not here describe the Hindu portion of Shaitnal society. Suffice it to say that Hindus form about 7% of the population and that most of the men are landless fishermen. The majority of the Muslims are cultivators; a small fraction are shopkeepers and businessmen.

My concern then is with the three Muslim groupings; (1) the gusti, a patrilineal lineage and its component families; (2) the mallot or samoj, a corporate social and Islamic association of great importance; and (3) the gram or village settlement. Another paper will discuss the networks of relationship created by kinship and marriage, and a third will describe social behaviour in relation to cholera, as well as the ideas and beliefs of Shaitnal people about the causes, spread, and proper care of illness. This paper begins a series, introduces the people of a typical rural community, and describes the social groupings that are basic to their life.

Environment and History:

In his well-known geography of India and Pakistan, Spate[1] divides the Bengal delta into five subregions. Shaitnal belongs in the division known as the eastern margin, which includes the Surma valley and the plains along the

Meghna River and the Chittagong coast. This embayment of lowland "forms the most amphibious part of Bengal during the rains."[2] The lower tracts are flooded to a depth of eight to fifteen feet, and homesteads are built on earth platforms fifteen to twenty feet high.

The climate is monsoonal. Winter is from December to February; the days are dry and cool, the nights are cold. From March until the arrival of the monsoon in June, heat and humidity prevail; this is the time of the "mango showers." Local depressions and conventional movements produce violent, thundery squalls--nor'westers--which occasionally reach hurricane force. From June through August, some relief from the heat comes with the rains and the increased cloud cover; during this period rain falls on an average of eighteen days per month, and accounts for more than half the annual total of ninety inches. Rain continues into September, but with fewer wet days. In October, the monsoon is in full retreat, and the temperature rises. Again, low pressure over the Bay of Bengal may unleash cyclonic storms.

The temperature and rainfall observations recorded at Comilla, forty-five miles east and in the same latitude as Shaitnal, are characteristic of the climate. (See Table I).

During the wet season, the rivers rise, and the fields are heavily flooded. At the height of the monsoon, little land is visible and travelling must be done by boat. "To say that travelling has to be done by boat gives... an inadequate idea.... Half a dozen huts are clustered together on a hillock a few yards square, and the inhabitants cannot proceed beyond that hillock, whether to visit their neighbors or their fields, to go to market or to school, without wading, swimming, or travelling in or on something that can be floated." [3]

Though little natural vegetation remains on the land, the countryside is far from treeless. Each settlement carries clumps of bamboo, the Indian date and the toddy-palm, banyan, pipol, tamarind, and mango trees. The aquatic flora is also rich; the ponds and canals are choked with reeds and sedges. "In the past two or three decades, the water hyacinth has spread with its usual disastrous proliferation, blocking many waterways to navigation and drainage."[4]

The agriculture of Shaitnal is dominated by jute and rice. Other field crops include millet, oil seeds, tumeric, chillies, onions, sugar cane, and pulses. In addition jack fruit, banana and payaya (paw-paw) grow abundantly. Vegetables such as pumpkin, cucumber, and gourds grow in kitchen gardens.

The inhabitants of Shaitnal believe that the thana was sparsely settled before the invasion of the Moghuls and the reign of Sultan Hussain Shah of Gour in the sixteenth century. The first settlers in the Union came from Barisal, Comilla, and Khulna about 250 years ago. The land was then part of the Zamidary of Kartikpur; the proprietor lived across the Meghna River near Faridpur. The rent collector (na'ieb), who was the proprietor's local

1. 1954: Chapter 19
2. Ibid, 532
3. Quoted in Spate, 1954:533
4. Spate, 1954:526

representative, lived just north of the Union of Sikirchar. Later, the land was transferred to the zamindar Sher Ali, who held all of Matlab thana. About one hundred years ago Matlab was divided into five smaller zamindaries; soon after, two families living at Shaitnal and Sengerchar purchased zamindary rights when the British auctioned an estate that had not met the revenue demand. Subsequently, other families purchased interests in these lands from the zamindars and became intermediary proprietors (talukdars), collecting revenue from the cultivators, paying a portion to the zamindar, who in turn met the fixed demand of the British government.

At Shaitnal, the landlords had great power, were respected and feared by the cultivators, many of whom were landless. The zamindars collected ten times the value of the fixed demand; they could raise rental charges at will, and often did so. Legislation to abolish the zamindary system, with compensation to landlords, was passed in 1950 soon after independence. This legislation took effect at Shaitnal in 1956. Today the prestige of the zamindar and talukdar families has diminished considerably. They have lost much of their income and now are less frequently called on to resolve disputes. Nevertheless, they are active in politics and still possess large personal land holdings, which give an adequate if not luxurious return.

Today the class structure of Shaitnal is in flux. The old bases of prestige--nobility of lineage, interest in land, adherence to traditional Islamic values--have been partly undermined. The newly emergent values are higher education, secure, remunerative urban employment, and success in business.

Social Groupings:

Though found in parts of Bengal, the compact village is not typical of Matlab thana. In Shaitnal settlements situated on river levees approach a linear layout but the majority have little form. The spacing of homesteads is so continuous that little pattern is discernable; but to describe this settlement as dispersed would be an abuse of language.

Shaitnal Union covers six and one half square miles, including canals and the fields which are completely inundated during the wet season. In this space live 14,000 people, an average of 2,200 persons per square mile. If fields, tanks and waterways are excluded, the density of inhabited land rises to 22,000 per square mile, equal to urban concentration. Few rural populations in the world are so closely packed.

1. The Gusti

The homestead or bari is the basic spatial and residential unit; the people who live there form the gusti, a patrilineal descent group. Each bari contains dwellings, kitchens, barns, often a bungalow for guests, and a dugout tank for water. The houses have high-pitched roof, thatched with grass, or made of corrugated iron if this is within the householder's means. The walls are made of split bamboo, reed or jute-stick matting, or more rarely of tin sheets or brick; the floor is hard-packed mud. Much of family living, including preparing and cooking food, takes place out of doors, either in an open kitchen partitioned from the central courtyard or in the square itself.

A Bengali's concept of home includes an entire homestead with several houses, a courtyard, tank, and outbuildings. In a Bengali homestead, most of the householders are closely related; they are ek gusti, one family in the extended sense. The gusti has a known genealogy of three or four generations, and all or most of the males are patrilineally related. The gusti owns in common such property as tanks for drinking and washing, latrines, and a bungalow kept for visitors. Generally, the senior male is head of the gusti, and he may intercede in family quarrels. Marriage within the gusti is permitted, but rarely occurs. A man who leaves his bari to work in towns continues to take an interest in his home; most urban jobholders return to the village frequently, particularly at Id and other holidays.[5]

Figure 1 shows the physical layout of a bari; the genealogy (Figure 2) indicates kinship ties among members of the gusti and the people who live in each house. The ten occupied houses form nine hearth groups; households 3 and 4 share a common kitchen. Two types of family occur: the simple or nuclear family, as in houses 2, 7, 9, 10 and 11, and the joint family, as in 1, 3 and 4, 6 and 8.

The simple family becomes a joint family when a son brings his wife to live in his father's house. The Bengali phrase ek shonga bhukto poribar means literally "joint meal family", indicating one of its essential characteristics. Bengali has no equivalent for simple family; people refer to the simple family as vunno, separate, in contradistinction to the joint family.

The second characteristic of the joint family is the possession of joint property, including land and rice granaries. The title to this property vests in the father during his lifetime; upon his death, title passes to his heirs as a joint estate. The joint family comprises either a man and his adult married son or sons with wives and children, or brothers with their families. The family head allots work, controls expenditure, and directs the sale or use of produce; the rice granary stands in his house, and his wife organizes cooking and distributes food among the women for their families.

As shown in Table II, the sample of 21 gusti is composed of 56 simple and 19 joint families. The simple family averages 4.5 members, the joint family 8.2. Each simple family lives in a single house; but 12 of the 19 joint families occupy more than one dwelling (Table III). Chart 1 shows examples of joint family structure and house occupancy. As the joint family expands by the marriage of sons and the birth of children, new houses are added.

The partition of a family estate does not automatically follow the death of the father; partition is often delayed for some years, for the heirs may have advantage in preserving the property intact. When the land is of considerable size, i.e., more than 10 acres, the brothers can manage more efficiently without division; one may specialize in keeping records and accounts, another may manage the labour, a third may take agricultural training. Such large estates are rare, however, and joint families with small holdings tend to break up quickly.

5. In a sample of 144 joint and simple families, 46 (6.5%) of the 706 members were living away from their homestead at the time of census. Ten were in Chittagong; 9 in Dacca; 7 in Narayanganj; 7 in Comilla; 4 in Sylhet; 3 in West Pakistan; and 1 each in Mymensingh, Faridpur, Rajshahi, Calcutta, Farajkandi, and Matlab. Thirty-one were males over age 15; 5 were females over 15; 5 boys and 5 girls under 15.

The joint family usually divides because of friction among the women who share the common hearth. One cause of trouble is dissatisfaction about apportioning food; according to its number, each family is entitled to a share. As the older brothers generally have larger families than the younger, the latter receive smaller portions. This annoys the younger brothers' wives, who know their husbands have a claim on the joint property equal to that of the elder brothers. The junior wives favor dividing the estate, the senior stand to lose. A younger brother's wife may also resent the way the family head allots work. Security is another motive: if her husband dies before the division, she and his children lose their rights in the property.

In Table II, the sex ratio in the simple family is 1.15 males per female. The bias increases in the joint family where the ratio is 1.66 males per female. This difference, significant at the 1% level (chi squared = 6.97, df=1, $p > .01$), reflects the difficulty women have in living together in the joint family. Despite the wide range in joint family size, - from 3 to 18 members, - no family has more than three married or widowed women members (Table II).

When the joint family breaks up, the result is not necessarily simple families; one or two simple families may detach themselves, leaving a smaller joint family behind. This situation occurs when the younger sons are immature or when some of the brothers take jobs away from home. Single men have less interest than married men in leaving the joint family. The unmarried need women to cook for them; because their sisters marry out at the age of 12, the men must rely on their mothers or wives of their elder brothers.

Thus, development of the family follows a cycle. When internal friction grows, the joint unit breaks up. Several simple families may result at once, or over the years sections may break away from the remaining joint family. In its turn, the simple family expands with the birth of children. When a son brings his bride to his father's home, the simple family becomes a joint unit, and the cycle begins anew.

Gusti are identified by quasi-hereditary titles which formerly indicated their position within a three-tiered heirarchy (Table IV). A son has the right to his father's title, though he is not obliged to assume it. Because these titles are not officially conferred, an ambitious man may acquire a higher title than that of his father. In the past the purchasing of a zamindary enabled a man to gain a higher title. Today, this is no longer possible; if not inherited, title must be achieved by the acquisition of large personal land holdings or by amassing wealth. A man seeking prestige adds the desired title to his signature on documents, such as wedding invitations and land deeds. If people accept his new status, they address him by the new title. Men who change their title are usually middle class; regardless of their income, aristocrats disdain title changes.

A title once indicated profession or occupation, as shown in Table IV. Though no longer a guide to all social behavior, the titles are still relevant in marriage. A man of high standing will marry a girl of lower title only if she is exceptionally beautiful and fair, rich or highly educated.

In a settlement of thirty gusti, only six or seven titles are used. Profession of a common title does not indicate cosaguinity; gusti with the same title usually are of separate origin.

In summary, the inhabitants of the bari form a patrilineal descent group, averaging twenty members. Approximately two-thirds of gusti members live in simple families which are domestically independent. The other third belong to joint families which share a common hearth. The males of the joint family possess rights in a common estate: the father holds the title during his lifetime, but the sons become coparceners upon his death.

2. The Mallot

After the gusti, the most important social grouping at Shaitnal is the mallot. Mallot is the local pronunciation of the Arabic millot. In reffering to mallot, Bengalis also use somaj, meaning society or association. Unlike the gusti or the village, the mallot has no name; instead in conversation people refer to it by the title or personal name of one of its prominent members. The mallot is a multi-purpose institution. The group eats together on festive occasions. Because of purdah, women cannot participate publicly in these gatherings; but when their husbands give a feast, the women help prepare the food. The mallot assembles at religious festivals such as Id; at sacrificial meals during the month of Ramadan; at marriages and deaths of members; and at rites of circumcision (musulmani). The mallot also meets for common prayer when flood, epidemic, or other calamity threatens. Mallot members visit each other in sickness. When help is needed to protect crops, members assist each other. Men with large holdings especially rely on this help. They do not pay the workers, but must provide goatmeat curry when the task is done.

Each family pays a monthly subscription (chanda) which is used to support a religious leader (mulla) who serves the group. At Shaitnal, the fee ranges from one anna to one rupee eight annas, according to the family income.

Mallots have no fixed size. At Shaitnal, they range from about 80 to 400 members, including women and children, and one north Dacca village of 5,000 has five mallots. The composition of three Segundi mallots is given in Table V. In this sample, the average number of members is 235, of whom 64 are males over the age of fifteen. (See Table V).

Families living in one bari usually belong to the same mallot, but this is not obligatory. A man may join a different mallot as a result of a quarrel in his bari. Although mallot membership is usually for life, there is no barrier to leaving and joining another. A person may also belong to two mallots at the same time. For example, if a man marries a girl with no brothers, her parents encourage him to settle on their land with the understanding that their daughter will inherit a share in her father's estate. A man in this position seeks admittance to his wife's mallot and also retains membership in his own. He then has two sets of obligations to fulfill and can call on both groups for help.

At marriage a bride becomes subject to the authority of her husband's mallot. If she commits a matrimonial offense, both her own and her husband's mallot mete out punishment. While a woman primarily belongs to her husband's

mallot, she retains certain rights and obligations in her own -- wife and mother in one, she is sister and daughter in the other.

The corporate character of the mallot is demonstrated in many kinds of behaviour. Although not a kinship group, the mallot members express unity in kinship idiom. A man says of his mallot: "We are like one gusti." Members address each other by kinship or personal names,[7] and the norms of kin behaviour are observed by all. The organization of festive meals clearly shows the corporate unity. When a wealthy or pious man sacrifices a cow, he invites all the men of the mallot to partake of the meal. They bring all or some of their sons, and daughters under the age of about eight. If they cannot afford to provide food for the whole mallot, three or four men unite to sacrifice a goat; they invite the senior male from each hearth group, whether it be a simple or joint family. By this means, they feed representatives of the whole group. Men who cannot afford meat for a large group purchase fish instead and invite only mallot leaders, the mulla, and a few close relatives. However few the invited guests, they represent the unit.

The unity of the mallot can also be observed when quarrels arise between a member and an outsider. His kinsmen immediately support him, while other members intercede. If mediation fails, the whole mallot rallies around the member. The fracas may lead to fighting with bamboo sticks.

Within the mallot, the rich give alms to the poor, all who can afford help others to weather a crisis. Charity is not limited to the mallot, but the obligation is stronger within it. As well as individual alms-giving, the entire group may give to the needy, using income from fines levied against delinquent members or from contributions of rich members.

The mulla is not a mallot official. His duties are to lead prayers at the mosque, to give religious instruction to the children, and to guide his followers in the way of Islam. One mulla may be supported by several mallots. His work is teaching rather than controlling his supporters.

While the mallot has no formally recruited officers, its leadership is seldom in doubt. The group is governed by a small number of informal leaders called matbars, who secure their position through force of character and social prestige and often by vying for power. The matbars adjudicate quarrels, fine offenders, administer corporal punishment, and represent the group in disputes with outsiders. Other leading men support their decisions and review their acts. The matbars can admit a man to membership and can expel anyone who offends the group. An ostracised person has a hard time. No one cooperates with him. He cannot enter the mosque. He receives no invitations. His existence as a social being is denied. He can, however, apologise and make amends; when he so does, the matbars may reinstate him.

Although the mallot has no fixed size, it has an optimal size which varies with local conditions. These include density of settlement; the presence of other institutions, such as courts to resolve disputes; and the

7. In certain kinship relationships, the senior person is expected to address the junior by personal name.

regional distribution of wealth and prestige. Informants say that in the past the mallot and the village were one unit, that as the settlements expanded, internal quarrels became more difficult to settle, and finally the mallot split forming new groups. The process of fission can be seen today. When a mallot becomes too large, any internal quarrel will divide it.

3. The Gram

Table VI shows the population of the eleven grams in Shaitnal Union. The mean size is 1237 members; the range is from 213 to 4168 members. Seven of the eleven units are divided into sections called para, which are socially significant only for Hindu fishing communities. The para is a neighbourhood within the gram; the inhabitants of one para rarely compose a mallot.

In English, the word "village" has mixed connotations, referring both to a physical settlement with a concentration of houses and to the people who live there. The Bengali equivalent, gram, shares this ambiguity. In addition gram can be used to include not only residential space, but also the fields adjacent to the settlement as fixed for revenue purposes. From the air it is difficult to see where one gram ends and another begins, so dense is the population, so amorphous the aggregation of houses. This difficulty does not exist in people's minds, however; everyone knows to which gram he belongs, and most people are familiar with the gram's geographical limits. But the social unity of the gram is questionable. The gram does not perform any collective activity; it owns no property in common; it has no official leader or administrative structure; it has no common centre or village square.

Nevertheless, certain ties occassionally unite the members of the gram; villagers do have a sense of common identity. Schoolboys compete in games and dramatic contests as representatives of one gram. The people living in a gram feel an attenuated kinship, though many among them have no cosanguinial and little social connection. Within the gram, it is polite to use kinship forms even when no true relationship exists. In conflicts, gram members support each other against outsiders.

Comment:

Although this paper has not been concerned with cholera per se, knowledge of social organization may be of use in studying cholera epidemiology. For example, the family system provides a ready-made unit for testing hypotheses about the precise role of food and water in the process of infection. Comparison of incidence of cholera in joint and simple families should reveal a higher male attack rate in the joint family, which has a preponderance of males whose food is prepared at a common hearth. If differences exist along familial lines, the role of the age factor might also merit study., Consideration of the structure of these social groupings may be useful in planning and interpreting field studies.

Summary:

This first report of field work at Shaitnal describes the three most important social groupings in rural Muslim Bengal. The gusti is a patrilineage, averaging 20 members who live in a single bari or homestead. Within

the gusti the component units are joint and simple families. The joint family cooks at a common hearth, stores rice in a common granary, and has an interest in a joint estate. After the death of the father, the joint family may either continue as a unit with the eldest son as head, or may divide into smaller units with each son receiving a share of the inheritance. Members of the gusti bear the same title, live in close proximity, own common property; and usually belong to the same mallot.

The mallot, the corporate group of greatest social significance at Shaitnal, usually numbers in the hundreds. Membership in the mallot is by birth or prolonged residence; but leaders of the mallot can accept or reject newcomers, and they can expel from the membership anyone who offends the group. Though not a kinship group per se, the mallot observes the forms and etiquette of kinship. The members unite on festive occasions; support a mulla for religious service and Islamic teaching; provide for the needy within their ranks; settle internal disputes, and unite against outsiders who threaten any member. The mallot is the largest corporate unit in the gram.

The gram or village is a social aggregate of limited importance; it performs no characteristic social activities, owns no property in common, and has no official leadership of administrative structure. The gram is significant in terms of social identity; its boundary is the limit of collective feeling, the dividing line between 'we' and 'they'.

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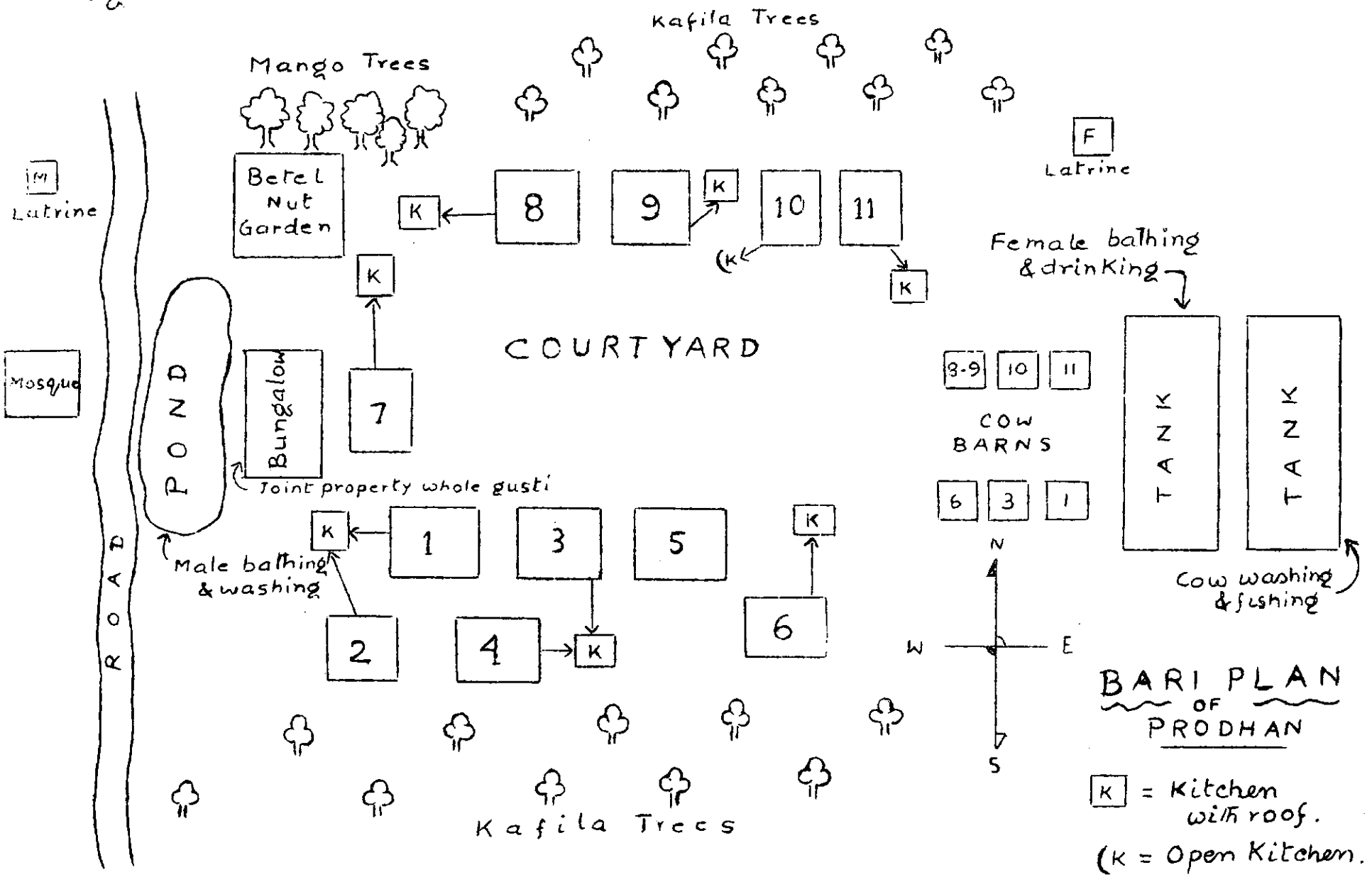
Figure - I

Another Bari (No Title)



BARI PLAN OF PRODHAN

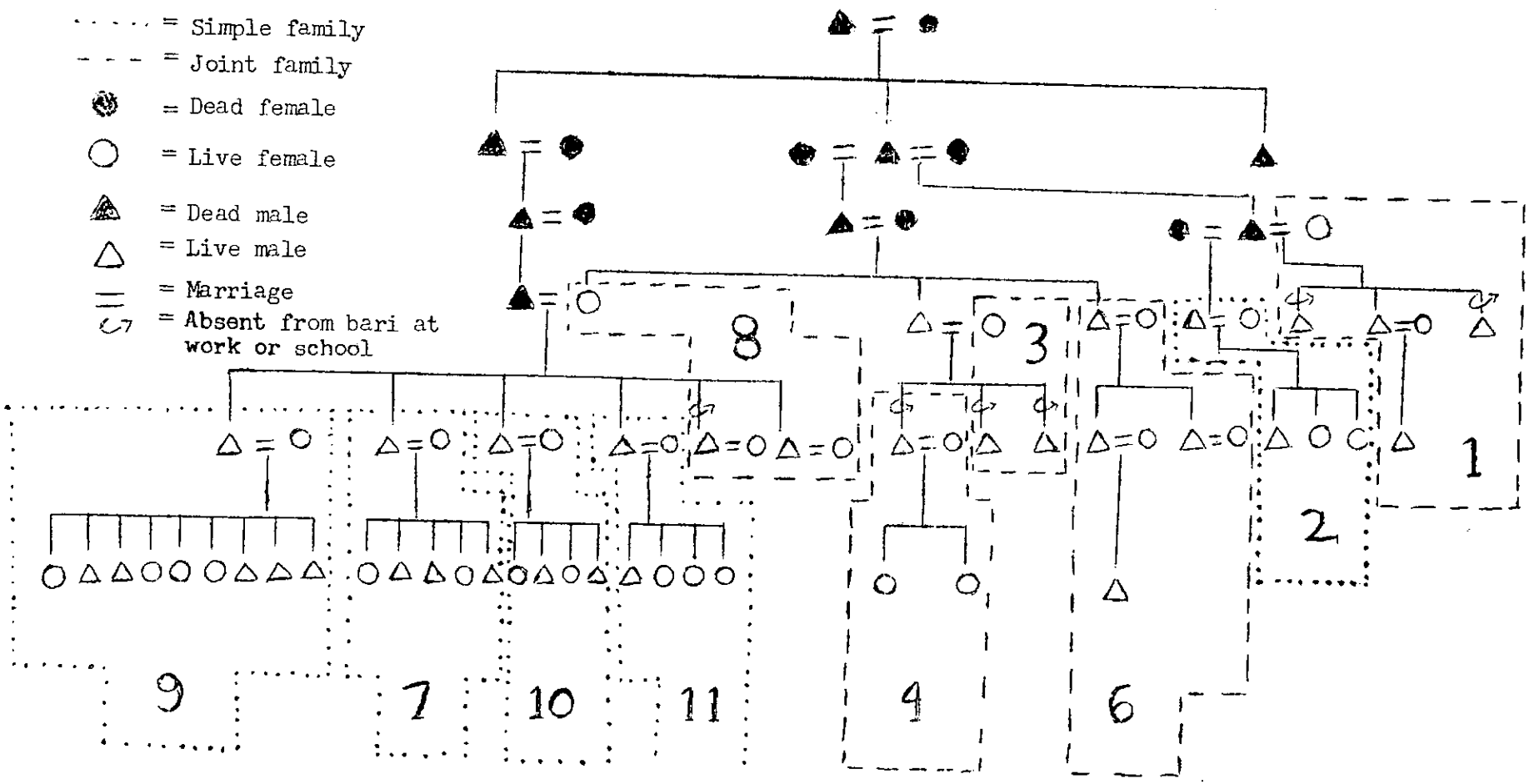
K = Kitchen with roof.

(K = Open Kitchen.

Another Bari (Bepari)

FIGURE 2

GENEALOGY AND HOUSE OCCUPANCY IN PRODHAN BARI

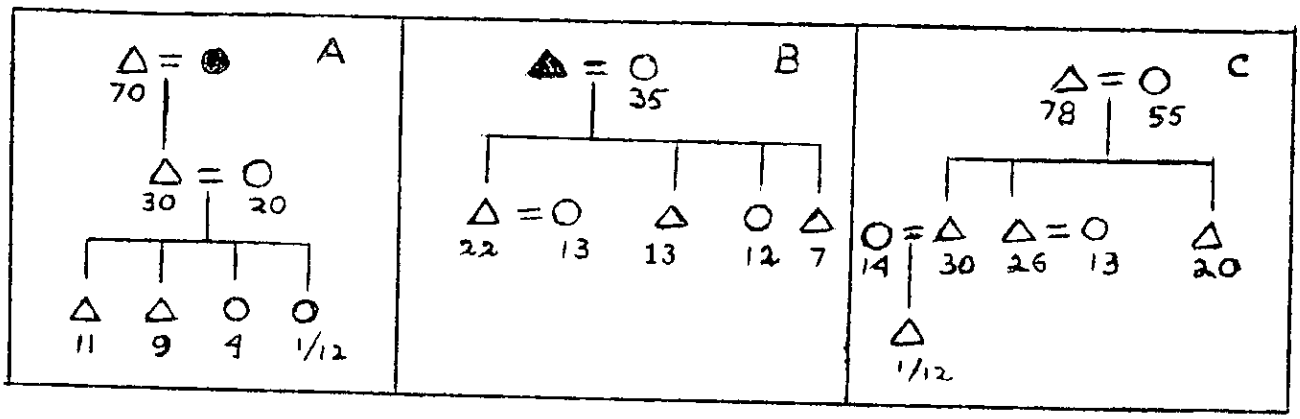


NB : 9 = House numbers in bari plan

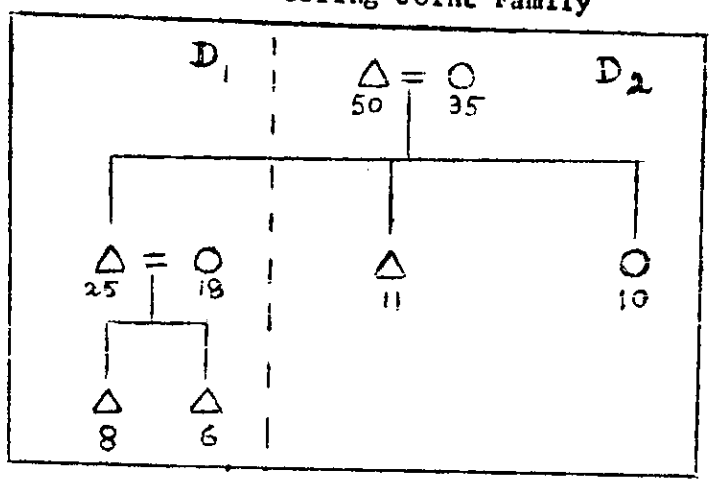
CHART 1

SOME EXAMPLES OF JOINT FAMILIES AT SHOTIKI

Single Dwelling Joint Families



Double Dwelling Joint Family



Triple Dwelling Joint Family

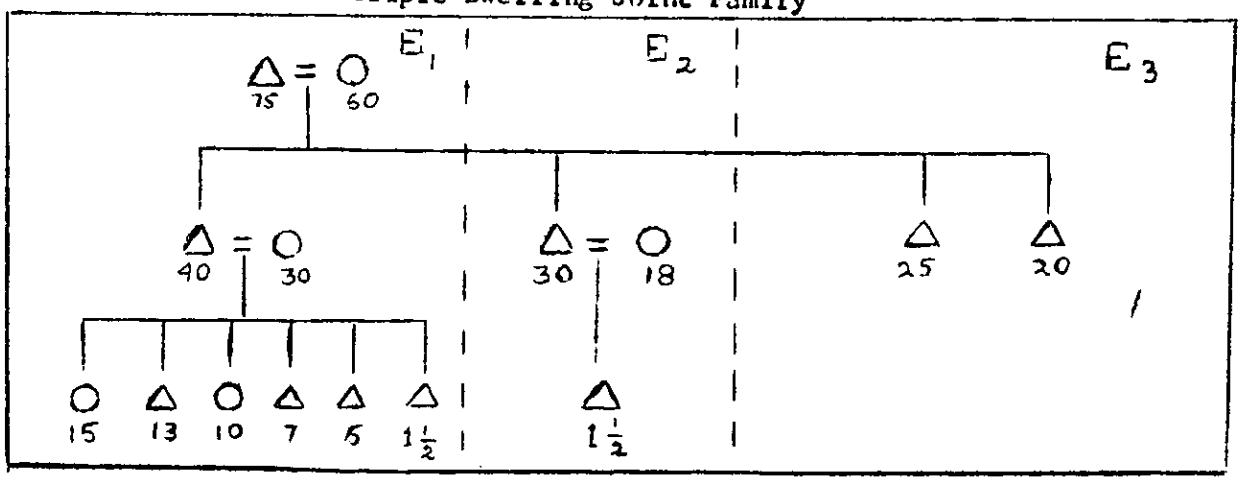


TABLE I

Temperature and Rainfall at Comilla, 1957-1960 monthly averages.

Month	Temperature Mean daily Max.	Min.	Range	Rainfall in inches	Number of rainy days
January	78.5	52.9	25.6	.32	0.8
February	82.2	57.5	24.7	1.22	1.9
March	89.7	67.1	22.6	3.01	3.5
April	92.3	73.3	19.0	5.85	6.5
May	91.1	74.9	16.2	12.39	11.8
June	88.3	76.8	11.5	18.28	17.1
July	87.7	77.1	10.6	16.42	18.8
August	87.5	76.9	10.6	17.18	17.8
September	88.6	76.9	11.7	11.35	13.7
October	88.2	73.4	14.8	6.72	6.6
November	84.0	64.1	19.9	1.29	1.6
December	79.2	54.8	24.4	0.30	0.4
				94.33	100.5

Source: Comilla District Census, 1961.

TABLE II

Composition of 21 Lineages in Shotiki (Gusti)

	Simple families	Joint Families	Totals
Number	56	19	75
Individuals	254	135	409
Mean Size	4.53	8.16	-
Modal Size	4	7	-
Range	1 - 10	3 - 18	-
Sex Ratio	1.15	1.66	-

TABLE III

Number of Houses Occupied by 19 Joint Families

Houses	No. Joint Families	No. of Persons	Mean Persons per House	Mean Joint Family Size
One	7	44	6.3	6.3
Two	9	67	3.7	7.4
Three	3	44	4.9	14.7
	19	155	4.6	8.2

TABLE IV

The Traditional Heirarchy of Some Titles in Use at Shaitnal.

Class	Secular Title	Religious Title
Uchchobungsha "high"	Chowdhury	Khondoker (those who first preached Islam)
	Mia (Zamindar) (i.e. landlord)	Mir (those who wrote amulets with Koranic verses)
	Talukdar (landlord by subinfeudation)	
	Bhuiya (land owner with large holdings)	
	Sarkar (writer or scribe)	
	Prodhan (leader or judge)	
Madhya bungsha "middle"	Khan	Mulla (imam and Koranic teachers)
Servants of the zamindars	(Dewan (advisor to zamindar)	Miji (minor religious teacher, assistant to the imam)
	(Halder) bodyguards and (Dhali) police for the (Laskor) zamindars	Munshi (minor religious teacher)
Nicho bungsha "low"	(Napit) barber	Fakir (popular saints not recognized by Islam)

TABLE V

The population of Three Segundi Mallots, 1964

Mallot	MALES		FEMALES		Totals	No. of simple & joint families	No. of lineages
	Under 15	Over 15	Under 15	Over 15			
A	26	35	27	44	132	31	5
B	93	100	98	81	372	70	12
C	53	57	38	54	202	43	5
	172	192	163	179	706	144	22

TABLE VI

The Population of Shaitnal Union 1961

Gram	Para	Males	Females	Total
Khaskandi		102	111	213
Shaitnal	(Boro Shaitnal	857	827	1684
	(Shaitnal Malpara	539	467	1006
	(Chotto Shaitnal	742	736	1478
	(All	2318	2030	4168
Segundi		635	590	1225
Kalipur	(Kalipur Bazar	315	269	584
	(Kalipur	256	278	534
	(All	571	547	1118
Lalpur	(West Lalpur	442	447	889
	(East Lalpur	406	343	749
	(All	848	790	1638
Imampur	(Imampur	268	309	577
	(Chengerchar	187	172	359
	(All	455	481	936
Shotiki	(Shotiki	130	121	251
	(Shotiki Bazar	117	110	227
	(Shotiki Malpara	187	134	318
	(All	434	365	796
Dhalikandi		116	117	233
Keshairkandi		312	286	598
Barivanga	(North Barivanga	233	226	449
	(South Barivanga	144	145	289
	(All	367	371	738
North Sengerchar	(Molla Kandi	236	229	465
	(Chan Sardar	324	323	647
	(Mulber Majir	308	279	587
	(Nij Sengerchar	116	134	250
	(All	984	965	1949
				13,612

Attitudes to Health and Disease in Rural East Pakistan

by

Shirley Glasse

Introduction:

Anthropological fieldwork was begun in November, 1963, to collect data relevant to the spread of cholera in a domestic setting. This required the study of local treatment given to cholera patients and of indigenous attitudes to causation and prevention of the disease. It became evident while collecting this information that to understand views about cholera it was necessary to study attitudes to health and disease in general. During the coming year additional data will be collected on attitudes towards cholera, personal hygiene, the handling, preparation, and distribution of food, and other social factors which may be relevant to the faecal-oral route of transmission of the disease.

This is an account of the treatment of disease in a predominantly Muslim rural community in East Pakistan. This report is not concerned with the extent to which non-Western medicine is magico-religious or rational. The aim is to reveal ideas implicit in the treatment of disease and to show that these ideas pervade wider realms of belief and action in the society.

Fieldwork was mainly carried out in two settlements of Shaitnal Union(1) named Shotiki and Shugundi. Shugundi is an entirely Muslim settlement of agriculturalists and small businessmen. Besides Muslims, Shotiki includes three Hindu caste groups - fishermen, a caste of Malis whose male members are employed as Union Council Choukidars, and weavers (though this traditional occupation is no longer followed by the caste members who now include in their ranks a primary school teacher, a kobiraj doctor, shopkeepers and a goldsmith). The informants therefore represent a stratified, heterogeneous group of people, differing in religion, occupation, and income. At one end of the scale the Muslim large landholders are the wealthiest and politically most powerful. Hindu fishermen are the poorest group.

Different levels of medical sophistication are found throughout the community. Middle and upperclass Muslims with business interests of their own or kinsmen in employment in the city have access to medical treatment in Dacca and Narayanganj. Poor Muslims or poor Hindus, if they could afford it, are timid about seeking aid beyond the boundaries of known territory. Rich and poor alike seek medical help from local practitioners.

In the Shaitnal area there are about 18 male practitioners with dispensaries in one of three bazaars(2) or practices operating from their own houses.

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(1) In Matlab Thana, District of Comilla. See R.M. Glasse for an account of the history and social structure of the area.

(2) Sengacor, Shotiki and Bapu Bazaars. For the purposes of this paper, practitioners at Kalipur to the north and Mohanpur to the south have been excluded, with the exception of one LMF Doctor who sometimes attends Shotiki on Bazaar days and who has been called to cases in Shotiki field-work area.

They include:

3 Allopaths(3) - Muslim - 2 with National Certificates. "National" practitioners study for 4 years after matriculation at the "National Medical Institute". They are unregistered and are at present fighting for recognition.
1 Licentiate Medical Fellow. The LMF course of 4 years training has recently been cancelled. Students still in training at the time of cancellation can enrol for an additional 2 years to take out a "condensed MBBS".

6 Allopathic 'quacks' - Muslim - a 'quack' is a practitioner with no formal qualification, who has acquired medical knowledge by working with other qualified or unqualified practitioners.

4 kobirajs(4) - Hindu - who give mixed Ayurvedic-Allopathic treatment.

1 'quack' - Muslim - who gives Ayurvedic-Allopathic-Unani treatment.

1 Homeopathic(5) 'quack' - Muslim - self taught from Homeopathic literature.

3 Homeopaths - Muslim - with HMB certificates, who also give Allopathic medicines.

There are in addition, three female Muslim kobirajis who use indigenous medicines, charms and amulets, one Muslim fakir who also dispenses both medicine and magico-religious cures, and a community of Bede(6) whose male members catch snakes and give treatment for snakebite, while the females provide amulets and perform cupping for body pain and for stomach complaints in children.

During the year too, on bazaar days, itinerant practitioners (mostly Muslim) attract an audience with drums and tricks, songs and the music of the ek-tara to sell a panacea compounded largely of Unani medicines(7). Two fakirs(8) were called in, from Comilla and Chittagong, to exorcise spirits possessing the insane,

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(3) Allopathy - The modern Western system of medicine.

(4) Kobiraj - Ayurvedic physician - loosely used to apply to any practitioner, Muslim or Hindu, who gives treatment with indigenous medicines and who has power to control spirits.

(5) Homeopathy - A system of medicine founded on the principle that 'like cures like', which prescribes minute doses of medicines that would produce in a healthy person the symptoms of the disease treated.

(6) Boat-dwelling, semi-nomadic Muslims, Bede are divided into 2 groups: a) one group, called Boido or Malboido, whose male members speciality is snakes and whose female members treat the sick. b) the others, Bede, who at Shotiki are fishermen, and entrepreneurs for the Hindu fishermen, and whose wives travel through the compounds selling bangles, soap containers and combs. The 2 groups say they do not intermarry.

(7) Unani medicine - The system of medicine developed by the Arabs incorporating Greek (Ionian, hence Unani), Iranian and Hindu ideas.

(8) Fakir - a Muslim religious mendicant. cf. Hindu Shadu.

and there was one itinerant fakir who said prayers for the sick, declared auspicious days on which a member of the business community might bank his money and settled a marriage dispute for the Mali caste group.

In addition a number of individuals in the community provide indigenous treatment - persons renowned for their skill in treating a single condition (for example, a Hindu Mali-Choukidar who on Tuesdays and Saturdays treats diarrhoea in breastfed children with 'blowing' and mantra(9), causing the mother's milk to dry), female murrubis (10) who minister to the younger women and infants of their compound, and intelligent, inventive women who retain a fund of indigenous cures told to them by others or who perhaps themselves fabricate new ones. They treat their own kin and generally give free advice to neighbours.

One example will illustrate a form of magical thinking involved in this kind of treatment - that is, give the patient a medicine which has similar properties to the disease, and he will recover. Aurina, a Muslim woman about 35 years of age, is the only living daughter of a father (deceased) said to have been a kobiraj. The father left his land half to Aurina and half to her stepbrother, and as Aurina has no children of her own she diverts her restless energies into running her small estate. Her fifth husband has come to live with her. He markets oilseed, jute and chillies in adjacent markets and Narayanganj, and from her home Aurina sells to her neighbours jutesticks, sweet potatoes and the fruit of her guava trees. She is an animated, rowdy conversationalist, given to earthy humour and boisterous story-telling illustrated in song and couplet. Aurina's aged mother recently suffered a condition in which water was said to have appeared all over her body. Aurina did two things about it. Firstly, she collected a long piece of Kolmi Sag (Ipomea reptans), an edible plant that grows in swampy ground, measured it to the length of her mother's body and hung it looped above the cooking place. As the plant dried, it was believed that all moisture on her mother's body would also dry. Next, from small pieces of banana tree-trunk she made two anklets for her mother to wear. During September the Hindu fishing community celebrated Gangima Puja, and they were joined in this by the Bede, who also depend on the river for their livelihood. At that time the fishermen made a miniature boat from a curved section of banana trunk, in the base of the boat placing paddy, one paisa, a little oil and sugar. At sunset there was a ceremony at the water's edge, the oil was lit and the small boat sent off into the river. It was this banana-boat which Aurina collected and brought home to her house. Pieces of it were then used as anklets in the treatment for her mother's oedema. Thus an object ritually associated with water was believed to have therapeutic efficacy for a state of water retention.

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(9) The blowing of air is a widely used therapeutic procedure among kobiraj, Bede, mullas; mantra - spells.

(10) Murrubi - senior females (or males) of the compound, who therefore command respect, if alert and active.

In addition, most adult women in Shaitnal, as in Dacca, have a broad knowledge of what might be called 'house medicines', generally used to treat themselves and their offspring, knowledge handed down in the family. For example, in Shaitnal, women dry vaccination and other sores with powdered turmeric, burnt paddy or a paste of powdered Chondon bark. Both Hindus and Muslims make poultices of heated cow dung and drink the urine of male cattle for liver troubles. Children with sore throats have their necks coated with white lime, and mothers wean offspring by rubbing their nipples with the juice of bitter leaves. Similarly, in Dacca, sprains are treated with a paste of heated turmeric and lime; children are cured of bed-wetting if they are rubbed at night low on the back (on what is believed to be the area of the kidneys) with mustard oil; and a little nut oil on the scalp, and two or three nuts eaten each morning, are believed to improve a dull memory. Thus, there is a pastiche of medical knowledge and belief. On one hand there is a fusion of ayurvedic, unani and homeopathic knowledge, stemming as it does from traditional thinking. On the other hand, allopathic medicine is partially accepted. As the list of practitioners showed, most doctors have incorporated the use of allopathic medicines in their treatment. The one exception is the self-taught homeopath who refuses to use saline to treat a cholera patient, and who criticises other homeopaths who 'mix' their medicine. All informants, even wealthy Muslims who often take their medical problems to Dacca, state that allopathic medicine is unsuitable for small children. It is thought to be too strong. Homeopathic medicine may have a good result or no result, they say. Allopathic medicine may have a bad result. Children also take homeopathic medicine more easily because it is given with sugar. One of the practitioners, a homeopath who also gives allopathic medicines, reserves his saline for adult cholera patients and treats cholera in children with homeopathic medicines only.

Both Hindus and Muslims manifest a willingness to try all kinds of medical, magical and religious treatment, to approach disease from as many vantage points as possible. In the course of the year, one Muslim family of father, mother and five children is known to have employed:-

1. A local homeopath for the smallest child's fever.
2. Dacca Medical College for cataracts on the father's eyes.
3. A Hindu neighbour for a poultice of leaves for the mother's infected finger.
4. A Muslim neighbour for the small son's fever, associated with facial and body swelling. The neighbour gave him wristlets of something said to be elephant's stool.
5. The Bede for an amulet for a female complaint of the mother's.
6. Powdered medicines from an itinerant bazaar Hakim for the same.
7. A Hindu kobiraj for saline when the mother's brother was visiting and suffered something like cholera.
8. The CRL fieldhouse for headaches, coughs, sores and worms.
9. Home treatments for smallpox sores (dried turmeric), fever (wristlets of Musikani Lota - Stephania hermandifolia) and many others.
10. Dietary restrictions and controls on those with colds, fever, dizziness, weakness.
11. Prayers and reading of the Koran for the recovery of the son with fever and fits. A goat was promised to the poor if the child recovered.

Beliefs about causation and treatment of disease differ according to the age, sex, and social status of the patient. Almost all infant maladies are thought

to be caused by Budh(11) - both Hindu and Muslims believe this. Therefore, babies with diarrhoeal disease are treated with amulets, charms and mantra and may not be taken to a doctor for consultation. Old people, especially elderly women, both Hindu and Muslim, feel themselves to be in the hands of God, and may refuse vaccination or any medicine if they fall ill. Poor people say they suffer from diarrhoea because they eat 'bad' food (the water from boiled rice, atta-ruti,(12) but that if rich people also have stomach trouble, then God has caused it and it is a matter to put right by behaviour.

The degree of belief in the supernatural varies from one disease to another. Leprosy is regarded as a punishment by God for sin. Temporary insanity and epilepsy are thought to be evidence of spirit possession. Worms are considered to result from eating large quantities of sweet potato. The appropriate treatment for these three states are consequently prayer, exorcism and medicine with a controlled diet.

Despite this apparent diversity of thought and action, there is throughout the community a basic cultural conviction that disease is a matter of discord. Harmony and order are essential to well-being. Health depends on physical, social, and spiritual harmony.

Faulty diet is probably the commonest explanation offered by both Hindus and Muslims for illness and the disorganisation of forces which control health. The Ayur-Veda deals at length with the action of foods that keep that keep the human body and mind in a state of perfect health. The whole of chapter 5 of the Koran relates to food. Thus, Muslim and Hindu literature recommend strict nutritional control.

> "The Holy Prophet recommended for a normal man the following quantity of food. One portion of his belly may be filled up with solid food and one portion with drink, and the remaining portion must be left empty." (13) ...."Excessive eating is a cause of bad luck" (14)... "The Almighty Allah knows our constitution perfectly well and He knows how to keep it best in health." (15)

> "It is said in Bhagwat Gita - that with a view to develop all good qualities, particular attention should be paid to diet. As this diet produces immunity against various disease and gives strength men will feel happiness and would have a desire to love other human beings" (16)... "Only good diet and sound digestive system are capable of keeping the body free from disease". (17)... "Half of the stomach should be filled with food, one fourth of it should be filled with water and the remaining one fourth should be kept empty for air, digestion of food and discharge of useless substances."(18)

The organs of the body are given a hierarchic order of importance.

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(11) Budh - ghosts, devils. (12) atta ruti - chapatis, made from wheat flour.
(13) Al Hadis, Vol. 2, p129 - Translation and commentary by Al-Haj Maulana Fazlul Karim.
(14) Ibid., p.130
(15) Ibid., p.154
(16) Kj.Dr. G.S. Varma: 'Miracles of Indian Herbs' p.269
(17) Ibid., p.270
(18) Ibid., p.271

"The stomach is the most important place of the body for health. It is, as it were, the fountain of health. If the stomach is healthy, the veins connecting with it carry healthy and pure blood throughout the body, and if the stomach is unsound, the veins carry disease.(19)

"The rest of the bodily organs will be performing their respective functions desirably well provided that the stomach remains in perfect order but as soon as any disorder finds its way in the stomach, the whole system is upset and some serious illness like cholera, gastritis, hyperacidity or anorexia sets in."(20)

A most detailed set of beliefs concerning the effect of specific foods on the body has been worked out in ayurvedic medicine. Foods are classified according to their 'heating' or 'cooling' effect on the body. The Koran, too, discusses foods with the properties of 'coldness' and 'heat'. These beliefs about the properties of food are firmly held by both Muslim and Hindu informants. For instance, meat, eggs, ghee, fish, honey and some oils (nut, mustard and coconut) are said to be 'hot'. Vegetables and fruit (especially the marrow 'lau', lemon, and cucumber) curd, and some oils, (til, lau and kodu seed) are regarded as 'cold'.

Illnesses too are classified as 'hot' and 'cold'. 'Hot' illnesses must be neutralised by 'cold' food and medicines, and vice versa. A person suffering from the effects of excessive heat (all forms of fever) should be bathed in cold water and given 'cold' food to eat. In general, a weak person must limit his consumption of 'hot' foods because he is incapable of digesting them. As his health improves, he may take more 'hot' foods.

There are detailed beliefs about the specific results of certain foods.

For several days after childbirth, women eat no meat, eggs, or fish or hot curries which would cause indigestion, but keep mainly to rice, bread, tea and cumin seed. These are easily digested, the latter especially having the property of making things dry.(21) Mothers increase their supply of breast milk by eating koi, magur and shing fish (Anabas testudineus, Clarias batrachus, Heteropneutes fossilis), and give the newborn child a first meal of honey and mustard oil, 'heating' foods, to give it strength and keep it free of colds.

There is a fund of information about foods that cause stomach upset and foods to restore dietary balance.

Jackfruit, sweet pumpkin, the seeds of kau fruit (Garcinia cawa) and some 'sag' (green leaf vegetables), especially pea 'sag', cause loose stool. English potatoes make stool firm, and prawns and hilsa fish (Hilsa ilisha) may constipate. Sour foods are thought to irritate wounds (circumcision and vaccination) and tamarind causes loss of sexual power. 'Hot' foods should be eaten in cold weather, and 'cold' foods in summer. For example, panta bhat, a 'cold' wet food, is suitable for summer mornings, but in winter dry foods (cirra - beaten rice and muri - puffed rice) are preferred.

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(19) Al Hadis, Vol. 2, p. 70
(20) Varma p. 150
(21) For several days after the birth of a child the mother may sit on heated earth or hold a pottery 'radiator' of burning cinders to her stomach to dry out her insides.

While faulty diet is probably the commonest explanation offered to explain disease, ill health may also be caused by immoderate or inappropriate behaviour in physical, social or even economic matters. One man of Shugundi whose right hand was maimed as a result of an infected wound, despite treatment in Bacca, is said to have suffered for his merciless business dealings.

Lack of harmony with the supernatural world is another potent source of sickness. Most people wear amulets, bracelets or toe-rings to ward off attack by spirits. If several children of one Hindu or Muslim mother die, the next survivor may be given an uncomplimentary name or symbolically 'sold' to a Hindu family for one paisa and 'purchased' again at the time of marriage. Thus, it no longer belongs to the mother and it is hoped that the jealous eye of an evil spirit will therefore not be interested to take it.

Ill health may also result from disordered relations between God and man. In times of epidemic, prayers and religious services are performed by both Hindu and Muslims to restore harmony with God.

Religious objects are thought to have a special efficacy to deal with matters of health. To hasten childbirth, a dried flower brought by a pilgrim from Mecca is dropped in water and the water given the mother to drink. A few drops of water from the burial place of a holy man will also speed a difficult labour. The Hindu Sacred Thread is used as a tourniquet for snakebite. The Koran is used as a weapon against disease - Sura 113 is believed to be especially efficacious against ills of the flesh, and 114 has power to counteract psychic afflictions. A mulla may recite portions of the Koran and breathe over a glass of water, then give a patient the water to drink. Or, an example of the symbolic use of writing, a verse from the Koran may be written on a plate or inside a bowl which is then filled with water to dissolve the writing, and this water is given to the patient. Amulets often enclose the written name of God, the Prophet or a holy place.

Good health then is a consequence of physical harmony, proper diet, and the ordered relations among men and between man and the supernatural. Medical care has a comprehensiveness which allows it to take account also of the prevailing season and climate, and the temperament and constitution of the individual. Men of hot temper should restr in themselves from eating excessively of 'heating' foods, lest their tempers become testier. In mid-October people at Shaitnal were speaking of a feeling of uneasiness, due to "the change in the atmosphere, and the change in the atmosphere of my body". (22) Others associated seasonal change with pain in the body and a lack of joy. The maintenance of good health is not a discrete form of activity. Al Hadis, in a section on unlawful food, discusses it in terms of health, food and morality.

> "As soundness of mind depends on soundness of body, therefore food and drink may be said to be one of the principle causes which lead a man to vice and virtue." (23)

A fakir may appropriately act as doctor, judge, business adviser, spiritual guide, seer and magician.

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(22) A secondary schoolteacher.
(23) Al Hadis, Vol. V p. 154

"Indian medicine considers disease as a state of disharmony in the body as a whole and a result not only of the external factors, nor merely of the external causes. Hence, according to it, treatment should aim at not only the finding of appropriate remedies but the employment of all available means to restore the normal balance of equilibrium." (24)

Harmony and discord are not ideas associated solely with health and disease. Harmony, balance and regulated relationships may be seen to be values on which the entire social order depends. Authority in the family rests with the father over sons, eldest brother over younger brother, husband over wife. The larger social units, both Hindu caste and Muslim class groups, form an ordered hierarchy, and rules about marriage and the traditional exchange of economic services help regulate relations among them.

Summary:

All levels in the community at Shaitnal employ a gamut of approaches to the problem of disease. Treatment may be had from a variety of ayurvedic, unani, homeopathic and allopathic practitioners, from kobirajs, fakirs, itinerant sellers of medicine, and the Bede. Many ailments are also treated with indigenous therapies or 'house medicines'.

Belief about the causation and proper treatment of disease varies according to the age, sex and social status of the patient. Whether illness is attributed to the Supernatural varies from one disease to another.

Despite this apparent diversity, there is a basic assumption that disease is a matter of discord, to be controlled by diet or by mending disordered relations with man, God and the Supernatural.

(24) Report of the Committee on Indigenous Systems of Medicine, 11, Ministry of Health, Government of India, 1948. p. 355.

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WATER STUDY IV (c)

by

Mr. S. Zafar Ahmed

The study of water was continued in order to define its part in the transmission and persistence of cholera in this endemic area (Table I). Chemical and bacteriological surveillance was maintained on the Buriganga and Sitalakhya Rivers (Graphs 1 and 2), the two principal water sources in the Dacca/Narayanganj area. V. cholerae Inaba organisms were isolated from the Buriganga on one occassion in February, and V. cholerae Ogawa was isolated once in August. This classical Ogawa organism was recovered where there were no known Ogawa cases. V. cholerae was not isolated from the Sitalakhya river on routine sampling; however, an epidemic of cholera broke out in late January among people who sought shelter from civil disturbances in the Laxmi Narayan Cotton Mills, situated on the banks of the Sitalakhya River.

Sampling points were established as shown in the map. The samples were obtained from the top 6 inches of the surface in mid river, with immediate plating of 0.1 ml on TTGA plates, and 50 ml was added to triple strength bile peptone broth. The findings are shown in Table II. All isolates were obtained by enrichment procedures only, indicating that the count was less than 10 per cc. but more than one per 50 cc. On 29th January, 1964, when 8 out of 10 points were positive, samples were taken at the same time from a depth of 10 feet; all were negative.

The infection apparently spread both above and below the point of outbreak, consistent with the fact that the river at this level is tidal and would be expected to carry organisms in both directions from the point of contamination. However, no cases were reported from this area after 5th February, 1964, despite extensive exposure to this contaminated water which many use for washing, bathing and drinking.

Previous studies on the survival of V.cholerae in water samples, from the local area were extended. As reported by others, survival is somewhat longer when competing organisms are removed by filtration or by boiling. Our studies of water samples have revealed no evidence that V. cholerae converted to a non-agglutinable vibrio which was able to survive; indeed the logical conclusion of these studies would be that contamination of water with vibrios yields only transient positivity. In boiled or filtered water, the organisms multiplied with a rise in titer for 2 or 3 days, after which counts dropped consistently to elimination.

John Snow clearly demonstrated the part water plays in the dissemination of cholera vibrios. What is not clear, however, is whether infection results from drinking contaminated water, or whether the contaminated water serves to inoculate some food in which the vibrios can multiply to achieve an infective dose for man. Our interest has been concentrated on the possibility that rice, the main staple local diet, might serve as such a culture medium.

The study of Bengal rice cookery (panta bhat) reveals differences in vibrio propagation based on the variety of rice used. Two types of cooking were used; in one the rice was boiled with an excess of water which was then decanted and, after the rice had cooled, the vibrio-containing water was added; in the other, only enough water was used in cooking to soften the rice and, after cooling, the contaminated water added. Recent Ogawa and Inaba isolates were used; after 18-20 hours in bile peptone broth, a 10^2 to 10^6 dilution was made in saline and this was then diluted 1:100 in distilled water, laboratory tap water or filtered river water. 200-300 mls of these vibrio-containing mixtures were added to cover the rice and the mixture permitted to stand overnight at ambient temperatures varying from 26° - 33° C. Periodic counts of the supernatant were made.

Three varieties of rice were used in our studies: Boro rice which is harvested in the spring; Aus, the fall variety which grows during the monsoon floods; and Aman, which is seeded in the spring but harvested in the winter. The Boro rice actually appeared to kill the vibrios; in 7 of 16 instances no vibrios could be isolated four hours after they have been added to the cooked rice (Table III). This absence of vibrios was especially marked (in 7 out of 8 instances) when the rice was boiled in an excess of water. Despite these negative results, one of the 16 Boro rice experiments showed a three-log increase in count of Ogawa organisms (from 420 to 6 x 10^3 vibrios per ml. of the supernatant).

Although the multiplication of Ogawa vibrios was poor in Aus rice, in all instances they survived for 8 to 12 hours. The addition of 0.1% or 0.5% sodium chloride resulted in marked increases in both Ogawa and Inaba counts in every instance. In a few experiments, Aman rice supported vibrio (Inaba) growth well, even without added salt; when salt was added the vibrio proliferation was the greatest observed.

Because a local cook was observed rinsing cooked rice in a pond or tank, studies were carried out in which cooked rice was rinsed in vibrio-containing water and then the water decanted. As shown in Table IV, these preparations also supported vibrio multiplication at room temperature.

The poorer vibrio growth seen in rice that has been boiled with an excess of water, and the stimulating effect of added salt, suggest that the potential hazard in Bengal rice cooking may be related to the salt content of the medium. This suggests that perhaps the rice grown in areas of high salinity may be more dangerous; in East Pakistan these areas also tend to have a higher cholera incidence. Studies are planned to determine whether salt content does in fact vary with place, rice variety, or season.

TABLE I

Water Report, October 1963 - September 1964.

		VIBRIOS			FECAL POLLUTION	
		No. of tests	% V.cholerae	% NAG	No. of tests	% Positive
Dug Wells	Surveillance	1878	0.7	60.0	605	92.0
	Case Investigations	150	12.0	66.0	87	84.0
	TOTAL	2028	1.5	60.0	692	91.0
Tanks	Surveillance	274	0	60.6	58	95.0
	Case Investigations	110	27.3	50.0	35	90.0
	TOTAL	384	8.0	60.0	93	92.5
Rivers	Surveillance	83	14.5	74.7	67	95.6
	Case Investigations	21	10.0	80.0	9	100.0
	TOTAL	104	13.5	75.0	76	96.0
Canals	Surveillance	88	1.1	70.0	27	92.6
	Case Investigations	16	0	70.0	7	100.0
	TOTAL	104	0	70.0	34	94.0
Tube Wells	Surveillance	145	0	7.5	137	11.0
	Case Investigations	179	0	1.6	93	9.6
	TOTAL	324	0	4.6	230	10.4
Municipal Supplies	Surveillance	6	0	0	10	0
	Case Investigations	136	0	2.2	71	10.0
	TOTAL	142	0	2.1	81	8.6
Storage Jars	Surveillance	-	-	-	-	-
	Case Investigations	767	2.2	6.4	636	50.6
	TOTAL	-	-	-	-	-
Sewage Effluent	Surveillance	52	8.0	65.0	-	-
Drain & Waste Water	Case Investigations	45	9.0	80.0	-	-
Latrines	Case Investigations	67	16.4	19.4	-	-
Manholes	Surveillance	12	0	33.0	-	-

* One municipal supply point at Agha Nasih Lane showed faecal pollution in 3 of 9 examinations in early August during the floods.

TABLE II

POSITIVITY OF THE LAKHYA RIVER, NARAYANGANJ FOR V.CHOLERAE INABA

27 January to 30 March, 1964
(Epidemic period 23 January - 5 Feb., 1964)
Mid-stream Sampling.

Points											
←Up Stream	F	-	+	-	-						
	E	-	+	-	0						
	D	-	0	-	0						
	C	-	+	-	0						
	B	-	0	-	-						
	*A	-	+	-	+						
Down Stream→	G	+	+	+	-	0	0	0	0	0	-	0
	H	+	+	+	-	0	0	+	+	-	-	+
	I	+	+	-	-	+	-	+	+	0	-	-
	J	+	+	+	-	0	+	0	0	-	+	+
Date of sampling		27/1	29/1	3/2	5/2	12/2	21/2	27/2	2/3	10/3	16/3	30/3

Keys:-

+ = Inaba isolated.
0 = No Inaba found
- = Not sampled.

Range of Findings.

Fecal contamination (E. Coli/100 mls)	-	230 to 930
pH	-	8.3 to 8.6
Chloride content in mg/l	-	5 to 14.
Alkalinity in mg/l as $CaCO_3$	-	166 to 176.
Sp. Conductance micromhos/cm	-	316 to 393.

TABLE III

Effect of Salt and Rice Variety on Vibrio Multiplication in 'PANTA BHAT'

Quality of Rice	Change in Titer after 8-12 hours	NO ADDED SALT		0.1% OR 0.5% SALT ADDED	
		Rice boiled with excess water	Rice boiled with minimal water	Rice boiled with excess water	Rice boiled with minimal water
Boro	Fall	8(5)	6(12)	-	-
	Rise x 10^1	-	1	-	-
	Rise x 10^3	-	1	-	-
Aus	Fall	3	4	-	-
	Rise x 2	1	1	-	-
	Rise x 10^1	-	1	-	-
	Rise x 10^2	-	-	1	1
	Rise x 10^3	-	-	3	1
	Rise x 10^4	-	-	1	7
Aman	Fall	-	-	-	-
	Rise x 10^1	-	1	-	-
	Rise x 10^3	-	1	-	-
	Rise x 10^4	-	1	-	-
	Rise x 10^5	-	-	-	1

( ) No vibrios recovered after 8-12 hours.

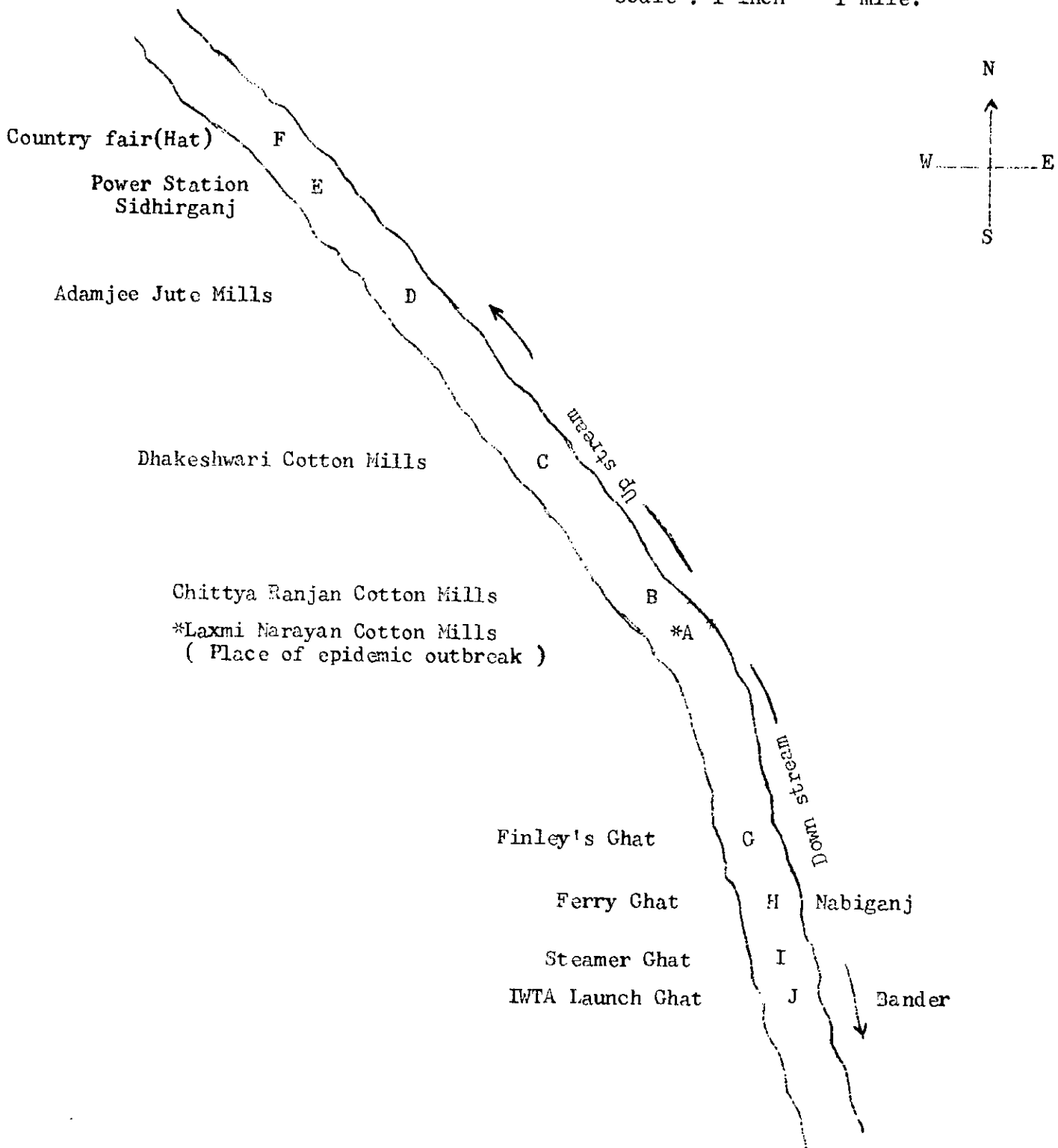
TABLE IV

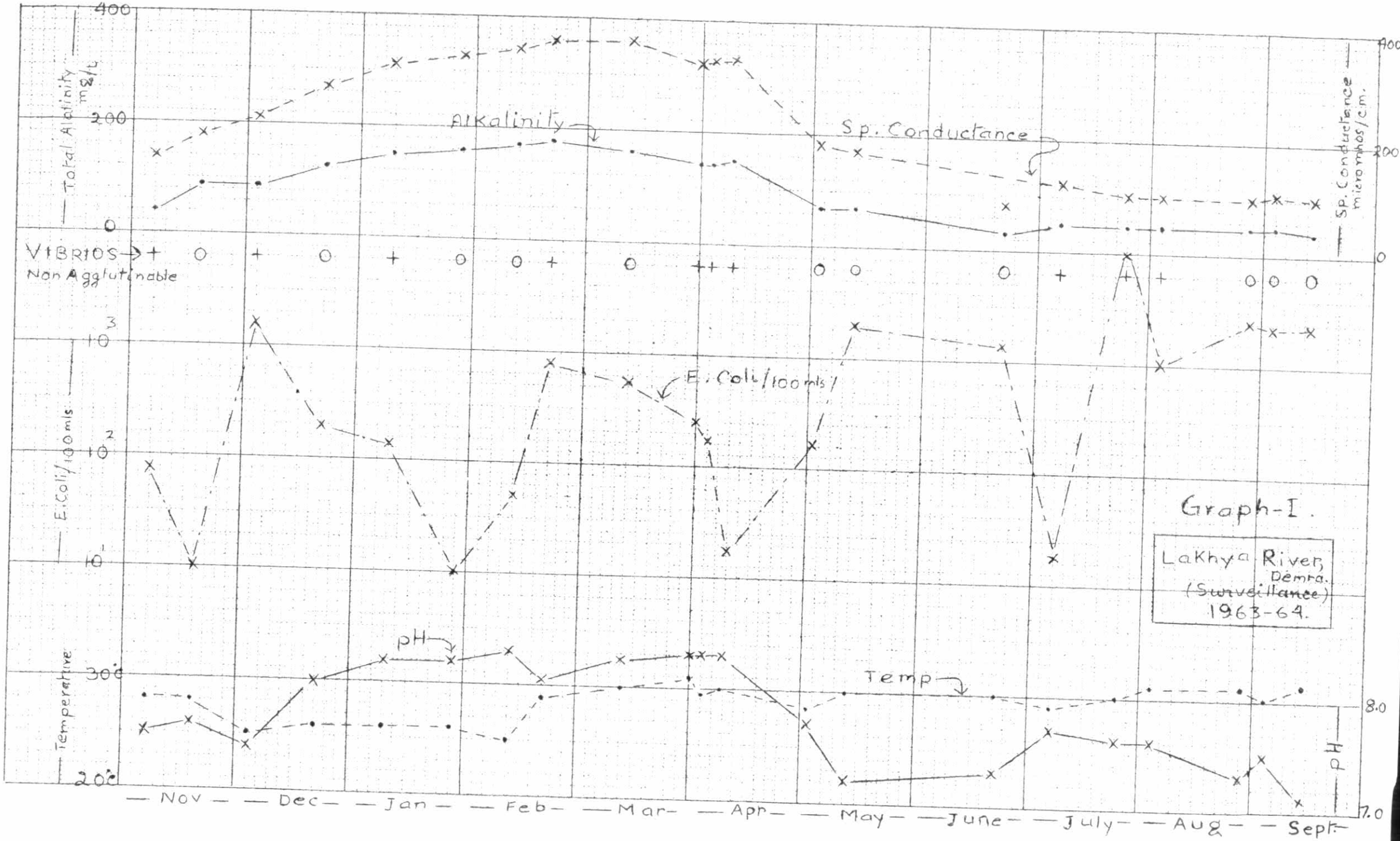
Multiplication of V.cholerae in rice rinsed with contaminated water.

Storage Time Hours	AUS RICE EXPERIMENTS			BORO RICE EXPERIMENTS			
	Count per gram of wet rice						
	1	2	3	1	2	3	4
0	3.4×10^5	9×10^4	2.5×10^2	1.8×10^5	7.5×10^5	-	10^3
4	10^6	5×10^5	2.5×10^3	3.5×10^5	1.25×10^6	5×10^2	-
6	1.6×10^6	5×10^6	1.5×10^4	-	3.3×10^7	10^3	-
8	2.8×10^7	4.1×10^8	10^5	-	8.5×10^7	-	-
12	1.6×10^8	1.8×10^9	6×10^5	5×10^8	3.1×10^8	1.5×10^5	1.5×10^5
24	1.2×10^9	1.1×10^{10}	6×10^6	5×10^8	5×10^8	5×10^6	1.5×10^7
30	2×10^7	3.2×10^8	plate covered with contaminant	3.8×10^7	-	-	2×10^5
pH Range (24 Hrs)	7.7 -6.1	-	7.7- 6.4	7.5- 6.0	7.7-6.2	7.7-6.8	7.8-6.2

Map of Lakhya River, Narayanganj, showing sampling points under surveillance both up stream & down stream from the point of cholera outbreak in January, 1964.

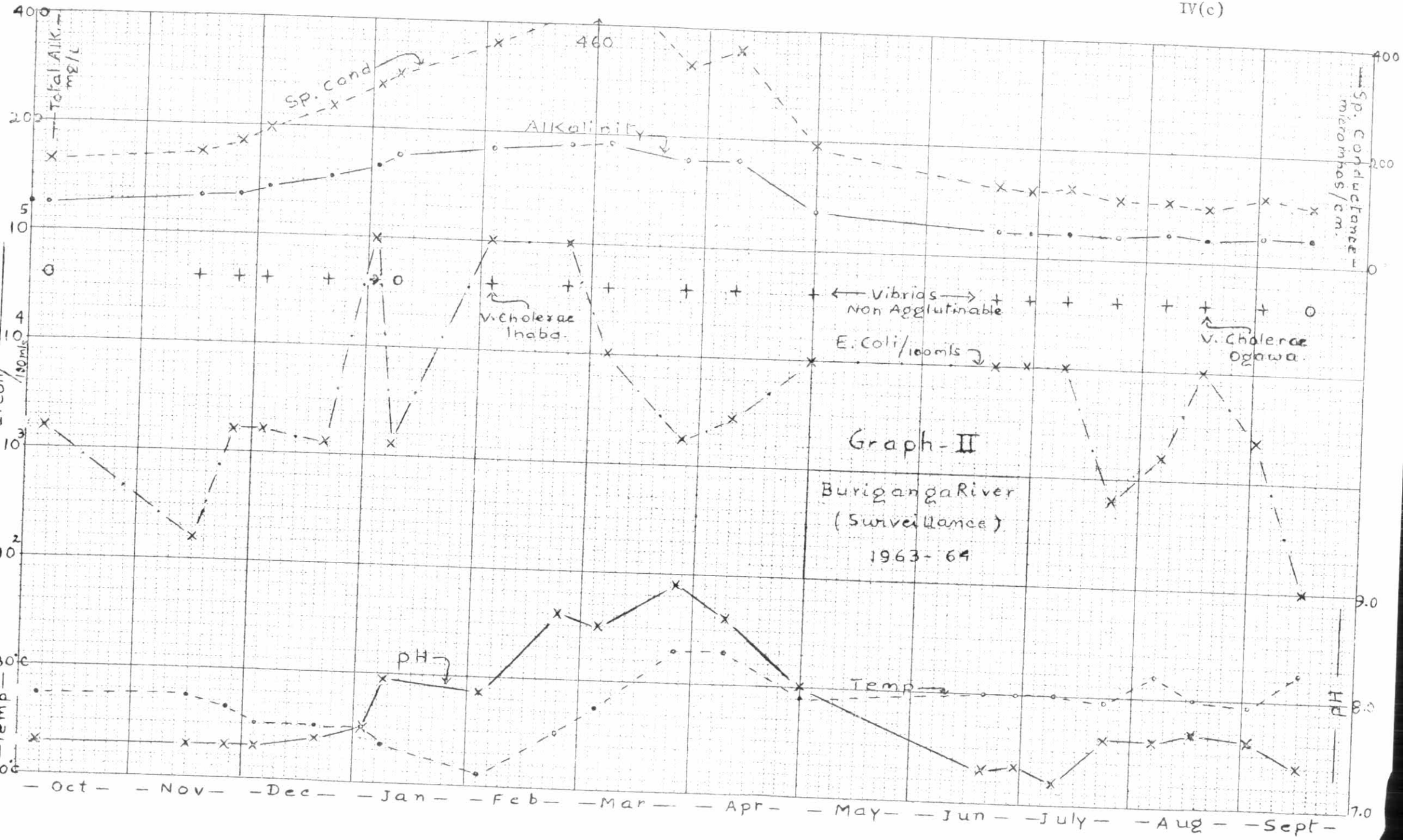
Scale : 1 inch = 1 mile.





Graph-I.

Lakhya River, Demra. (Surveillance) 1963-64.


Total Alk. mg/l
SP. Cond
Alkalinity
Sp. Conductance micromhos/c.m.
Vibrias
Non Agglutinable
V. Cholerae Inaba.
V. Cholerae Ogawa.
E. Coli/100mls
pH
Temp
Graph-II
Buriganga River
(Surveillance)
1963-64
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
July
Aug
Sept

Family Study

by

Drs. Oseasohn, Khan, Ahmed, Islam and Rahman and Mr. Zafar

Introduction:

Data collected from 85 families observed during 1962-63 were presented in part in the 1963 Annual Report. Further analyses were performed and findings summarized as follows: multiple cholera cases were observed in 33 of 85 families. Intrafamilial attack rates were higher among children than adults. Spread of cholera within these families was suggested by the distribution of intervals between primary and subsequent cases, the relationship between length of home stay of index cases and increased numbers of subsequent cases, and the effect of family structure on intrafamilial attack rates. (This material has been accepted for publication by the Bull. of the WHO).

The present investigation was designed to describe 1) inapparent infection as well as diarrheal disease associated with V. cholerae, 2) the relationship between infection, water use, and sanitary facilities, and 3) the spectrum of disability associated with V. cholerae infection.

Method:

Families of CRL hospitalized cholera patients living in Dacca were selected for study within two days of onset of illness in bacteriologically positive index cases. Families were defined as units consisting of two or more persons who lived and ate together. Families were observed daily for 10 days by teams comprised of one physician and one health visitor. At the first visit a census was taken and data were collected on housing, water use, and sanitary facilities. Family members were queried daily about the occurrence of diarrhea or any symptom which interfered with usual duties. When either diarrhea or disability was encountered, a detailed history of illness was obtained. Disability was classified as follows: 1) none, 2) inability to perform usual duties, 3) inability to stand without aid, and 4) inability to respond to queries. Rectal swabs were used to collect daily fecal samples from all family members for typable V. cholerae. Swabs were placed in bile peptone transport media and incubated overnight at 37° C. Sub-cultures were made on gelatin agar and Monsur's bile peptone tellurite medium and incubated overnight at 37° C. Suspicious colonies were tested for agglutination by specific antisera. Cholera cases were defined as persons with acute diarrheal disease associated with recovery of V. cholerae from one or more fecal samples; all other isolations of V. cholerae were regarded as instances of inapparent infection. All water sources and storage facilities were sampled daily for bacteriologic examination by the methods reported previously (Annual Report, 1963).

Results:

Twenty-seven families were selected for study between November, 1963 and October, 1964. Although the number of familes with multiple cases is small, certain trends are suggested by their distribution within families.

One or more instances of subsequent infection was found in 11 of those 27 families. The distribution of diarrheal and inapparent infection in families is shown in Table 1. The age and sex distribution of persons with infection and the population at risk are shown in Table 2. The total infection rate was higher among those below 15 years of age than among those 15 and over. Infection rate did not increase with family size (Table 3); nor did the rate differ between persons exposed to index cases for lengthy intervals (12+ hours) and those exposed for shorter intervals (Table 4). A significantly higher rate was observed in families in which the index case was female than among those in which the index was male (Table 5).

The distribution of first positive cultures by day of appearance among infected persons is shown in Table 6. The bulk of subsequent infections appeared within 2-3 days of onset of illness in the index cases. No new infections were observed after the seventh day. The relationship between positive cultures and appearance of symptoms among 18 cholera cases is shown in Fig. 1. Several subjects exhibited positive cultures for intervals up to 5 days prior to onset of gastrointestinal symptoms.

Disability associated with cholera infection was examined in 22 subjects 3 or more years of age (Table 7). Nine of the 22 were severely disabled.

Family water sources and storage facilities for contamination with V. cholerae have been studied since January 1964. Findings in 20 consecutive families are summarized in Table 8. Initially, vibrios were present in water samples in 12 of the 20 families. By the seventh day none of the water tested revealed cholera organisms. The relationship between water findings and appearance of multiple infections in families is shown in Table 9.

Discussion:

Suitable case material was found only infrequently during the past year, and family studies have progressed slowly. The burden of endemic cholera appears to fall more heavily on children than adults, a finding observed previously in Dacca families (1962-63) and consistent with age - incidence data in a variety of infectious diseases.

The data on vibrios in water and infections in families do not provide any answer to the riddle about which came first. Studies in large European epidemics in the last century have established that cholera can be spread by water. However, water does not appear to be a major factor

in the spread of cholera within families in this endemic setting. Water samples (Table 9) were positive with equal frequency in households with and without multiple infection. In addition, despite up to 6 days of exposure to organisms, a pronounced secondary wave of infections was not observed.

These family studies will be continued during the coming year.

TABLE 1

Distribution of Type of Cholera Infection in 27 Families

Type of Infection	Number of Families
None	16
Diarrheal disease alone	4
Inapparent infection alone	2
Both	5
TOTAL	27

TABLE 2

Frequency of Cholera Cases and Carriers and Persons at Risk in Families of 27 Hospitalized Cholera Patients

Age (yrs.)	Number of Cases	Number of Carriers	Total Number Infected	Number at Risk	Infection Rate (%)
<15	12	5	17	71	23.9
15+	6	5	11	83	13.3
TOTAL	18	10	28	154	18.2
SEX					
Male	7	7	14	85	16.5
Female	11	3	14	69	20.3
TOTAL	18	10	28	154	18.2

TABLE 3

Infection Rate and Family Size

Family Size	No. Infected/No. at Risk	Infection Rate (%)
2-5	8/21	38.1
6-9	19/93	20.4
10+	1/40	2.5
TOTAL	28/154	18.2

TABLE 4

Infection Rate and Length of Stay at Home Before Hospitalization of Index Cases

Interval (hours)	No. Infected/No. at Risk	Infection Rate (%)
<12	22/125	17.6
12+	6/29	20.6
TOTAL	28/154	18.2

TABLE 5

Infection Rate and Sex of Index Cases

Sex of Index Case	No. Infected/No. at Risk	Infection Rate (%)
Male	8/87	9.2
Female	20/67	29.9
TOTAL	28/154	18.2

TABLE 6

Distribution of First Positive Cultures by Day of Appearance Among 28 Infected Persons

Day	Cases	Carriers	Total
1	9	4	13
2	5	1	6
3	0	1	1
4	3	1	4
5	0	1	1
6	1	0	1
7	0	2	2
8	0	0	0
9	0	0	0
10	0	0	0
TOTAL	18	10	28

TABLE 7

Disability Associated with Cholera Infection Among 22 Persons 3 Years of Age or Over

Level of Disability	
ACUTE DIARRHEA	
Unresponsive	2
Unable to stand	7
No disability	4
No symptoms	9
TOTAL	22

TABLE 8

Water Findings in 20 Cholera Families

Vibrio Culture	Days after onset of illness in index cases				
	1-2	3-4	5-6	7-8	9-10
Positive	12	5	3	0	0
Negative	8	15	17	20	20

TABLE 9

Water Findings and Multiple Infections in Families

Cholera Vibrios in Water	Multiple Infections		
	Present	Absent	Total
Present	6	6	12
Absent	1	7	8
TOTAL	7	13	20

FIGURE 1

Bacteriologic Findings and Appearance of Symptoms in 18 Cholera Cases

Ind. No.	Age (yrs)	Findings*	Days after onset of illness in index case									
			1	2	3	4	5	6	7	8	9	10
1-3	<15	Culture	0	** +								
1-4	<15	Culture	+	** +								
6-2	15+	Culture	** +									
6-7	<15	Culture	0	0	0	0	0	+	+	+	not	tested
7-4	<15	Culture	** +									
7-5	<15	Culture	+	0	0	** +						
8-2	15+	Culture	+	0	+	+	+	+	0	0	0	0
8-3	<15	Culture	0	0	0	** +						
34-2	15+	Culture	0	+	+	+	** +					
34-4	<15	Culture	0	+	+	+	+	0	+	+	0	0
37-3	<15	Culture	** +									
39-3	<15	Culture	0	0	0	0	+	+	+	+	0	0
39-4	<15	Culture	+	+	Moved away from home							
39-8	<15	Culture	0	0	0	0	+	0	0	0	0	not tested
41-7	<15	Culture	0	+	0	+	+	+	+	+	+	+
42-2	15+	Culture	0	+	+	0	+	0	0	0	0	0
42-3	15+	Culture	+	+	+	0	0	0	0	0	0	0
42-7	15+	Culture	0	0	0	+	0	+	+	0	0	0

* cultural findings - presence of absence of *V. cholerae* in daily rectal swabs (+ or 0)

shaded area indicates presence of diarrhea for vomiting; no entry indicates no symptoms

** hospitalized

Kavar Bazar Diarrhea Morbidity Study

IV(e)

by

Director of C. R. L.

John P. Craig, M.D., Muslimuddin Khan, M.B.B.S., D.P.H.
Ranjit K. Barui, M.B.B.S., Syed Zafar Ahmed, M.Sc.

One of the many unresolved problems in the natural history of cholera concerns the whereabouts of the infective agent between outbreaks of clinically manifest disease. In places and at times when cases of cholera diarrhea are continuously present, it is reasonable to postulate that new cases are infected by direct or indirect exposure to discharges from existing cases. Similarly, pandemic spread has usually been ascribed to a direct case-to-case dissemination along trade routes across the world. In the endemic area of Bengal, however, many local areas, free of recognized disease for months or years, suddenly experience sporadic cases, or more often sizeable outbreaks, in the absence of obvious introduction or contact with frank clinical cholera from outside the community. It is undoubtedly true that negative findings must be interpreted with caution, especially in an area where accurate histories of movements and contact are difficult to obtain; nevertheless, the facts call for continued search for sources of infective vibrios other than clinical cases of cholera.

Inference from other diarrheal diseases would suggest that environmental reservoirs and persistent inapparent infection in man would be the most likely means of maintaining cholera in an endemic area. Repeated search for inapparent infection with cholera vibrios has led most workers in the past to conclude that the so-called carrier state is transient, usually persisting for no longer than two weeks, and that vibrios are rarely, if ever, found in routine rectal swab surveys even in communities in cholera-endemic areas, unless clinical disease is also present in the community at the same time. It must be recalled, however, that the methods employed have depended on the presence of viable vibrios in the terminal one or two centimeters of the intestinal tract; experience with typhoid bacilli reveals that the flora of this locus is not always representative of the preceding ten meters of the enteric canal and its several tributaries.

The occurrence of a sharp outbreak of cholera in a refugee camp near Dacca, East Pakistan, in January, 1964, provided an unusual opportunity to investigate the fate of vibrios following the disappearance of clinical disease.

The camp was organized in the center of the village when the need for protection during communal disturbances forced the residents to flee from their homes. They were congregated under extremely crowded conditions for more than a week. Hence, the movement of these refugees from their homes to the camp did not involve contact with persons outside their own village.

Immediately after the cholera outbreak had subsided, a longitudinal study of diarrheal disease and enteric infection was begun in families in which cholera had occurred, and in control families which had not suffered from cholera.

Although the original and primary aim of the study was to conduct a long-term search for cholera vibrios in a community during a time when clinical cholera was absent, it was clear that the epidemiology of cholera must be viewed as only

one aspect of the epidemiology of diarrheal disease in general. Hence, the original scope of the study was broadened to include a description of diarrheal illness in a community in East Pakistan, and a search for association between diarrheal disease and a selected group of organisms usually considered to be enteric pathogens.

The study, which is still in progress, has been designed to answer the following questions:

1. What are the seasonal incidence and age and sex distribution of diarrheal disease in families with and without recent cholera.
2. What is the incidence of infection with Vibrio cholerae, non-cholera vibrios, Shigella and Salmonella in these groups, and what association exists between infection and diarrheal illness?
3. What is the time and place distribution of contamination of water sources with cholera and non-cholera vibrios and with fecal E. coli, and what association exists between such contamination and diarrheal illness among water consumers?
4. More specifically, in the event cholera reappears in the village, do inapparent infection with V. cholerae and/or contamination of water sources precede or follow the appearance of clinical cholera?

The interim report which follows is presented in two sections; the first describes the cholera outbreak in January; the second describes the design and methods of the diarrhea morbidity survey and the preliminary findings of the first seven months, from February 1 to August 30, 1964.

I. THE CHOLERA OUTBREAK

On January 15, 1964, in the wake of communal disturbances, over 6,000 men, women and children, representing an estimated 90% of the population of a village near Dacca, East Pakistan, fled from their homes into the central area of the village to seek mutual protection. Although family units were partially maintained for cooking and eating, the extreme overcrowding resulted in a marked increase of personal contact and a complete breakdown of sanitary facilities. Abundant opportunity arose for contamination of the limited water supplied in open earthenware jars before covered tanks with gravity outlets were installed.

Two cases of cholera appeared on January 17 and, between January 18 and February 11, a total of 95 cases were admitted to the Pakistan-SEATO Cholera Research Laboratory. V. cholerae Ogawa was isolated from all 95 cases. The dates of onset of the 95 cases are shown in Table I. Subsequent family histories suggest that two fatal cases in children occurred in the camp. A member of the CRL staff visited the camp two to four times daily between January 18 and January 31, to search for cases, and daily visits were made throughout February to obtain complete family histories. It is therefore unlikely that any cases of frank cholera were missed. Mild diarrhea associated with V. cholerae was certainly overlooked as attested by the fact that cholera vibrios were isolated in three instances from ambulatory children with very mild diarrhea. No attempt was made, however, to determine the incidence of inapparent infection or mild diarrhea associated with V. cholerae during the epidemic period.

Food rationing officials estimated the total camp population to be 6,422 representing 1,022 families with an average family size of 6.3 members. In Table II, attack rates in the total camp population are compared by age with attack rates in affected families. In the total camp population, the attack rate in children was more than twice as great as in adults, but within affected families the excess in children was slight. On first examination this discrepancy appeared to be attributable to the excess of adults in the total camp population, as compared to the population of affected families, and suggested that families with fewer children had lower attack rates. However, the data in Tables V and VI do not indicate that attack rates among affected families were influenced by family size. This does not preclude the possibility that unaffected families may be smaller and have fewer children.

Teams of field workers from the CRL compiled family rosters of all affected families, defining a family as a group of people living in the same dwelling, who cook and eat together. The 95 confirmed cases were distributed in 74 families comprising 595 individuals with an average family size of 8.0. The data indicated that affected families were somewhat larger than nonaffected families. The occurrence of multiple cases in families is shown in Table III.

The attack rates in members of affected families, by age and sex, are shown in Table IV. No cases were seen in infants. Among families the attack rates rose slightly with age, but the paucity of cases among adult males resulted in overall lower attack rates among adults and among males.

Secondary attack rate cannot be accurately determined in cholera, since present knowledge does not allow us to distinguish between those simultaneously infected but with different incubation periods, and those arising secondarily following exposure to a primary case. Hence, in this discussion, the terms "subsequent case" and "subsequent case rates" have been used.

It was of interest to determine whether, under conditions of refugee camp life, attack rates in members of affected families and frequency of multiple cases in families would be related to family size as has been shown in fixed family settings. (See Family Studies Section, this report.) Multiple cases occurred in 17 of the 74 affected families as shown in Table II. Attack rates in family members and subsequent case rates by family size and number of children in the family are given in Tables V and VI. The attack rates and subsequent case rates are higher in smaller families and families with fewer children. In the case of family size, the difference in attack rates is significant ($p. < .02$).

In contrast to these findings, previous observations indicated that subsequent case rates were highor in larger families. Perhaps this difference is related to the epidemic setting of the present study. In the crowded refugee camp family integrity had partially broken down, whereas the earlier observations were made on sporadic cholera occurring in fixed family settings more widely dispersed in time and space. Hence, family size might reasonably be expected to exert less influence on case distribution in a refugee camp situation.

The hours of onset of the 95 confirmed cholera cases are shown in Table VII. As has been noted previously*, onsets tended to be clustered in the early morning

* Rumpf, T., 1898 Asiatic Cholera, Twentieth Century Practice, Vol. XIV p. 363, William Wood and Co., New York.

hours. Onset was defined as the time of first episode of vomiting or diarrhea, whichever occurred earlier; patients were rarely questioned concerning prodromal symptoms and, when they were, the severity of later symptoms often obscured their memory. Concentration of onset time at a certain period of the day may be associated with time of exposure or with cyclic changes in gut function. Careful observations of onset time would be worthwhile to increase knowledge of both the epidemiology and patho-physiology of cholera.

II. LONGITUDINAL FAMILY STUDY

The study commenced on February 4, 1964, as soon as the families in the refugee camp had returned to their houses. The study is still in progress, and this report deals with the seven month period ending August 30, 1964.

Personnel and Methods

Three teams of house visitors, each consisting of one male and one female worker, visited the study area daily from Monday through Friday. The village was divided into three zones, with one team assigned to each zone. The cholera-affected families were located, and for each affected family a neighboring family which had been unaffected by cholera during January and February, 1964, was selected as a control. The families were not selected by a random system since willingness to cooperate in a long-term study was a necessary prerequisite. In general, affected families were willing to allow weekly visits and rectal swab collections, but some failed to cooperate and were deleted from the study. Hence, both affected and control families can best be defined as groups of families who were willing to cooperate in such a study.

At first visit to each family, a record was made of the name, age, sex and family relationship of each individual, and the address and date of entry into study. (Appendix A) An occupational and residential history of each family member was taken and the numbers of days per week on which each member regularly left the village was recorded. (Appendix B) A family water consumption record was made showing source of water used for drinking, cooking, washing dishes, bathing, laundry, and latrine. (Appendix C) Water records were given to a team of two sanitary inspectors from the Water Section, CRL, who were permanently assigned to the study. Water surveillance activities are described below.

Each family was visited once a week by the field teams. Inquiry was made concerning births, deaths, illnesses, occupational changes, and changes in water usage since the last visit. Rectal swabs were obtained from each willing member, streaked directly on SS plates and placed in bile-peptone broth, pH S.O. After overnight incubation, bile-peptone was subcultured to tellurite-taurocholate-gelatin agar plates (TTGA). During the first five months of the study, swabs were first streaked on TTGA and then placed in selenite broth. However, experiment with a sample group using both SS plates and selenite broth showed that Shigella isolated on SS plates frequently failed to appear following selenite enrichment. Hence, the frequency of Shigella isolations in the first five months was not comparable to that of later months.

Between 45 and 60% of all members of study families submitted to swabbing in any given week. Cooperation was greatest among females and among children. Two

samples of weekly collections were examined to estimate the age and sex distribution of persons swabbed. Of a total of 1,186 persons under observation, specimens were collected on 647, or 55%. Forty-four percent of males, 65% of females, 85% of children 0-4, 65% of children 5-14 and 37% of persons over 15 were swabbed.

On June 1, 1964, a diarrhea clinic was opened in the village, staffed daily by a physician and aid nurse. All residents of the village were encouraged to attend. Two rectal swabs were obtained on all cases of diarrhea. One swab was streaked directly on TTGA and placed in bile-peptone, pH 8.0; the other swab was streaked directly on SS agar and placed in selenite broth. Because it served the entire village, the clinic provided qualitative information from a population approximately ten times that of the study families, and thus alerted the investigators to the kinds of illness and kinds of organisms present in the community.

The clinic also provided medical care for the study families and promoted greater willingness to cooperate in a long-term study. No chemotherapeutic or antibiotic agents were dispensed in the clinic. All patients whose illnesses appeared to require specific therapy were admitted to CRL. Others were treated symptomatically.

Water surveillance consisted of weekly sampling of all dug wells located on study-family premises, and of all tube wells, tanks, and canals in the village.

All water samples were cultured for vibrios by adding 50 ml of water to 25 ml triple-strength bile-peptone enrichment medium. Fecal contamination of each tube well was estimated at the time of entrance into the study by culturing for heat-resistant Escherichia coli. A weekly record of the results of sampling was kept on each dug well, tube well, and tank. (Appendix D) Isolation of cholera vibrios from any water source was followed by quantitative estimation of vibrios daily for eleven days by direct streaking of 0.1 ml of the water sample on TTGA plates.

The following procedures were done in the event of isolation of V. cholerae from any individual: (1) each member of that individual's family was swabbed every other day, for a total of six swabbings; (2) if there was a dug well on the family premises, daily water samples were collected for eleven days, and cultured for vibrios by both direct plating and by bile-peptone enrichment; (3) all members of the family who used the same dug well for any purpose were also swabbed as in (1); and (4) all dug wells on all premises contiguous to those of the index case were cultured as in (2). Isolations of Salmonella, Shigella or non-cholera vibrios from study-family members were followed up by swabbing on the third or fourth day as well as by routine weekly swabbings.

Births, deaths and illnesses were reported on simple forms completed by field teams or the clinic physician. (Appendices E, F and G) For each study family, a separate file was maintained and included: (1) A copy of the family roster, (2) results of all weekly rectal swab examinations (Appendix H), and (3) reports of all births, deaths and illnesses occurring in the family members.

RESULTS:

During the first seven months of the study, a total of 1,477 persons were seen. Of these, 77 families were in the cholera-affected group, and 135 were control families. Because of emigration and failure to cooperate, many of the original affected families were lost. New control families were added during each month of study. A total of 19,797 person-weeks of observation were made -- 9,478 in affected families and 10,319

in control families.

Age and sex distribution and family size of affected and control families indicated that no significant differences in family structure existed. The median size of affected families was 8.0 members, and of control families 7.2.

Cholera

During the period covered by this report, two unassociated cases of cholera-like diarrhea were seen in the entire village. The first case, with an onset during the week of February 24, was an adult female member of a family which had had cholera in January. No cholera vibrios were isolated throughout her hospital stay; hence, she was considered a case of non-vibrio cholera. The second case, with onset during the week of March 30, was an adult male who had recently immigrated. V.cholerae Inaba was isolated. No previous isolations of Inaba sub-type had ever been recorded from this village, although Ogawa strains had been isolated from sporadic cases in late 1963, prior to the refugee camp outbreak. No subsequent cases appeared, but Inaba was isolated during routine weekly rectal swabbing from a healthy male child during the week of April 6. This child had had clinical cholera in January from which V. cholerae Ogawa had been isolated. The detection of two Inaba infections in the same village within two weeks, in the absence of prior or subsequent Inaba cases, suggested that they might be associated in some way. They resided approximately one half mile apart, however, and intensive questioning of members of both families revealed no direct or intermediate contact between the two cases at any time. A chain of undetected infections could not, of course, be excluded. Water supplies used by both families remained negative.

Other Illnesses

In the following discussion, data from cholera affected and non-affected families are combined, as numbers were small, and the chief purpose in maintaining separate groups was to compare their future cholera experience.

Monthly incidence of diarrheal and non-diarrheal disease, expressed in cases per 1,000 person-weeks, is given in Table VIII. Diarrhea was the chief cause of reported illness during all months except August, when an acute, febrile non-diarrheal illness reached minor epidemic proportions in the community.

The age and sex distribution of these illnesses is shown in Table IX. Diarrheal illness was concentrated in very young children and incidence fell with increase in age. The sexes were affected equally in all age groups.

A summary of all isolations of enteric pathogens and their association with diarrhea is given in Table X. Age, sex, and seasonal incidences of infection have not yet been determined. Nearly half the Shigella isolations were made from persons with diarrhea whereas Salmonella and non-cholera vibrios were more often found in healthy persons.

The frequency with which various organisms were isolated from patients with diarrheal illness is shown in Table XI. As in most studies of this kind, no recognizable pathogen was isolated from the vast majority of diarrheal illnesses. Shigella, heretofore rarely isolated from natives of East Pakistan, proved to be fairly common, and to be associated with diarrhea with unexpected frequency.

Salmonella, on the other hand, was rarely associated with diarrhea. Ten of the twelve Salmonella isolates, including two S. typhi, were isolated from healthy individuals.

Since Shigella was the only organism isolated in appreciable numbers, an attempt was made to estimate the frequency of inapparent infection based on total swabs collected. The results are shown in Table XII. The inapparent infection rate, based on the total number of swabs collected from persons without diarrhea during the week of collection, was only 0.4%, whereas among persons with diarrhea, 16.9% of cultures yielded Shigella. The number of swabs obtained from persons without diarrhea represented about one-half the maximum possible collections, based on the total person-weeks of observations. It is possible, therefore, that so-called carriers could have been concentrated in the unswabbed half. The more likely assumption, however, is that Shigella was associated with diarrhea in this community and that inapparent infection was not common.

Of the 82 Shigella isolated, the largest number were Group B, but Groups A, C, and D have also been found. In addition, several strains were isolated which had all the bacteriologic characteristics of Shigella, but failed to agglutinate with A, B, C, D or Alkaligenes-Dispar serum. These have been tentatively included with Shigella in this report; they are being investigated further.

The age, sex and seasonal distribution and association with disease will be determined for each of the kinds of organisms isolated. The diarrheal incidence among family members associated with infected persons is also being studied.

Non-cholera vibrios of several Heiberg groups were isolated frequently from dug wells throughout the seven-month period. Relatively few human isolates were made, and these were rarely associated with diarrhea. The role of non-cholera vibrios as pathogens is of considerable interest, however, and the Rayar Bazar community provides a unique opportunity to observe the association of diarrhea and the presence of these organisms in drinking water. Accordingly, a study designed to investigate this question has been initiated.

SUMMARY

A longitudinal diarrhea morbidity study was started in February, 1964, among families residing in a village in which a sharp outbreak of cholera had occurred during the preceding month. A total of 77 cholera-affected families and 135 control families were observed during the first seven months. Surveillance consisted of weekly rectal swabbing for bacterial enteric pathogens and recording of diarrheal and non-diarrheal illness.

The first part of this report describes the cholera outbreak in January and early February, 1964. The second part is a report of preliminary findings for the period of February 1 to August 30, 1964. Diarrheal illness accounted for more than half of reported morbidity, and was concentrated among children 0-4 years of age. The bacterial agents isolated and their association with diarrheal disease are briefly discussed. As planned, the study will continue until June 30, 1965, for a total of 17 months of observation.

TABLE I

Confirmed Cholera, Kayar Bazar, January-February 1964.
by Date of Onset

Date of Onset	Number of Cases
January 17	2
January 18	3
January 19	2
January 20	11
January 21	14
January 22	11
January 23	5
January 24	11
January 25	6
January 26	9
January 27	10
January 28	3
January 29	3
January 30	2
February 5	1
February 11	1
Unknown	1
TOTAL	95

TABLE II

Attack Rates in Refugee Camp Population and in Affected Families, Rayar Bazar, Dacca, January-February, 1964

Age	Estimated persons at risk *	Persons at risk in affected families	Confirmed cholera cases	Attack rates in camp (%)	Attack rates in affected families (%)
9 or under	1,687	228	42	2.48	18.4
over 9	4,735	367	53	1.11	14.5
TOTAL	6,422	595	95	1.47	16.0

* Based on food ration records.

TABLE III

Distribution of Cholera Cases by Family
Rayar Bazar, Dacca, January-February 1964

Number of cases per family	Number of families	Total Cases
0	948*	0
1	57	57
2	13	26
3	4	12
TOTAL	1,022*	95

* Based on estimates from food ration records.

TABLE IV

Incidence of Confirmed Cholera among Members of Affected Families, by Age and Sex, Rayar Bazar, Dacca, January-February,1964.

Age	Persons At Risk			Cholera Cases			Intrafamilial Attack Rate (%)		
	M	F	T	M	F	T	M	F	T
< 1	15	8	23	-	-	-	0	0	0
1-4	37	32	69	6	5	11	16.2	15.6	15.9
5-14	114	108	222	20	23	43	17.5	21.3	19.4
15+	142	139	281	8	33	41	5.6	23.7	14.7
TOTAL	308	287	595	34	61	95	11.0	21.2	16.0

TABLE V

Attack Rates by Family Size,
Rayar Bazar, January-February,1964

Number of persons in family	Number of Families	Persons at risk	Total Cases	Intra-familial attack Rate (%)	Subsequent Cases	Persons at risk	Subsequent Case Rate (%)
< 8	37	217	46	21.2	9	180	5.0
8 or more	37	378	49	13.0	12	341	3.6
TOTAL	74	595	95	16.0	21	521	4.0

TABLE VI

Attack Rates by Number of Children in Family,
Rayar Bazar, January-February,1964

Number of children < 10 years in family	Number of families	Persons at risk	Total Cases	Intra-familial attack Rate (%)	Subsequent Cases	Persons at risk	Subsequent Case Rate (%)
< 4	48	339	60	17.7	12	291	4.1
4 or more	26	256	35	13.7	9	230	3.9
TOTAL	74	595	95	16.0	21	521	4.0

TABLE VII

Hour of Onset of 95 Confirmed Cholera Cases, Ravar Bazar, Dacca, January-February, 1964

Hour of Onset	Number of Cases
0001 - 0200	5
0201 - 0400	10
0401 - 0600	22
0601 - 0800	19
0801 - 1000	5
1001 - 1200	4
1201 - 1400	4
1401 - 1600	4
1601 - 1800	5
1801 - 2000	7
2001 - 2200	2
2201 - 2400	7
Unknown	1
TOTAL	95

TABLE VIII

Monthly Disease Incidence, Cases per 1,000 Person-Weeks, for all Families, Rayar Bezar, Dacca

February - August, 1964

Month of Onset	Person-Weeks of Observation	Diarrheal Disease		Non-diarrheal Disease	
		Cases	Rate	Cases	Rate
February	3,342	27	8.0	5	1.4
March	3,011	5	1.6	3	0.9
April	2,451	4	1.6	0	0
May	2,552	28	10.9	8	3.1
June	2,422	37	15.2	21	8.6
July	3,225	68	21.0	45	13.9
August	2,794	48	17.1	65	23.2
Unknown		8		13	
TOTAL	19,797	225	11.3	160	8.0

TABLE IX

Disease Incidence by Age and Sex, Cases per 1,000 Person-Weeks. All Families, Rayar Bazar, Dacca.

February - August, 1964

Diarrheal Disease

Age	MALE		FEMALE		TOTAL	
	Cases	Rate	Cases	Rate	Cases	Rate
0-4	40	26.7	36	22.5	76	24.5
5-14	50	13.1	45	12.9	95	13.0
15+	22	4.7	32	6.8	54	5.7
TOTAL	112	11.1	113	11.5	225	11.3

Non-Diarrheal Disease

Age	MALE		FEMALE		TOTAL	
	Cases	Rate	Cases	Rate	Cases	Rate
0-4	14	9.3	21	13.1	35	11.3
5-14	53	13.9	27	7.7	80	11.0
15+	18	3.7	27	5.7	45	4.7
TOTAL	85	8.4	75	7.6	160	8.0

TABLE X

Agents Isolated from Study Family Members
Rayar Bazar, Dacca.

February - August, 1964

Agent	Associated with Diarrhea	Not Associated with Diarrhea	Total Isolates
Shigella	36	46	82
Salmonella	2	10	12
V. cholerae Ogawa	2	0	2
V. cholerae Inaba	1	1	2
Non-Cholera Vibrios	2	17	19

TABLE XI

Bacteriologic Findings in All Diarrheal Illnesses in Study Families, Rayar Bazar, Dacca.

February - August, 1964

Organism Isolated	Frequency	% of Total Diarrheal Illnesses
Shigella	36	16.0
Salmonella	2	0.9
V.cholerae Ogawa	2	0.9
V.cholerae Inaba	1	0.4
Non-cholera Vibrios	2	0.9
E. histolytica	2	0.9
No enteric pathogen	168	74.7
No culture taken	12	5.3
Total Illnesses	225	100.0

TABLE XII

Association of Diarrheal Illness and Isolation of Shigella
All Study Families, Rayar Bazar, Dacca.

February - August, 1964

Symptoms Within one Week of Swabbing	Number of Visits	Rectal Swabs Collected	Percent Swabbed	Shigella Isolated	% Shigella of total Swabbed
Diarrhea absent	19,572 *	10,359	52.9	46	0.4
Diarrhea present	225	213	94.7	36	16.9
TOTAL	19,797 *	10,572	53.4	82	0.8

* Based on total person-weeks of observation.

FAMILY ROSTER OF CHOLERA CASE 1964

Location of House

Name	CRL No.	Age	Sex	Family Relationship	Refugee Camp	Date & Hour of entering to camp	Date & Hour of onset of cholera	Date & Hour of Admission-CRL

RAYAR BAZAR FAMILY STUDY
OCCUPATIONAL AND RESIDENCE HISTORY

Family Number_____

Date form completed_____

If family leaves Rayar Bazar, give,

a. Date_____

b. Destination_____

Location of house in Rayar Bazar (para or section of village)

a. At present_____

b. Before Jan. 15, 1964:
Same House? (Yes-No) _____
If no, give previous location:
_____

Questions to be answered for each family member:

1. Present Occupation
2. Has person changed job since Jan. 15, 1964? (yes-no)
3. Place of work (thana, mohalla, para & street)
4. How many days per week does he or she work outside Rayar Bazar?
5. For Footnotes. If necessary, indicate footnote by number and write explanation on reverse side of sheet.

Ind. No.	1	2	3	4	5

Appendix : C

RAYER BAZAR FAMILY STUDY

Family Water Consumption Record

Family Number _____ Date this form was completed _____

Name of Head of Household _____

Individual Number of Head _____

Location of House in Rayer Bazar (Para) _____

Chief Sources of Water for this family:

Usage	Tank No.	Dug Well No.	Tube Well No.	River or Canal	Other
Drinking					
Cooking					
Washing Dishes					
Washing Clothes					
Bathing					
Latrine Use					

If dug well is used for any purpose by this family, give location of well with respect to house

_____

_____

How many families use water from this well? _____

Is this well used by any other study family?(Yes or No) _____

If yes, give family number _____

If any changes in water usage take place after this form is completed, please fill in the following:

Date Change	Nature of Change

Appendix D

RAYAR BAZAR FAMILY STUDY

DUG WELL RECORD

Code Number of Well_____ Index Family Number_____

Other Study Families using same well_____

Location (Para) in Rayar Bazar_____

Location of Well in Household Compound_____

_____

Distance from Latrine (indicate unit of measure)_____

Sketch of household with location of well and latrine:

Date Sample Obtained	Water Lab. Number	Vibrios				Coliforms		pH F / Lab
		NAG		AG		E. coli 45.5	MF coliforms	
		Broth	Plate	Broth	Plate	MPN/100 ml.	per 100 ml.	

RAYER BAZAR FAMILY STUDY

BIRTH REPORT

1. Family and Individual number of Mother _____

2. Name of Mother _____

3. Individual Number Assigned to Newborn _____

4. Name of Newborn _____

5. Date of Birth _____ _____ _____ _____
   day month year hour

6. Was this a normal delivery? _____ (Yes or No)

   If no, describe the abnormality _____

   _____

   _____

7. Is Mother now well? _____ (Yes or No)

   If no, describe illness _____

   _____

   _____

8. Is infant now well? _____ (Yes or No)

   If no, describe illness _____

   _____

   _____

   _____

   _____

   _____

Signed _____

Date _____

Appendix F

RAYER BAZAR FAMILY STUDY

DEATH REPORT

1. Family number of deceased _____

2. Individual number of deceased _____

3. Name of deceased _____

4. When did deceased become ill? _____ day _____ month _____ year _____ hour

5. When did death occur? _____ day _____ month _____ year _____ hour

6. Was the illness leading to death associated with diarrhea? _____ (Yes or No)

7. Was the deceased hospitalized? _____ (Yes or No)

8. If yes, at what hospital? _____

9. Describe illness in detail _____

_____

_____

_____

_____

_____

_____

_____

_____

Signed _____

Date _____

Appendix :G

RAYER BAZAR FAMILY STUDY

ILLNESS REPORT

Date of visit/attending clinic _____ Clinic entry number_____

Study family? (yes or no) _____ Family number _____ Indiv.No._____

Name _____ Age _____ Sex _____

S/O _____ Head of Family _____

Address _____

If diarrhea: Date of onset _____ Hour of onset _____

Total number of stools since onset _____ Average daily number _____

Rice-water stools/loose motion/blood/mucus/vomiting/tenesmus/fever/

prolapsed rectum/abdominal pain/distension/tenderness

Other complaints: _____

Remarks: _____

Is patient able to perform usual duties? _____, confined to bed? _____

Provisional diagnosis _____

Examined / interviewed by : _____

CRL Admission No. _____ Date of admission _____ Hour _____

Was rectal swab obtained? (Yes or no) _____ Specimen number _____

Laboratory results:

Appendix H

RAYER BAZAR FAMILY STUDY
Bacteriologic Report

Family Number _____ Date Collected _____ Signature _____

Ind. No. _____ Bact. No._____ Result:	Ind. No._____ Bact. No. _____ Result:
Ind. No._____ Bact.No._____ Result:	Ind. No. _____ Bact.No._____ Result
Ind. No. _____ Bact No._____ Result:	Ind. No. _____ Bact.No._____ Result:
Ind. No._____ Bact.No. _____ Result:	Ind.No._____ Bact.No._____ Result:
Ind. No._____ Bact.No._____ Result:	Ind.No._____ Bact.No._____ Result:
Ind. No. _____Bact.No._____ Result:	Ind. No._____ Bact.No._____ Result:

Vibrio Infections of Ruminants

by

Miss Anisa Saad and Dr. Robert S. Gordon

Introduction:

The continuing prevalence of cholera in East Pakistan stimulated a study of ruminants as possible reservoirs of V. cholerae. The investigation was based on the observation made by Abou Gareeb (1960) of non-agglutinable vibrios. He raised but rejected the hypothesis that silent animal reservoir for V. cholerae might possibly exist among cattle when true cholera vibrios are not found among human beings. In July 1962, study of ruminants began in our laboratory and continued for a period of seven months. To determine whether the findings were reproducible, cows were restudied in August 1964.

Materials and Methods:

Blood was obtained from 564 cows, 161 goats, 57 buffaloes and 6 sheep at the time of slaughter in the Dacca abattoir. Sera were provided from 44 cows at the Agricultural College, Mymensingh, East Pakistan, by courtesy of Dr. William Dieterich. Blood specimens were drawn from 30 calves at the Institute of Public Health, Dacca, prior to their use for the production of Smallpox Vaccine. Sera were obtained by Dr. Robert Gordon from 46 cows in the abattoir in Hechingen, Germany.

Rumen contents of 479 cows, 274 goats, 56 buffaloes and 3 sheep were obtained in the Dacca abattoir immediately after the rumen was removed using as sterile technique as the conditions permitted.

Rumen contents were cultured aerobically in alkaline peptone water (peptone 1% NaCl 1%, pH 9.0). Overnight cultures were streaked on gelatin agar plates and incubated at 37° C for 18 to 20 hours. Slide and tube agglutinations were performed on suspected colonies of vibrios using 'O' Group I and type specific sera provided by Dr. Goodner. Biochemical procedures were used to classify isolated vibrios into Heiberg groups. The sera were screened for V. cholerae agglutinins by standard tube agglutination technique (Goodner 1960). Living suspensions of Ogawa 463, 465 and Inaba 462, 466, all isolated in this laboratory in 1962 from cholera cases in Khulna, were used an antigen.

Brucella agglutinins were determined by the rapid slide agglutination test (Schubert 1953) with antigen provided by Colonel Robert Yaeger, V.C.

Absorption tests were performed on two cow sera with high anti cholera agglutinin titer. Living organisms, Ogawa strains 465 and Inaba strain 466; NCV 5 and Escherichia coli were used in the absorption tests (Goodner 1960).

Results:

Vibrios were isolated from the rumen of all species examined in this study; from a few animals, two strains, biochemically different were isolated (Table I). No isolated organisms agglutinated in 'O' Group I sera; biochemically all fell into Heiberg Group I and II. Only three sheep were examined - the rumen of one was found to contain Heiberg Group I non-cholera vibrio.

Serological Studies:

Antibodies which agglutinated living cholera vibrios were found in all species of rumens examined (Table II). They were most frequently seen and at higher titer among cattle. In 1962, 23% of cows tested had titers of 1 to 300 or greater as compared to 10% among goats and 2% among buffaloes. In 1964, antibodies were again demonstrated which agglutinated the classical cholera vibrios, although at lower titer. Pertinent, however, is the observation that 20% of serum from 46 German cows available agglutinated cholera vibrios at a titer of 1 to 100.

From 10 animals, both a non-cholera vibrio isolate and a serum were available; 3 of those had no agglutinins either against cholera or the homologous non-cholera vibrios; in 3, antibodies were present against both organisms to equal titer, and, in the other 4, the anti-cholera titers were higher than those against the homologous non-cholera vibrio.

The persistence of these antibodies was examined in a calf with a titer of 1:2000 Ogawa and 1:1000 Inaba which was purchased in November 1962. At the present time it still has agglutinating titer of 1:320 against both antigens.

Because of the antigenic cross between vibrios and Brucellae, the sera were examined for Brucella agglutinins. Only 8 out of 594 cows had anti-Brucella titers of 1:80 or higher (Table III). Comparison of anti-Brucella and anti-cholera titers in 580 of these sera is shown in Table IV. No association is evident.

Five cow sera with titers of 1:1000 or greater against V. cholerae were tested against 45 non-cholera vibrios to see whether these agglutinins are directed against all vibrios. Patterns of agglutination fell into every possible variety as seen in Table V.

The result of absorption of two cows and a vaccinated goat is given in Table VI. The cow sera were obtained in 1962, when both agglutinated Ogawa and Inaba to a titer of 1:1000. At that time, Calf 4 agglutinated NCV 5 to a titer of 30, while cow 106 had a titer of 1000 against this organism. The agglutinating antibodies were completely absorbed from the cow sera by Ogawa, Inaba and non-cholera vibrio antigens. In contrast, absorption with the same NCV strain had no effect on the specific agglutinins of the goat which had been vaccinated with frozen and thawed Ogawa organisms; only absorption with Ogawa vibrios completely removed agglutinins.

Discussion:

These results are interesting in two ways. Firstly, rumen vibrios usually reported are obligatory anaerobes; these were typical aerobic growing non-cholera vibrios. Secondly, there was no evidence in this study that infection of the cow with V. cholerae occurs in the endemic area. The complete absorption of the agglutinating antibody by non-cholera vibrios suggests that this may be a common flagellar or other factor.

TABLE I

Isolation of non-cholera vibrios from rumen contents of different species of ruminants.

Animal Source	No. examined	Rumens positive for vibrios		NCV strains isolated		
		No.	%	GroupI	Group II	Total
Cattle 1964	200	76	38.0	27	51	78
Cattle July - January	279	30	10.75	15	18	33
Goats July - January	274	16	5.83	7	10	17
Buffaloes July - January	56	8	14.28	4	4	8
Sheep	3	1	-	1	-	1

TABLE II

Agglutinins in ruminant sera for living *Vibrio cholerae* antigens.

Ruminants	Agglutinins Titer										
	<1:30		1:30		1:100		1:300		1:1000		Total
	No.	%	No.	%	No.	%	No.	%	No.	%	
Cattle 1964	92	23.1	194	48.7	48	21.9	22	5.5	3	1	398
Cattle July 1962-Jan 1963	18	7.5	70	29.2	96	40.0	49	20.4	7	2.9	240
Goats July 1962-Jan 1963.	43	26.7	68	42.2	34	21.1	16	9.9	-	-	161
Buffaloes July 1962-Jan 1963.	21	36.8	21	36.8	14	24.6	1	1.8	-	-	57
Sheep July 1962-Jan 1963.	-	-	5	83.3	1	16.7	-	-	-	-	6
German Cows October 1962	3	6.5	34	73.9	9	19.6	-	-	-	-	46

TABLE III

BRUCELLA ABORTUS AGGLUTININ TITERS IN RUMINANTS.

Ruminants	Total	Antibrucella Titers						
		Less 1:20	1:20	1:40	1:80	1:160	1:320	1:640
Cattle	594	470	101	15	5	2	-	1
German cattle	46	45	1	-	-	-	-	-
Goats	113	110	2	1	-	-	-	-
Buffaloes	47	47	-	-	-	-	-	-
Sheep	4	4	-	-	-	-	-	-

TABLE IV

Comparison of Brucella and V.Cholerae Agglutininis in 580 cow sera.

Anti-cholera titers: Serum dilution	20	20	40	80	160	320	640
1000	8	0	1	0	0		
300	44	10	6	2	0		
100	120	40	4	2	1		
30	203	34	2	0	1		
30	90	9	1	0	1		1

Serum dilution
Antibrucella agglutinins

TABLE V

Agglutination titers of cow sera against 45 NCV isolated from rumenents.

Number of NCV strains with given pattern	S E R U M				
	Cow 13	Cow 30	Cow 106	Cow 137	Cow 4
13	0	0	0	0	0
2	0	0	0	30	0
1	0	0	30	30	0
1	0	0	100	100	0
1	0	0	100	100	30
1	0	30	0	30	0
2	0	30	0	0	0
3	0	30	30	30	0
1	0	30	0	0	0
1	0	30	30	30	100
1	0	30	0	0	30
1	0	30	30	100	30
1	30	30	30	100	100
1	30	30	0	30	30
2	30	30	30	30	0
1	30	100	0	30	0
1	30	30	0	30	0
4	30	30	30	30	30
1	30	100	100	100	1000
1	100	30	30	100	0
1	100	30	30	30	30
1	100	300	300	300	0
1	100	100	100	300	30
1	300	300	300	300	300
1	1000	1000	1000	1000	30
Ogawa	1000	1000	1000	1000	2000
Inaba	1000	1000	1000	1000	1000

TABLE VI.

Agglutinin titers of sera absorped with whole living organisms

Agglutination antigens / Absorbing antigen	SERUM											
	Calf 4				Cow 106				Ogawa immunized Goat			
	Og 465	In 466	NCV 5	E.coli -	Og 465	In 466	NCV 5	E.coli -	Og 465	In 466	NCV 5	E.coli -
None	640	640	0	20	320	320	0	0	5120	5120	0	0
V.cholerae Ogawa 465	0	0	0	20	0	0	0	0	0	0	0	0
V.cholerae Inaba 466	0	0	0	20	0	0	0	0	640	0	0	0
NCV 5	0	0	0	20	0	0	0	0	5120	5120	0	0
E. coli	1280	640	0	0	-	-	-	-	10240	5120	0	0

References

Abou Gareeb, A. H., International Journal of Prophylactic Medicine and Social Hygiene, 4:(1) 1960

Schubert, J. H., Journal of Laboratory and Clinical Medicine 41:(5) 1953, 776-781

Goodner, K., SEATO Conference on Cholera, December 1960, pages 104-110.

Cholera Vaccine Field Trial in Rural East Pakistan

First Six Months of Observation

by

Dr. R. Oseasohn and Dr. A. S. Benenson

Introduction:

Cholera vaccine was the first anti-bacterial vaccine introduced for the control of communicable disease in man. Despite widespread use, convincing proof of its efficacy is still not available. Accordingly, in November 1963, the Pakistan-SEATO Cholera Research Laboratory initiated a controlled field trial of cholera vaccine in rural East Pakistan. The study was designed to answer three related questions: (1) can a parenterally injected vaccine prevent the diarrhea associated with Vibrio cholerae? (2) if so, for how long? and (3) does vaccine prevent inapparent infection among family contacts of persons with diarrhea associated with V. cholerae? This report deals with the experience of the first six months of this field trial.

Methods:

A group of 23 riverine villages in Matlab thana, an administration subdivision of Comilla District about 40 miles from Dacca, was selected for study because of continued high prevalence of cholera in the recent past. These communities are located along the Gumti River which allowed easy access by boat for case finding and treatment. This is one of East Pakistan's most densely populated areas; the population density exceeds 1500 per square mile.

Local health officers had prepared a census of the population of these villages, during a smallpox control program in 1961. On the basis of this census, the population was estimated to be 24,000. These census lists were revised by a house-to-house survey, and each individual within each village was numbered in consecutive order with a letter prefix identifying the village. Interest in the immunization program was aroused by discussion with town elders and distribution of explanatory leaflets in Bengali.

The cholera vaccine used was a commercial preparation containing a mixture of both Inaba and Ogawa strains. The vaccine was prepared by harvesting surface agar growth in 0.5% phenolized saline, and diluting the two components to an optical density equivalent to 8000 million V. cholerae organisms per ml. The finished vaccine had a relative mouse potency of 4.40 against the Ogawa, and 32.4 against the Inaba reference vaccines of the National Institutes of Health, Bethesda, Maryland*. The vaccine contained 1.3 mg total nitrogen per ml, 0.08 mg trichloracetic acid-precipitatable nitrogen per ml, and 0.9 mg nitrogen per ml precipitated by 1.5 volumes of acetone**. Typhoid, paratyphoid A and

* These assays were performed by Dr. John C. Feeley, Division of Biologics Standards, National Institutes of Health.

** Chemical determinations were performed by Dr. John Barbaro, Department of Immuno-chemistry, Walter Reed Army Institute of Research, Washington.

paratyphoid B vaccine was used as the control. The organisms were grown on agar surfaces, heat-killed, suspended in 0.5% phenol, and diluted by nephelometric density so that the finished vaccine contained 1000 million S. typhi and 750 million of both Paratyphoid A and Paratyphoid B organisms per ml. The two vaccines were distributed in identical bottles, one labelled by a red letter "A", and the other by a blue "B". The materials were coded by one observer who did not reveal the identity of the test materials throughout the field trial. The dosage schedule was 0.5 ml for those over 12 years of age, 0.25 for those 2 to 12, and 0.1 for those under 2. After 7027 persons had been inoculated, the dose was reduced to 0.4 ml. for those over 12 years of age because local reactions had interfered with the participation of adults.

Immunizations were performed during a 6-week period beginning on 1 November, 1963. The vaccination team proceeded along the river by boat. Jet injectors were used almost exclusively to perform the inoculations. Odd-numbered persons received vaccine "A", and even-numbered persons, vaccine "B". Family members who appeared at the time of vaccine administration, but who had been overlooked during the census review a few days earlier, were added to the lists and given alternately vaccine "A" and "B". All recipients were given a card, one side of which showed the following: village, vaccine trial number, name (in Bengali) age, sex, and date of inoculation. On the reverse side of the card, a notice in Bengali requested that the study team be notified promptly in case of diarrhea, and that the patient come at once to the central field hospital for treatment if symptoms suggested cholera. (Appendix A).

Medical care was provided by this laboratory in a facility situated in Matlab Bazar, a centrally located market community. A physician and nurse were in constant attendance at the hospital which was housed first in a small tin shed and later on a barge provided by the Government of East Pakistan. Patients were treated with intravenous fluids and tetracycline (W. B. Greenough III, R. S. Gordon, Jr., I. S. Rosenberg, B. I. Davies and A. S. Benenson 1964).

Case finding was organized as follows: The 23 villages were divided into 3 groups. Surveillance within each group was under the supervision of an experienced field worker. All supervisors had received graduate training in social sciences at Dacca University and had been engaged in community studies related to cholera for at least one year prior to the vaccine trial. The villages within each group were assigned to 7 field workers who were assisted by a local midwife in each village. Families were visited twice weekly to inquire about diarrheal disease and encourage prompt use of the field hospital in the event of illness. Whenever a new case of diarrhea was discovered, details of the illness were recorded on a simple report form (see Appendix B), and a fecal sample collected by means of a tellurite-impregnated rectal swab which was placed in bile-peptone transport media. Specimens were sent to the laboratory in Dacca, usually within 24 hours, for further bacteriological investigation. The laboratory procedures employed have been described previously. (Monsur, K. A., 1963). Fecal samples obtained from hospitalized patients were streaked directly on tellurite-taurocholate-gelatin agar in addition to the use of the liquid transport media.

Disability associated with diarrheal disease was classified as follows: no interference with usual duties, inability to perform usual duties, inability to walk, and inability to respond. Whenever persons were observed with diarrhea

who showed inability to walk or to respond, fecal samples were collected on at least two consecutive days from vaccinated contacts, and they were queried concerning diarrhea.

The area supervisors were responsible to investigate and report each death occurring among vaccine recipients during the study interval in their respective area (see Appendix C). Whenever death was associated with diarrhea, fecal samples were collected from household contacts if none had been obtained from the deceased.

Results:

The total population of the 23 villages was 27,639, of which 14,059 (50.9%) participated in the trial (Table 1). An excess of males (114.7 males per 100 females) was observed. This finding is consistent with previous estimates showing sex ratios in the 17 districts of East Pakistan ranging from 106.5 to 116.8 males per 100 females (Census of Pakistan,1951). Participation was greatest (61.8%) among 5 to 14 year olds. Males appeared to be less co-operative than females (47.5% versus 54.8%); this may be an artefact related to the purdah system.

Vaccine "A" was given to 7013 persons and "B" to 6956. The two groups did not differ by age, sex, history of prior cholera or past anti-cholera inoculation (Table 2).

The frequency of diarrhea episodes occuring in the two groups within the first 6 months of the trial is shown in Table 3. During the first 7 days after inoculation, V. cholerae Inaba was isolated from 6 persons with diarrhea. One had received Vaccine "A" and 5 Vaccine "B". These have not been included in the subsequent analysis. Findings are summarized by 3-month intervals according to the presence or absence of vibrios on culture, and whether the individuals were observed in the community or hospital. Twenty-eight instances of cholera infection were seen in the first 3-month period, and 13 in the second. In both time intervals, the number of infections occuring in the "A" group exceeded that in the "B" group. The frequency of diarrhea episodes from which vibrios were not isolated was similar in both groups and increased during the period of study. The age distribution of persons with vibrio-associated diarrhea is shown in Table 4. Thirty-four of the 41 infected individuals were less than 15 years of age, and 17 of these 34 were less than 5 years of age. The incidence rate during the 6-month interval was 4.8 per 1000 among vaccine "A" recipients and 1.0 per 1000 among recipients of vaccine "B".

Only 5 asymptomatic infections were discovered among 131 inoculated family contacts studied during the first 6 months of the trial. One of the 5 had received vaccine "A" and the remaining 4, vaccine "B".

Forty-seven deaths occurred among vaccine recipients (Table 5). Two of the 47 died less than 7 days after inoculation; one was a 2-year old female vaccine "A" recipient with chronic bloody dysentery from whom V.cholerae was not recovered; and the other was a 16-year old male with bacteriologically confirmed cholera one day after vaccine "B" was administered.

Fatal illnesses in 12 of the remaining 45 were associated with gastrointestinal symptoms ("diarrhea", "loose stools", dysentery", and "mucus and blood stools") (Table 6). Rectal swabs were obtained from either patients or family contacts in 10 of the 12, and V. cholerae was isolated from only 1 of the 10. This patient (P1069) was a 5-year old male who was given vaccine "A" on 29 November and developed typical cholera on 9 December. His mother refused to permit hospitalization.

Discussion:

While previous reports (W. M. Haffkine, 1895; R. Adiseshan, C.G. Pandit and K. V. Venkatraman, 1947) indicated that cholera inoculation reduced the incidence of the disease, the evidence was not convincing. These earlier studies (Greenwood, M., Jr. and G. U. Yule, 1915; C. M. MacLeod, 1960) were critized because of faulty experimental design, such as the lack of a suitable control group, failure to observe a strict double-blind technique, the conduct of trials during an epidemic resulting in unequal exposure to infection in the groups being compared, and inadequate diagnostic data. In this study a double-blind technique with a suitable control group was used: the two test groups were comparable in every respect measured. Intensive surveillance was maintained, and only bacteriologically confirmed instances of cholera infection were included. The riverine villages which were selected provided a typical endemic setting.

Cholera reporting in most endemic areas has been incomplete and based largely on deaths certified by nonmedical officials. The present study provided an opportunity to determine more accurately the frequency of diarrheal diseases associated with Vibrio cholerae in a defined population. Infections were more frequent among children as might be expected in an endemic situation.

A significantly lower incidence of diarrhea associated with V. cholerae was observed in the group which received vaccine "B", which proved to be the cholera vaccine. Thus, the data collected during the first 6 months of the trial indicate that cholera vaccine can be an effective prophylactic procedure. The duration of effectiveness, however, remains unknown, and surveillance is being maintained to answer this question. The data concerning the effect of inoculation on inapparent infection are too scanty as yet to permit conclusions.

Because this study was designed to ask whether a parenterally injected vaccine can protect against cholera, the most potent commercial preparation available was used. Hence the inferrence cannot be made that all cholera vaccines will afford protection.

Conclusion:

In a controlled field study of cholera vaccine, the incidence rate of diarrhea associated with V. cholerae over a 6 month period was 4.8 per 1000 among those receiving a control (TAB) vaccine, and 1.0 per 1000 among those receiving a commercial cholera vaccine of high potency. The disease in this endemic area was concentrated among children.

TABLE I

Participants in Vaccine Study by Age and Sex

Age Group	MALE			FEMALE			TOTAL		
	No. of Inhabitants	Vaccinated No.	%	No. of Inhabitants	Vaccinated No.	%	No. of Inhabitants	Vaccinated No.	%
<5	2607	1273	48.8	2665	1263	47.4	5272	2536	48.2
5-14	4417	2638	59.7	3355	2160	64.4	7772	4798	61.8
15+	7731	3088	39.9	6837	3632	53.1	14568	6720	46.2
Not recorded	11	4	-	6	1	-	17	5	-
TOTAL	14,766	7003	47.4	12,863	7056	54.9	27,639	14,059	50.9

TABLE II

Vaccine Groups by age, sex, previous cholera and prior cholera inoculation.

	VACCINE A		VACCINE B	
	No.	%	No.	%
Males:				
Age < 5	621	17.3	652	19.0
5-14	1342	37.5	1296	37.9
15+	1613	45.1	1475	43.1
Unknown	4	0.1	-	-
TOTAL	3580	100.0	3423	100.0
Females:				
Age < 5	667	18.9	596	16.9
5-14	1072	30.4	1088	30.8
15+	1783	50.6	1848	52.3
Unknown	1	-	-	-
TOTAL	3523	99.9	3533	100.0
Total:				
Age < 5	1288	18.1	1248	17.9
5-14	2414	34.0	2384	34.3
15+	3396	47.8	3324	47.8
Unknown	5	0.1	-	-
TOTAL	7103	100.0	6956	100.0
History of previous cholera	415	5.9	414	6.0
No history of previous "	6577	94.1	6440	94.0
Total with known history	6992	100.0	6854	100.00
Prior cholera immunization	5465	78.4	5347	78.2
No history cholera "	1508	21.6	1488	21.8
Total with known history	6973	100.0	6835	100.0

TABLE III

Frequency of Diarrhea Episodes During First Six Months

V. cholerae culture* result and source of patients	Dec. 63 - Feb. 64		Mar. 64 - May 64		Dec. 63 - May 64	
	Vaccine A	Vaccine B	Vaccine A	Vaccine B	Vaccine A	Vaccine B
Culture positive						
Community	10(3)	3(1)	5	1	15	4
Hospital	14(1)	1	5	2	19	3
TOTAL	24	4	10	3	34	7
Culture negative						
Community	708	684	1508	1447	2216	2131
Hospital	13	12	49	38	62	50
TOTAL	721	696	1557	1485	2278	2181

* All isolates were Inaba serotype with the exception of 5 Ogawa isolates shown in parentheses.

TABLE IV

Frequency of diarrhea associated with V. Cholerae during first 6 months by age and vaccine group.

AGE GROUP	TOTAL			
	VACCINE A		VACCINE B	
	No.	Rate	No.	Rate
<5	15	11.7	2	1.6
5-14	13	5.4	4	1.7
15+	6	1.8	1	0.3
TOTAL	34	4.8	7	1.0

TABLE V

Deaths in Vaccinated Persons During First Six Months

Time Interval	Deaths Associated with Gastrointestinal Symptoms		Total Deaths	
	Vaccine A	Vaccine B	Vaccine A	Vaccine B
Dec. 63 - Feb. 64	4	2	11	7
Mar. 64 - May 64	4	2	15	12
Dec. 63 - May 64	8	4	26	19

TABLE VI

Findings in 12 Deaths Associated with Gastrointestinal Symptoms

Patient No.	Vaccine Group	Age & Sex	Date	V. cholerae isolated: Patient	V. cholerae isolated: Family	Remarks
P1069	A	5M	12/18	Inaba	-	typical cholera illness; family refused hospitalization.
B81	A	5F	12/18	Not tested	neg. (1 of 7) tested	typical cholera illness after contact with cholera.
1955/A	A	3M	1/3	"	neg. (2 of 2)	typical cholera illness.
C1855	A	4M	1/25	"	n.t	died of severe diarrheal illness in another village
D799*	A	3M	4/8	neg.	neg. (7 of 12)	chronic - bloody diarrhea with dehydration, died in transit to CRL Hospital in Dacca.
R625	A	60M	4/24	neg.	neg. (1 of 1)	chronic dysentery with repeated (3) cultures.
0495	A	50F	5/4	neg.	neg. (7 of 7)	-
C1259	A	55F	5/7	neg.	n.t.	chronic illness.
A1046	B	2F	1/10	neg.	n.t.	probable pneumonia.
B836	B	2M	1/21	n.t.	n.t.	chronic bloody dysentery.
D430	B	2F	4/17	neg.	n.t.	past history of dysentery and diarrhea; details of terminal illness uncertain; mother refused hospitalization.
S86	B	3M	4/22	neg.	n.t.	chronic dysentery in addition to "cold and cough."

* This patient was hospitalized; all others were observed in the community.

APPENDIX A.

(Translation)

If you have loose motion, report at once to the Health Assistant of your area or to the Sanitary Inspector of Matlab for taking proper care. If you think it is cholera please bring the patient at once to the Medical Officer at Matlab deputed by the Cholera Research Laboratory for prompt treatment.

APPENDIX B

SPECIMEN REPORT

Vaccine trial number of persons from whom sample was taken .....

Date sample was taken ....
day month year hour

Was this the first ..... 2nd ..... 3rd .....4th .....5th .....6th .....7th....
swab in this illness?

Was swab taken because of diarrhea? Yes ..... No.....

If yes, date of onset of diarrhea? ....
day month year

If yes, was person unable to perform usual duties? Yes..... No.....

If yes, was person unable to walk? Yes..... No.....

If yes, was person unconscious? Yes ..... No.....

Was swab taken because another family member had diarrhea? Yes ..... No.....

What is vaccine trial number of family member with diarrhea? .....

LABORATORY REPORT:-

APPENDIX C

DEATH REPORT

1. Vaccine trial number of deceased .....

2. Was the deceased hospitalized? Yes ..... No.....(Check)

3. Was a culture obtained? Yes ..... No.....

4. When did the deceased become ill? ....
day month year hour

5. When did death occur? ....
day month year hour

6. Was the illness leading to death associated with diarrhea? Yes... No...

7. Describe the illness in detail.....

.....

.....

.....

.....

.....

Signed.....

Date .....

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TETRACYCLINE IN THE TREATMENT OF CHOLERA

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ANTIBIOTICS are well known to be vibriocidal in vitro and in vivo; but, despite this, no beneficial effect of antibiotic therapy on the clinical course of cholera has been reported (Pollitzer 1959). If the clinical syndrome of cholera is the result of the presence of *Vibrio choleræ* in the gastrointestinal tract, removal of the organism should result in observable clinical benefit. During December, 1962, cholera cases entering the ward facilities of the Pakistan-SEATO Cholera Research Laboratory included many instances of severe and long-continued diarrhœa, associated with continued excretion of large numbers of vibrios. The management of these cases with replacement therapy required large quantities of fluids and much professional care. In the hope of shortening the course of the illness, we elected to administer a potent vibriocidal agent in addition. We here present the results observed in seventy-seven patients with bacteriologically proven cholera, all of whom received fluid and electrolyte replacement. In thirty-eight, tetracycline was also administered

intravenously and by mouth, while the other thirty-nine received no antibiotic or other vibriocidal agent. Treated and control cases were not strictly alternated, but the groups were sufficiently alike to make comparison valid.

Patients and Methods

Proven cholera cases admitted to the ward of the Pakistan-SEATO Cholera Research Laboratory during the last week of December, 1962, and the first 4 months of 1963 were analysed. Any case requiring fluid replacement on admission or passing at least 1% of the body-weight in liquid stool during a 24-hour period was included. Only patients given tetracycline both intravenously and orally were considered in the antibiotic-treated group. Thirty-nine cases which met these criteria fell into the tetracycline-treated group, and forty fell into the control group. There were two deaths in this series. One, in the tetracycline-treated group, was that of a 40-year-old man who had been in vascular collapse for about 72 hours before admission. One 2-year-old child in the control group died as a result of insufficiently rapid initial rehydration. These two cases have been excluded from further analysis.

On admission, and daily thereafter, a rectal swab was plated directly on gelatin agar (Smith and Goodner 1958) and bile-tellurite agar (Monsur 1961) as well as being put through enrichment media for vibrios (alkaline bile-tellurite peptone broth). Isolates were verified by slide agglutination, using both group and specific Ogawa and Inaba antisera. All isolates were non-hæmolytic, did not agglutinate chicken cells, and were lysed by Mukerjee's phage IV (Finkelstein and Mukerjee 1963). No other pathogens were identified in any of these cases.

Serum-proteins were measured by refractometry in a Bausch and Lomb serum-protein meter. Serum-CO_2 content was determined by the Van Slyke manometric technique on blood drawn under oil. White blood-cell counts, differential counts, microhæmatocrits, and photoelectric hæmoglobin determinations (cyan-methæmoglobin technique) were done on all cases in the acute and convalescent periods. Urinanalysis and stool examinations for ova and parasites were carried out during convalescence.

Patients were treated on specially designed cholera cots which permitted total collection of stool and urine. Total intake and output were summarised at 6-hourly intervals. The accuracy of intake and output measurements was verified by weighing patients at least once daily.

All patients were treated by fluid replacement along the lines suggested by the balance studies of Phillips and his collaborators (Watten et al. 1959, Phillips 1962). An initial infusion of non-pyrogenic isotonic saline was followed by a bicarbonate-saline-potassium mixture consisting of 5 g. sodium chloride, 4 g. sodium bicarbonate, and 1 or 2 g. potassium chloride per litre.

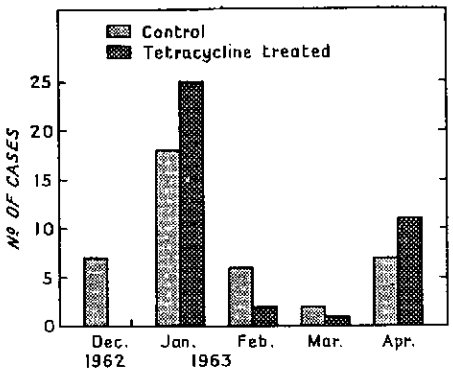

Fig. 1—Number of tetracycline-treated and control cases, by month of admission.

The goal was to eliminate hypovolæmia as soon as possible, to correct acidosis, and then to maintain a balance between intake and output of fluids and electrolytes. No glucose was given by any route. In the majority of cases it was necessary to use the femoral vein for initial fluid administration and drawing of blood samples. Some cases received vitamin C, thiamine, and/or B-complex vitamins parenterally.

In those cases treated with tetracycline, a dose of 100 mg. (stabilised with 250 or 300 mg. of ascorbic acid) was added to the 1st or 2nd litre of saline infused, and given rapidly over a period of 15-20 minutes. An oral schedule of 250 mg. every 6 hours was begun immediately on admission unless vomiting prohibited it, in which case the schedule was delayed for 6-12 hours. Intravenous doses were repeated approximately every 8 hours as long as medication could not be given reliably by mouth.

The treatment groups were not established in a prearranged manner; but over the course of the epidemic, approximately equal numbers of patients were included in the tetracycline-treated and control groups (fig. 1). After we had gained an impression that tetracycline therapy was beneficial, cases deemed to have a poor prognosis because of extremes of age or severity of symptoms were usually included in the tetracycline-treatment group. In the analysis of records, the various parameters (listed in tables I and II) were examined to test the comparability of the two groups. The only points of comparison showing statistically significant discrepancy were vitamin therapy and initial intravenous fluid requirement. By all criteria examined, the group treated with tetracycline contained the more seriously affected patients.

Results

The effect of tetracycline was manifest in the course after the 1st day of treatment. Both total volume of stool and requirement for intravenous fluids after the first 24 hours

TABLE I—CLINICAL COMPARABILITY OF TETRACYCLINE-TREATED AND CONTROL GROUPS

—	No. in treated group	No. in control group
Total numbers	38	39
Males	26	28
Females	12	11
V. cholerae Inaba	32	35
V. cholerae Ogawa	6	4
Other disease present	4*	5*
Intestinal parasites present	24	28
History of previous cholera †	3	3
Cholera immunisation †:		
Within 2 yr.	14	14
Over 2 yr. ago	2	3
None	19	17
Unknown	3	5
Treatment before admission:		
None	17	18
Sulphaguanidine and i.v. fluids	19	21
Other	2	0
Radial pulse weak or absent	17	17
Stuporous or unconscious	18	20
Temperature greater than 99°F during hospital stay	8	10
Vitamin therapy after admission:		
Vitamin C	38‡	11
Vitamin B	25	11
None	0	17

* Other diseases: 1 case each of asthma, pneumonia, chronic bronchitis, and previous gastrectomy in the treated group; and non-toxic goitre, herpes labialis, emphysema, bronchitis, and pregnancy in the control group.
† Undocumented statement by patient or relative.
‡ The preparation of tetracycline for i.v. use contained a therapeutically significant quantity of ascorbic acid.

were strikingly less in the tetracycline-treated group (fig. 2). Tests of significance indicate t values of 3·9 and 18 respectively, and $P < 0{\cdot}001$ for each comparison. Fig. 3 shows that duration of diarrhœa (defined to include any 24-hour period in which more than 1% of the body-weight in liquid stool was passed) was significantly shortened in the tetracycline-treated group as compared to the control group ($t = 3{\cdot}65$; $P < 0{\cdot}001$).

The mean duration of bacteriological positivity of the stool was greatly reduced in the treated group ($t = 6{\cdot}6$; $P < 0{\cdot}001$), with no case having a positive culture for more than 3 days (fig. 4).

Since vitamin therapy was not the same in the two groups the seventeen in the control group who did not receive vitamins B or C were compared with the twenty-two who had received these vitamins. The same variables used to evaluate tetracycline were examined. No influence of vitamin therapy was demonstrable.

TABLE II—CLINICAL COMPARABILITY OF TETRACYCLINE-TREATED AND CONTROL GROUPS

	Treated group		Untreated group		Significance of difference	
	Mean	95% confidence limits	Mean	95% confidence limits	*t*	P
Age (yr.)	17	13–21	18	14–21	0·1	>0·1
Body-weight (kg.)	27	21–33	33	29–36	1·6	>0·1
Hours of diarrhœa before admission	18	13–23	21	15–26	0·7	>0·1
Plasma-protein on admission (g. per 100 ml.)	10·1	9·5–10·6	9·7	9·1–10·4	0·8	>0·1
Plasma-protein convalescent (g. per 100 ml.)	7·0	6·6– 7·3	6·9	6·5– 7·3	0·18	>0·1
CO_2 on admission (mEq. per litre)	12·4	10·9–13·9	12·3	10·7–13·8	0·14	>0·1
Hæmatocrit on admission	47	45–50	44	41–47	1·5	>0·1
Hæmatocrit convalescent	35	34–37	34	32–36	0·65	>0·1
White cells on admission (c.mm. $\times 10^{-3}$)	23	20–26	20	17–23	1·2	>0·1
Stool-volume in first 24 hr. after admission (ml. per kg.)	100	68–133	72	42–104	1·2	>0·1
Intravenous fluids required in first 6 hours after admission (ml. per kg.)	85·2	74–96	62·6	48–77	2·1	<0·05

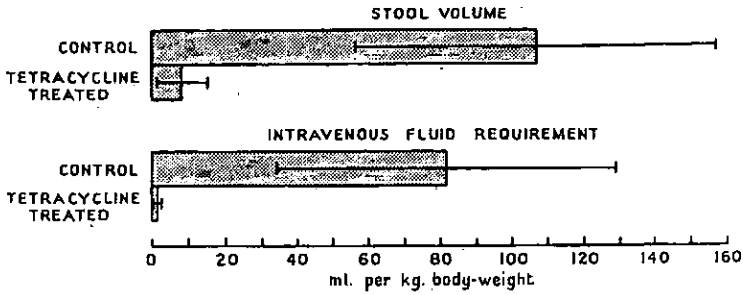

Fig. 2—Mean stool-volumes and intravenous fluid requirements after first 24 hours of therapy (95% confidence limits of mean indicated).

Because of the difference in initial intravenous fluid requirements of the two groups, the relationship of this variable to the subsequent course was examined in those cases not treated with antibiotic. The coefficient of

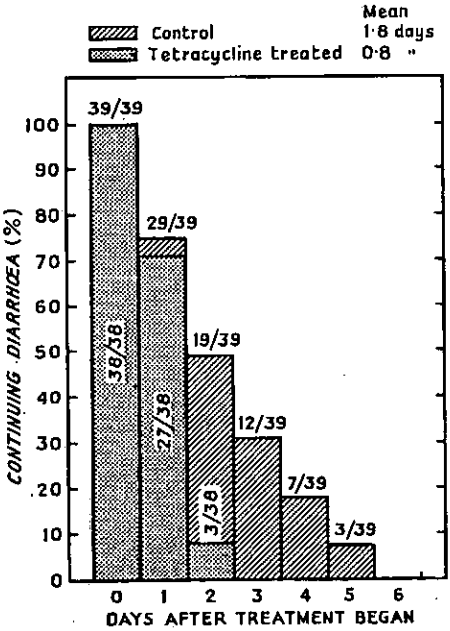

Fig. 3—Duration of diarrhœa after start of treatment.

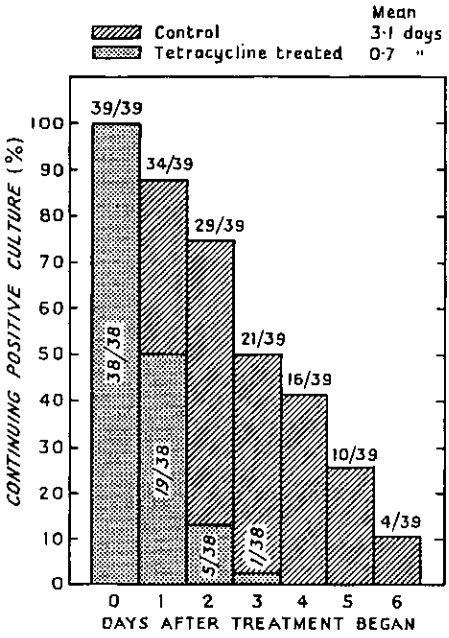

Fig. 4—Duration of positive culture after start of treatment.

correlation was positive and highly significant ($P < 0{\cdot}001$) for the comparison of intravenous fluid requirement during the first 6 hours with each of the following variables: stool-volume after the first 24 hours, intravenous fluid requirement after the first 24 hours, and duration of diarrhœa after hospital admission. The coefficient of correlation for the comparison of initial intravenous fluid requirements with the duration of positive cultures was lower, but still significant ($P < 0{\cdot}05$). These correlations indicate that patients who appear to be severely ill on admission to the hospital are most likely to have a long and severe course.

Discussion

The present study was not planned in advance as a controlled trial, and no placebo medication was available. Although the cases treated with tetracycline and the controls were not strictly alternated, the only evident resulting bias lies in the direction of including cases of higher risk and greater severity in the tetracycline-treated group. As these severe cases are the ones most

prone to prolonged illness, the bias in case selection tends to obscure the therapeutic effect of the antibiotic. The ability of antibiotic to eliminate the organism from the stool has been recognised in the past (Pollitzer 1959) and is evident in the results of the present study. A favourable effect on the course of disease, however, has not previously been reported. A preliminary account (Carpenter et al. 1963) indicates that confirmatory data will be forthcoming.

It is generally agreed that replacement of fluid and electrolytes is essential in the treatment of cholera patients. Such replacement is the only necessary therapy for patients in whom the natural course of the disease is short. For patients who suffer lengthy purging, however, management can be difficult and costly, and death is common unless sophisticated medical attention is available, along with laboratory support and adequate sterile supplies. Unless difficult cases can be recognised in advance, a potent antibacterial agent should be given to every patient from the beginning. This will not only reduce the number with lengthy diarrhœa, with resultant savings in sterile fluids and hospital bed space, but will also obviate returning patients who are still bacteriologically positive to the community.

Summary

Tetracycline as an adjunct in the treatment of cholera was evaluated by comparing the clinical course of patients who had received replacement of fluids and electrolytes alone with those who had been given tetracycline as well. The antibiotic eliminated bacteria from the stool, shortened the duration of diarrhœa, and decreased the requirements for intravenous fluids.

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TREATMENT OF CHOLERA IN 1964

By

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Treatment of Cholera in 1964

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TREATMENT OF CHOLERA IN 1964

Introduction

It is a paradox that cholera, a disease which can be treated with out-standing success by simple medical techniques, continues to take an annual toll of life in East Pakistan and neighbouring areas. The exact magnitude of the problem and its annual cost cannot be determined, as many cases occur in isolated rural districts and are not reported. Deaths occur not only among the elderly and weak, but among productive laborers, among mothers with dependent children, and among students who have not yet made use of their education. These are persons whose services should not be lost to a developing nation. In addition, fear of cholera may have an adverse effect upon travel, business, and tourism resulting in indirect economic losses which cannot be evaluated with certainty. Until adequate measures are developed and put into effect, the primary burden of preventing suffering, grief, and economic losses due to cholera will continue to fall upon the hospitals and physicians to whom the affected individuals turn for care.

Why are not all cholera cases in East Pakistan receiving adequate treatment today ? It is not because existing knowledge is inadequate, as will be shown in this article. Rather, the chief problem is that the necessary materials for the cure of cholera, and personnel skilled in their use, are not provided at the time and place they are needed. Cholera outbreaks occur suddenly, without warning, and the number of people affected is often so great that the available medical facilities are overwhelmed. Before extra help can be brought, the epidemic has done its damage and subsided. Treatment of epidemic cholera must be made as simple and cheap as possible, so that no time or money will be wasted on unessential medicines, and so that paramedical or even unskilled personnel can effectively assist doctors in ensuring survival of the victims.

Pathogenesis of Cholera

The treatment of cholera is based upon an understanding of the pathologic physiology of the disease, with the therapist counteracting, by specific procedures, the abnormalities that develop. The

main features of the pathophysiology of cholera were known in England over a century ago, long before the causative microorganism had been discovered. Basing his findings on the work of earlier scientists, Dr. John Snow in 1854 could ascribe the collapsed state of the cholera sufferer to the loss of water, salt, and alkali from the blood into the stools. He argued against the idea of a general toxemia in cholera, because all the morbid signs and symptoms of the disease could be relieved by the intravenous injection of saline solutions containing sodium bicarbonate. Thus cholera was long ago recognized as a primary affliction of the digestive tract, with all other signs and symptoms being consequences of the diarrhea and losses of body fluids.

These pioneer studies have been followed by many further investigations, culminating in the precise balance studies carried out in recent years by Phillips and associates. (Watten, **et al.** 1959, Watten, **et al.** 1962, Phillips, 1962). These investigations have revealed that in cholera, large volumes of liquid stool are formed that contain, on the average, about 140 mEq. (3.2g) of sodium, 15 mEq. (0.6g) of potassium, 100 mEq. (3.6g) of chloride, and 45 mEq. (2.7g) of bicarbonate per liter. The evacuations contain only insignificant quantities of proteins, sugars, or other body constituents. The copious stool is formed whether or not the patient takes food or fluids by mouth. As a result, there is a net loss of water and electrolytes from the body, particularly from the circulating blood. The blood volume is diminished, while its viscosity is greatly increased by the elevated concentrations of plasma proteins and erythrocytes left behind. The only electrolyte whose concentration in the plasma is greatly altered is bicarbonate, as salt and water are lost in the stool in isotonic proportions. Because the stools contain much more bicarbonate per liter than does plasma, a relative deficiency of this ion results, and the patient becomes acidotic. The decreased blood volume and elevated blood viscosity result in impaired circulation, and in shock which may progress to death in severe cases. Failure of circulation is the cause of cyanosis, coldness of extremities, suppression of urine, and many of the classical signs and symptoms of cholera. Acidosis causes vomiting and labored respiration, and may alone be the cause of death if allowed to persist while dehydration is corrected.

The causative organism of cholera, Vibrio cholerae, was discovered by Robert Koch in 1883. This bacterium has been studied intensively since that time, and diagnostic methods have been developed which make its specific indentification easy (Monsur, 1963 ; Benenson, **et al**, 1964). There can be little doubt that the presence of this organism in the intestine of cholera victim is the cause of the diarrhea and all its consequences, but understanding of the underlying biochemical mechanisms still eludes investigators. Unlike many other enteric pathogens, the cholera vibrio does not invade or destory tissue, and is not usually found outside the lumen of the digestive tract. Presumably, the bacterium pro-

duces a toxin which affects the intestinal epithelium resulting in the outpouring of fluid, but this hypothetical toxin has not been identified.

Principles of Cholera Therapy

On the basis of these concepts, a therapeutic program for acute cases has been formulated and found to be effective in practice. The first need is to correct losses of water and electrolytes which have been incured before the start of treatment. In general, the volume of stool which the patient has passed is unknown, so that the required volume for replacement has to be determined by clinical examination of the patient (see below) or laboratory study of his blood, as recommended by Phillips (1926). We **must emphasize that the quantity of fluid to be given to each case of cholera must be individually determined and that there is no fixed dose.** Once the dehydration present on admission has been overcome the patient will require further intravenous fluid in a quantity that will depend on the quantity and duration of his diarrhea. While he is in the hospital, his stool output can be measured, and intravenous fluid given to equal the output.

Acidosis should be corrected at the same time as dehydration. Acidosis and dehydration develop together and are usually of parallel severity, as both are consequences of the loss of an alkaline stool of the average composition noted above. We recommend the use of a single solution for the treatment of cholera which provides sodium chloride and sodium bicarbonate or sodium lactate in a ratio approximating that in which they are lost in the stool. If enough of this fluid is given to rehydrate the patient and maintain him in a satisfactory balance, the acidosis will be simultaneously relieved.

Although potassium, calcium, magnesium, and other substances are lost in the stool in cholera, the replacement of these components by vein has not been shown to be essential. Body pools of these substances are not dangerously depleted in a case of cholera which is properly managed, with the exception of potassium, which usually can be given satisfactorily by the oral route.

It is important that intravenous replacement solutions be clean, free of pyrogens, and given through scrupulously clean infusion sets. Unless these precautions are followed, rigors and febrile reactions will ensue. Such pyrogenic reactions cause intense discomfort to the patient, and give rise to apprehension on the part of relatives and staff. However, the occurence of such a reaction should not be allowed to interrupt necessary intravenous therapy. The offending bottle and infusion set should be removed at once, and a new bottle of non-pyrogenic solution substituted.

Intravenous rehydration, and restoration of electrolyte balances in the cholera patient will prevent death, and must be continued for as many days as the disease persists. In severe cases given this treatment alone, the purging may continue as long as 7 or 8 days, and the requirements for professional care and sterile intravenous fluids may be very great.

Therefore, it is advisable to attack the cause of the diarrhea, the cholera vibrio, with an antibacterial agent capable of putting a rapid end to the infection. Practical trials of different agents and doses have been too few in the past, and we are not yet in a position to state which is the drug of choice in cholera. However, **tetracycline** has been shown to be effective in two independent studies (Greenough **et al.** 1964, Carpenter **et al.** 1964). Other agents which are known to be capable of killing V. **cholerae** in the test tube are likely to prove useful, but have not yet received adequate clinical trial.

A wide variety of medicines, other than replacement fluids and antibacterial drugs, have been advocated and are being used in treating cholera. None of these have been scientifically proven to be of value, and some may be harmful. All are indirectly harmful by diverting medical attention and financial resources from the procedures that should be employed. Some of the most commonly used medications are :

(1) Epinephrine, coramine, and other cardiac stimulants. These are given by injection in the vain hope that they will restore circulation, but are more likely to do harm by increasing vasoconstriction and further limiting blood supply to such vital organs as the kidneys. Adequate circulation is impossible until the blood volume is restored to normal by infusions. When sufficient fluid replacement is given, the pulse and blood pressure rapidly return to normal, and there is no need for a stimulant or vasopressor agent.

(2) Atropine and other anticholinergic drugs. The notion that these will lessen intestinal motility and decrease the diarrhea has no scientific basis. The loss of fluid from the blood into the intestine would be just as dangerous to the patient even if expulsion of the fluid **per rectum** could be stopped.

(3) Oxygen. The rapid breathing of the cholera patient is due to acidosis, not anoxia. It should be treated with intravenous bicarbonate or lactate rather than oxygen. Cyanosis in cholera is the result of impaired circulation, rather than damage to the lungs. Cyanosis cannot be relieved by oxygen alone, but responds immediately to adequate fluid therapy. Oxygen is costly and is not indicated in the therapy of cholera.

(4) Desoxycorticosterone and other steroid hormones. The use of these agents, for their salt retaining properties, is unnecessary. Cholera patients retain salt well when treated with adequate intravenous fluid replacement. The addition of steroids is of no demonstrated benefit to the patient, and in view of their many toxic effects, potentially harmful.

(5) Sulfaguanidine. This drug is used in an attempt to combat the infection, but is probably inadequate. Examination of a few strains of **Vibrio cholera** recently isolated from patients in Dacca revealed that these were relatively resistant to sulfa drugs. Present evidence is not sufficient to settle the question, but until further research can be done on the sulfonamides, the more powerful antibiotics should be the antibacterial agents of choice.

(6) Bismuth, kaolin, and other related mixtures. These agents are supposed to lessen the diarrhea by local mechanical or absorptive effects. There is no evidence that they have any effect in the overwhelming diarrhea of cholera.

Treatment of cholera at the Pakistan-Seato cholera research laboratory :

The standard procedure for the treatment of acute cholera now in use at the hospital ward of the Cholera Research Laboratory is as follows :

(1) The newly arrived patient is seen by a physician who makes the diagnosis on the basis of history, physical examination, and the appearance of the stool when it is passed. Patients with other illnesses (such as gastrointestinal hemorrahge) may complain of vomiting, diarrhea, and collapse; such cases are transferred to an appropriate medical facility. If the doctor is satisfied that the illness is consistent with cholera, and that the patient will require intravenous fluid, he is admitted.

(2) The cholera victim is placed on a specially constructed cholera cot for the duration of his acute illness. This cot is made of canvas or jute, and has a hole under the buttocks. A rubber sheet on the cot with an attached sleeve that fits through the hole channels all liquid stool into a calibrated bucket where it can be measured. Accurate records of fluid intake and output are maintained at the bedside, and the medical and nursing staff make frequent checks of the balance between intake and output.

(3) Intravenous replacement solution is given by vein, rapidly at first in collapsed cases, and then more slowly to complete hydration and maintain it. The initial rate of fluid administration should be at least 50 ml. per minute in an adult in shock, continued until radial pulse is restored. The rate may then be reduced to perhaps 25 ml. per minute until the patient is adequately rehydrated as judged by a full, slow pulse with normal blood pessure, restoration of skin turgor, loss of the drawn and sunken-eyed facial appearance, return of full consciousness, and loss of restlessness. **Initial rehydration in a typical collapsed adult case will usually require about 3 liters of fluid.** If too much fluid is given, the patient will develop obvious edema, first of the face and subsequently in dependent regions.

Unless there is pre-existing heart disease, a modest degree of overhydration only causes peripheral edema and does no serious harm. The heart and lungs should be examined by auscultation at frequent intervals, especially if heart disease is suspected. When the patient has been restored to a normal state of hydration, the rate of infusion is reduced so that the amount given is determined by, and equal to the output of stool and/or vomitus. Many cases begin active purging again about 3 hours after initial rehydration, and fluid requirements during the first 12 to 24 hours in hospital may reach 20 liters in an adult.

In severely dehydrated and collapsed patients, particularly in infants and young children, the performance of a venipuncture in the arm may be difficult or impossible. In such cases, cut-downs could be done, but this procedure takes time that may be at a premium. We prefer

to place a needle temporarily in the femoral vein in adults or the external jugular in children. After enough fluid has been given to restore circulation to normal, insertion of a needle into an arm vein for maintenance therapy is usually not difficult.

Other routes for fluid administration cannot be recommended with confidence. Hypodermoclysis is not satisfactory ; the rate at which the fluid is absorbed is too low to maintain balance. Infusions into bone marrow or into the peritoneal space may be of value in certain cases where venipuncture cannot be effected, but these procedures require further evaluation.

The simplest effective replacement solution is one containing 100 mEq. (5.9 grams) of sodium chloride, and 50 mEq. (4.2g) of sodium bicarbonate or lactate (5.6g) per liter. Prepared solutions of this or similar composition are available commercially in some places; elsewhere the solution must be prepared by the user, either from the basic ingredients or from commercially available normal saline and isotonic sodium lactate. If the latter preparations are used, they need not be mixed before administration. Adequate mixing will occur in the patient's circulation if one unit of isotonic lactate is given for every two units of saline. A slightly more complex solution, which has been used extensively at the Pakistan -SEATO Cholera Reserach Laboratory, * is one prepared from 5 grams sodium chloride, 4 grams sodium bicarbonate, and 1 gram potassium chloride, dissolved in a liter of pyrogen-free distilled water. This solution offers 133 mEq. sodium, 13 mEq. potassium, 99 mEq. chloride, and 48 mEq. bicarbonate per liter.

(4) Antibiotic therapy is instituted as soon as is practical. Until further clinical trials have defined the optimal agent and dosage, we recommend the use of tetracycline. An oral schedule of 250 mg every 6 hours may be begun immediately on admission unless vomiting prohibits it, in which case the initiation of therapy may be delayed for 1 to 3 hours. Another schedule which may be even more effective includes "loading" doses of 500 mgm by mouth at admission and 6 hours later, followed by 250 mgm 6 hourly. Present evidence indicates that supplementation of oral therapy with intravenous tetracycline does not further hasten the patient's recovery or the eradication of vibrios from the stool.

(5) As soon as vomiting subsides, the patient is allowed to take water by mouth. If intravenous solutions containing no potassium are used, oral potassium supplements should be started at once. One should administer about 15 mEq. of potassium (1 gram of potassium chloride, or 250 ml. of coconut juice) for every liter of stool passed. Two to three grams of potassium chloride may be dissolved in each liter of drinking water without making it unduly bitter. Natural fruit juices, especially the juice of the green coconut, are rich in potassium and more palatable.

(6) Within the first 24 hours of admission, the majority of patients regain their appetites and may be given bland foods.

Food is not withheld from any patient, especially a child, when he complains of hunger. Bananas, bread, milk, and juices are easily tolerated, and provide extra potassium, calcium, and other nutrients. Early administration of such a bland diet does not appear to aggravate or prolong diarrhea.

(7) As soon as diarrhea ceases, the patient is transferred to an ordinary bed for convalescence. Dietary intake is increased as rapidly as the patient desires. In our experience, relapses have been very rare when antibiotic therapy was discontinued as early as the third day, but further experience will be required before this practice can be generally recommended. We prefer to keep the patient under observation and on antibiotics for a minimum of four days when circumstances permit.

Complications of Cholera

With the exception of pre-existing conditions or diseases, virtually no complications will occur when the typical acute case of cholera is treated from the outset by the procedure outlined above. A variety of annoying symptoms may occur during convalescence, but these are not serious and require no special treatment. They include weakness, burning discomfort in the abdomen and transient hearing loss in a few cases. However, if no treatment is available until late in the course of illness, or if inadequate treatment is given and the patient is referred to hospital later, a number of serious complications may ensue. Of these, the most serious is acute renal failure.

Acute renal failure may develop after shock from any cause, and cholera is no exception. Renal failure may be prevented by adequate rehydration given at the outset of collapse, but cannot be so easily reversed once it is established. The treatment is the same as that for acute renal shut-down in other illnesses and consists of the following :

(1) Limitation of fluid and electrolyte intake to equal losses from the body by stool, urine (if any) and vomitus, plus an allowance for insensible water loss. The insensible loss may range from less than one liter for a small adult in cool weather to several liters in hot climates where sweating occurs. Daily weights are of great value in the control of overall body-fluid balance.

(2) Administration of a diet low in nitrogen and potassium, but high in carbohydrate and fat to diminish body catabolism. Butter, sugar, and rice are good foods for this purpose, whereas meat, fish, fruits and vegetables should be avoided. If anorexia, nausea, or vomiting prevents oral intake of carbohydrate, at least 100 grams of glucose per day should be given in a very slow intravenous drip.

(3) Use of artifical substitutes for renal function in severe cases. Peritoneal dialysis has been succesfully employed at the Cholera Research Laboratory, and is a simple enough procedure to be used in any hospital.

Acidosis may persist after dehydration is corrected in cases of cholera treated with normal saline alone. Such patients feel ill, often appear mentally

obtunded, may be unable to eat, and have a tendency to develop edema. Excessively deep and rapid respiration is a universal sign, as the body tries to rid itself of excess acid in the form of carbon dioxide. In the worst cases, pulmonary complications may develop. The basis of this respiratory syndrome is not clear, but its manifestations are increasing dyspnea, development of moist rales in the lung bases, and, with further progression, the occurrence of fatal pulmonary edema. In our own limited experience, these cases fail to respond to the administration of digitalis or oxygen, but are benefited by the administration of sodium bicarbonate in massive doses. As much as 20 mEq. of bicarbonate per kg. body weight has been required to return plasmaCO_2 content to the normal range. Whether this large amount could be administered by mouth is doubtful; our experience has been with the intravenous administration of an isotonic solution of sodium bicarbonate (12 grams per liter). In patients so treated, the excess of fluid and sodium might have been expected to increase pulmonary congestion; none the less, its administration was followed by rapid improvement and relief of symptoms.

Hypokalemia may occur in cholera if potassium replacement is not given either by vein or by mouth. It is less common in cases treated with antibiotics, as diarrhea does not long continue in this group. Muscular weakness is the main symptom; hypokalemia may also induce ileus, abdominal distension, and drowsiness. The principal danger lies in the chance of the development of fatal cardiac arrhythmias; fortunately, this outcome is rare. Hypokalemia need not occur at all if the therapy outlined here is utilized. If it does occur, it should be treated immediately by the oral administration of potassium-containing fluids (see above), or by the incorporation of potassium salts into infusions.

Tetany occurs rarely in cases of cholera when acidosis is corrected very rapidly or if an excess of alkali is given. This complication is easily recognized clinically and may be treated by injections of calcium gluconate by vein.

Hypoglycemia severe enough to cause symptoms may occur in cholera patients if food is withheld. In our experience, the majority of such cases are children. This complication must be considered if a patient, particularly a child, who has not yet been able to take food seems unusually drowsy or exhibits irrational behaviour. Such a patient should receive intravenous glucose; if he improves rapidly, he may be presumed to have been hypoglycemic. Further attacks may be prevented by giving food by mouth, or adding glucose to the infusion fluid.

Pre-existing diseases or conditions may complicate the course and treatment. Of these, the most common and troublesome in our experience is pregnancy. Cholera in late pregnancy frequently precipitates a premature delivery, and fetal loss is common. During the past year, six cases of cholera occurring in late pregnancy have been admitted to the Cholera Research Laboratory. All the mothers responded satisfactorily to treatment, but only two continued their pregnancy. The remainder

went into labor and delivered stillborn fetuses between one and four days after onset of cholera. Until more can be learned of the causes of fetal loss in cholera, we cannot recommend any particular procedure for dealing with these cases. However, a pregnant female suspected of having cholera should be hospitalized and given vigorous therapy as early as possible.

A patient with edema who subsequently develops cholera may present an unusual problem. During the epidemic of January, 1964, two such cases entered the CRL ward. In one, a 40-year-old housewife, the edema was due to myxedema of ten years' duration, previously unrecognized and untreated. In the other, a 50-year-old man, it was attributed to congestive heart failure, presumably **cor pulmonale** on the basis of chronic bronchitis and emphysema. In each of these cases, the clinical estimation of fluid requirements was difficult, and subsequent laboratory tests showed that too little had been given. In each case, however, satisfactory recovery occurred.

Results of therapy of Cholera

Results of some of our earlier experiences with the treatment of cholera have been published elsewhere (Greenough **et al,** 1964): these data contributed to the formulation of the present regimen. A short-lived but severe cholera outbreak occurring in Dacca and Narayanganj in January, 1964, afforded an opportunity to test current ideas under conditions of stress.

During the two-week period from 18 January through 31 January, 1964, 175 cholera suspects were admitted to the ward. Rectal swab cultures yielded V **cholerae** in 151of these. On the peak day of the outbreak, 32 new patients were admitted, of whom 30 were bacteriologically proven to be cholera. Although the normal bed capacity of the ward is only 20, its census rose as high as 54. Convalescent patients had to remain on the floor until tents could be set up to accommodate them. Any patient who in the doctor's opinion would require intravenous fluid was admitted ; none were turned away for lack of space. During the outbreak, 39% of the patients treated were males, and 61% females. Small children weighing under 10 kg. formed 18% of the admissions, while 5% of the patients gave their age as 50 years or more. Although the patient population included many at the extremes of age, in whom the prognosis of cholera has been considered to be serious, no hospital deaths occurred. The Epidemiology Section of the Cholera Research Laboratory has initiated a follow-up study, and 93 of these patients have been visited after discharge from the hostal. It was learned that one five-year old boy died at home after discharge, but circumstances are not known in detail. This patient, whose cultures yielded V. **cholerae,** had no diarrhea while in the hospital. He was not anuric, and was discharged improved. The death was clearly not due to acute cholera but may have represented some undiagnosed complication. Aside from this individual, all patients remained well after discharge, with no elapses or other complications.

The average requirements for bed space, antibiotics, and intravenous fluid in this experience may be of value in planning for future epidemics. The average case was discharged in 2½ days. The standard dose of tetracycline was 250 mgm every six hours, with initial intravenous doses given to some patients. All drugs were discontinued when the patient left the hospital. For the average adult case, therefore, the total amount of antibiotic used varied from 2.5 to 3.0 grams, depending on whether an intravenous dose or an initial oral loading dose was prescribed. The average body weight for the whole group was 24.8 kg., the average intravenous fluid requirement, 5.2 liters.

Although many laboratory determinations were performed on these patients, the results were utilized for research purposes only. None of the findings were available to the attending physicians early enough to influence their decisions concerning patient care. Satisfactory therapy can thus be given without any laboratory assistance.

Summary :

The treatment of cholera is simple, and is based on two premises :

1. Water and electrolytes lost in the stool must be replaced by intravenous infusions containing sodium choride and sodium bicarbonate (or sodium lactate).

2. The duration of the infection must be shortened with antibiotic therapy. Tetracycline is of proven value. If these measures are carried out as recommended, the mortality for cholera can be reduced to the vanishing point, even under trying epidemic conditions. The principal remaining problem in cholera therapy is a logistic one : that of making the necessary materials and skilled personnel available at the time and place of an outbreak of the disease.

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C H O L E R A

METHOD OF PAKISTAN-SEATO CHOLERA RESEARCH LABORATORY

BY

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CURRENT THERAPY

The copious "rice-water" diarrhea which characterizes cholera may result in the loss of as much as 1 leter of body fluid per hour. This causes a typical clinical syndrome marked by dehydration, acidosis, and shock. Vascular collapse can result within 2 hours of the abrupt onset of disease. Death may occur within 4 hours, but more often after 24 hours of illness. Vibrio Cholerae can often be isolated in nearly pure culture from the stools of early cases. Sometimes, cases clinically indistinguishable from cholera occur from which no pathegen can be isolated. On the other hand, V. cholerae infection may also be associated with mild, self-limited diarrhea, or an asymptomatic carrier state. Thus, although isolation of the causative organism may be useful for investigation of the disease, or control of an outbreak, it is not useful as a guide to treatment of the individual case.

The signs and symptoms of cholera are readily explained by profound losses of water and electrolytes. Recent careful studies of the composition of cholera stool are those of Watten, et al. (J. Clin. Invest., 1959). Cholera stool is isotonic and contains on the average 137 mEq/l Na, 16 mEq/l K, 107 mEq/l Cl and 45 mEq/l HCO3. Other ionic components are present in approximately the

same concentration as in plasma. Insignificant amounts of plasma proteins and glucose are found.

The treatment of cholera is fundamentally simple. The basic objectives are to replace losses of water and electrolytes by infusions, and to destroy the causative organism. If fluid and electrolyte losses are promptly and adequately replaced there should be no deaths or serious complications. Satisfactory replacement can be effected with isotonic solutions containing approximately 100 mEq/l sodium chloride and 50 mEq/l of either sodium bicarbonate or sodium lactate. Although losses of potassium are large, intravenous replacement has not yet been shown to be essential to survival. Moreover, potassium replacement may be easily accomplished by the oral route.

Bedside observations suffice to determine the quantity of replacement fluid to be given. The most reliable and convenient indices are the quality of the radial pulse, blood pressure (particularly pulse pressure), facial appearance, auscultation of lungs, urine output, and if possible, body weight. It is important to recognise that in uncomplicated cases there is considerable latitude on either side of the state of "ideal hydration" consistent with a favorable result. 177 consecutive cases of cholera were successfully treated

in Dacca at the Pakistan-SEATO Cholera Research Laboratory and 62 cases in Calcutta by Carpenter and associates without using laboratory data to judge intravenous fluid needs. Thus, although laboratory measurements such as plasma protein, plasma specific gravity and electrolyte concentrations, etc., are useful for studying the disease, they are not essential to guide the therapy of an uncomplicated case of cholera. This is particularly important since cholera so often occurs in situations and on such a scale that it is not feasible to have adequate laboratory support.

Studies both at the Pakistan-SEATO Cholera Research Laboratory (Lancet 1:355, 15 February, 1964) and by Carpenter and associates (Bulletin of Calcutta School of Tropical Medicine 12, 30, January 1964) have shown that early administration of tetracycline to cholera cases will not only kill V. cholerae but also significantly shorten the duration of diarrhea. Some cases treated by fluid replacement alone may continue to pass large volumes of liquid stool for 7 or 8 days, and require as much as 70 liters of intravenous fluid replacement. An agent capable of shortening the duration of disease greatly simplifies management.

Although cholera is an infectious disease it is not highly contagious.

Medical personnel attending cholera patients are protected by observing simple rules of hygiene such as washing hands after being in contact with infected material. Elaborate isolation techniques interfere with patient management and are not necessary. Vaccination of ward personnel is recommended although its efficacy remains unproven despite many years of use.

Treatment Method:

1. Place the patient on a cot which has a hole located under the patient's buttocks. This can be made of canvas or burlap on a wooden frame; a rubber sheet with a sleeve through the hole channels the stool into a bucket. This permits collection of total output and avoids contamination of the area. The bucket should be capable of containing 7 liters. A dip stick or the bucket itself should be calibrated to measure the volume contained.

2. A single isotonic solution containing 50 mEq/l of sodium bicarbonate or sodium lactate and 100 mEq/l of sodium chloride will be effective replacement for all uncomplicated cases. If such a solution is not available isotonic saline can be alternated with isotonic sodium lactate or bicarbonate in a ratio of 2:1. Physiologic concentrations of other solutes may be beneficial in infusion solutions but have not been shown to be necessary for survival.

3. Establish an intravenous route through which fluid can be given as fast as 1 liter in 15 minutes. In adults, if a peripheral vein cannot be located either the external jugular or femoral vein may be used. In infants and children a 21 gauge thin-walled scalp vein needle is helpful for placement in an external jugular vein. If all else fails the subclavian vein can be used.

4. Infuse fluid as rapidly as possible until a good full radial pulse has returned and pulse pressure is at least 20-30 mm Hg. Subsequently, fluid is given at the same rate as the stool output. Further regulation of intravenous fluid should be based on observation of the patient's radial pulse, blood pressure, lung bases, facial appearance, urine output and, if possible, body weight, as well as the stool volume.

5. Oral fluids should be given to cover insensible water losses and provide supplements of potassium, carbohydrate, etc. Green coconut water, frequently available in cholera prone areas, contains on the average 70 mEq/l of potassium, and 2.5 gms % reducing substances. Water, other fruit juices, bananas, milk and other soft foods or liquids may be given as soon as the patient desires, which is often shortly after rehydration.

6. Tetracycline, 100 mgm I.V., should be given immediately and repeated 6 to 12 hours later. As soon as the patient can swallow, a dose of 500 mgm every 6 hours should be administered for at least 48 hours. It is very likely on the basis of more recent studies that the intravenous component is unnecessary and may be omitted without loss of effect. Other vibriocidal antibiotics have not yet been properly tested; they may also work.

7. Vasopressor agents, cardiac stimulants, anti-cholinergic drugs and steroids are not indicated and may be harmful.

Complications:

1. Acidosis, although a feature of all cases, may be extreme, particularly in instances of prolonged vascular collapse or when previous treatment with saline alone has been given. Profound hyperventilation occurs and rales suggestive of pulmonary edema may develop. In such cases infusion of isotonic sodium bicarbonate solution is effective in treating the acidosis. When the acidosis is corrected, hyperventilation and pulmonary rales diminish despite the added fluid volume and sodium.

2. Oliguria lasting 11-36 hours is a feature of some cases of cholera. If shock has been prolonged and therapy inadequate, renal failure ensues which

may last for several weeks. The treatment of such cases is the same as for acute renal failure due to other causes.

Laboratory measurements, such as CO_2, electrolytes and BUN, NPN, or creatinine are desirable in treating the complications mentioned in (1) and (2) and greatly improve the accuracy of the therapy rendered.

3. Hypokalemia may be severe if the diarrhea is prolonged and potassium replacement neglected. In such cases ECG changes occur and renal tubular damage has been demonstrated by renal biopsy.

4. Hypoglycemia, sometimes resulting in coma, has been seen, particularly in the pediatric age group. Oral administration of carbohydrate in the form of soft foods or juices is recommended and will avert this complication.

5. Hypocalcemia and tetany have been seen rarely and require intravenous calcium.

6. Burning abdominal pain, transient hearing loss, and dizziness may occur in convalescence but resolve spontaneously.

Summary:

In cases of uncomplicated acute cholera, prompt and adequate replacement of fluid and electrolyte losses will ensure survival. Bedside observation

suffices to control fluid replacement. It has been shown that both sodium chloride and either sodium lactate or bicarbonate are essential components of intravenous replacement solutions. Potassium losses are considerable but can be successfully replaced by mouth. Tetracycline administered promptly in an adequate dosage will eradicate V. cholerae and shorten the duration of illness.

RAPID IDENTIFICATION OF VIBRIO CHOLERAE BY DARKFIELD MICROSCOPY

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Synopsis

A rapid, simple, reproduceable method for recognizing the presence of Vibrio cholerae in diarrhea cases is described. Employing darkfield microscopy and specific antisera, the method is capable of accurate recognition and serotyping of vibrios. In 80% of cases, a definitive diagnosis is available within five minutes. Elaborate equipment and long term training of technicians are not required for reliable results. The method is easily performed by one person in the field.

RAPID IDENTIFICATION OF VIBRIO CHOLERAE BY DARKFIELD MICROSCOPY

The genus Vibrio is named for the unique rapid to-and-fro motility which is characteristic of this group of organisms. In 1884, Robert Koch compared this vibrating, swarming movement to the exceedingly rapid progress of a host of gnats. In 1919, Sanoerelli (quoted by Pollitzer, 1960) reported that vibrio movement was three to ten times more rapid than that of other common motile bacteria. However, diagnostic methods based on this characteristic have not been advocated since 1896 (Dunbar, quoted by Pollitzer); in fact, the value of direct microscopic examination of material from cholera patients is deprecated in the literature generally available (Pollitzer).

Although proper treatment of the cholera patient does not depend on early recognition of the causative organism, early diagnosis is important for checking the spread of cholera and studying the disease. Ordinary bacteriological culture techniques usually require 18 to 24 hours before the specific diagnosis can be established. For the recognition of carriers, the added enrichment procedure prolongs the period to 36 or 48 hours. The fluorescent antibody technique (Finkelstein, 1959) makes a definitive diagnosis possible within one hour of taking a sample from the suspected case, and the carrier can be recognized within 7 to 8 hours. This technique, however, requires costly equipment and a highly skilled observer. The present study was undertaken to determine whether early, reliable diagnosis could be achieved by simpler techniques based on the vibrio's characteristic motility.

Materials and Methods:

Equipment: The following equipment was used for these studies: A Baker research microscope equipped with a Trilux condenser, which provides both phase contrast and darkfield illumination; a Leitz Ortholux microscope with phase contrast condenser; and a Cooke-McArthur microscope equipped with darkfield condenser. 10X and 40X objectives were used with 8X or 15X oculars. Microscope slides and cover slips were chemically clean. The group and type-specific cholera antisera contained no bacterial preservative, as determined by lack of effect on heterologous vibrio strains. The titers of the sera used were such that a 64-fold dilution was the end point in tube agglutination with living vibrios.

Media:

Bile peptone broth with a pH of 9.2, and tellurite-taurocholate gelatin agar (TTGA) plates were prepared by the method of Monsur (1963). The non-suppressant broth was 1% trypticase (Difco) and 1% sodium chloride in distilled water; this had a pH of 7.2.

Examination Technique: A small drop of the test material was placed on a clean slide by a bacteriological loop, a capillary pipette, or other means; a clean cover slip was applied, and the slide examined under darkfield or phase illumination at 400 to 600X magnification. If organisms with the typical motility of vibrios were observed, two other preparations were set up: one was mixed with an equal volume of Inaba antiserum, and the other with anti-Ogawa serum; cover slips were applied. If motility ceased in one of these preparations within

three to five minutes and no change had occurred in the other, specific identification was completed. If the contrast between the two preparations was not definite, an additional mount was prepared with vibrio O group I antiserum. If this mixture had no effect on the vibrio motility, the organisms were presumed to be non-cholera vibrios.

With enrichment technique, the rectal swab tube containing bile peptone broth was held at 37°C., and the fluid was examined after various intervals with final examination after at least 18 hours of incubation.

The sensitivity of the darkfield method was measured by performing simultaneous darkfield readings and bacterial counts on 10-fold dilutions in enrichment broth of cholera stool or broth culture of vibrios. At least two observers made darkfield readings on coded samples; the readings were repeated after overnight incubation. Counts were performed by dropping a measured volume on the surface of TTGA plates and enumerating the colonies after overnight incubation.

Clinical material: Rectal swabs or fecal samples were obtained from 138 patients admitted to the ward of this laboratory. By the laboratory's normal bacteriologic routine, 107 of these patients were shown to be infected by V.cholerae. This routine consists of daily culturing directly on gelatin agar and TTGA plates, with subculturing to these media after overnight enrichment in tellurite bile broth (Monsur, 1963). The other 31 cases were bacteriologically negative for V. cholerae on all examinations.

The rectal swabs (plain cotton wool) were placed in test tubes containing 0.3 to 0.5 ml of bile peptone broth and held at 37° C. Every time material was removed for examination, a drop was streaked on a TTGA plate, which was examined the following day independently by the diagnostic bacteriologists.

The swabs of 53 patients were taken and examined within five minutes of the patient's admission; swabs from 33 were examined within five minutes and again after 4 to 30 hours incubation at 37°C. Swabs from 52 other cases were first examined microscopically after incubation for ore than three hours; these were re-examined at 18 hours or more if a specific diagnosis had not been established.

Rectal swabs were taken daily from 111 patients (89 of the V. cholerae positive and 22 of the negative cases) to observe the persistence of vibrios. If a specific vibrio type had already been established, typing was not always repeated. The majority of these patients had been treated with tetracycline (Greenough, et al 1963).

Results:

With either darkfield or phase illumination, the motility of vibrios is easily visualized and clearly different from that of other organisms we have found in fecal samples. At 400-600X magnification, the motility of the vibrio is so rapid that it cannot be held in the microscopic field. Some vibrios are observed to move in a gyrating, centrifuge-like manner. In the dark field, material positive for vibrios evokes a mental image of many shooting stars in a dark sky. At a magnification of 100-150X, Koch's analogy of swarming gnats is apparent, with busy milling in a scintillating field. The characteristic

motility is perhaps clearer in the darkfield than the phase contrast microscopy. Because darkfield equipment is more generally available and less costly than that for phase contrast, our further studies were done with darkfield only.

In 16 separate dilution studies, the highest dilution in which vibrios were recognized had an average bacterial count of 4.3×10^5 vibrios per ml., with a range in individual observations from 2.3×10^4 to 1.7×10^6. After at least eight hours incubation at 37°C., re-examination of these dilutions suggested that the sensitivity of the method with the enrichment step allows detection of an individual vibrio in the starting material. Similar results were obtained with the suppressant bile peptone broth at pH 9, and with the non-suppressant broth at pH 7.2.

Specific or group antisera immobilized cholera vibrios within three to five minutes; in many instances, motility had ceased before the mixture could be examined. The differences in the two preparations were unequivocal if the vibrio density was sufficient; in case of doubt, longer enrichment usually clarified the situation. Occasionally, though the difference between the two preparations was definite, some vibrio motility persisted. In several of these cases, both cholera and non-cholera vibrios were later found on the culture plates.

The swabs of 62 of the 107 V. cholerae positive patients were examined within five minutes; 79% were correctly detected by the direct technique. When the material was examined after enrichment, the darkfield findings agreed almost perfectly with the cultural results. Of 71 V. cholerae cases, 94% were recognized by darkfield examination, as compared to 96% recognized in the same material by a highly selective cultural method (Table I).

Subsequent daily swabs were taken from 89 of the positive cases; again, agreement with the cultures was excellent. More specimens were detected as positive after enrichment by darkfield than by culture (32% compared to 29%); the validity of these positive results is attested by two instances in which re-examination of the plates disclosed rare vibrio colonies obscured by overgrowing organisms. (These instances are tabulated as positive by both techniques). One case reported as V. cholerae Ogawa by darkfield, but NC by culture, developed a significant rise in antibody titer against Ogawa. (Footnote (a) Table I).

All cases negative for V. cholerae were negative on all examinations(Table I). It is pertinent that non-cholera vibrios were recognized as such in three admission specimens of V. cholerae negative cases, and in the subsequent specimens on two other cases. These were not confused with V. cholerae.

Correlation was excellent between the darkfield technique and matching cultures. However, discrepancies between darkfield results and those of routine bacterial methods were noted in significant number when the rectal swabs were tested after patients had one or two days of tetracycline therapy. Ninety-five plain swabs incubated in 0.3-0.5 ml of bile peptone broth were negative by darkfield and matching culture; V. cholerae was demonstrated in 26 of the tellurite impregnated swabs taken at the same time and incubated in a relatively large volume (1.5 ml) of bile peptone broth. It is likely that dilution of antibiotic by the excess medium permitted isolation of V. cholerae in these tetracycline treated cases.

Discussion:

A simple darkfield microscopic technique can makeavailable to the clinician, investigator, or quarantine officer a specific diagnosis of cholera within minutes in approximately 80% of vibrio-caused diarrheal cases. When enrichment (in either selective or non-selective media) is added, nearly all cases recognized by culture are detected. Definitive information is available within 8 to 18 hours; in contrast, 48 hours may be required for a final negative report with cultural techniques.

The characteristic motility of the vibrio has long been appreciated; in 1960, Majid Khan (1962) used the hanging drop to diagnose cholera, but reported that this technique had real value only after six hours incubation in alkaline peptone water. The use of darkfield illumination facilitates the visualization of typical motility; the addition of the specific antisera minimizes the risk of false positive results; indeed, in sone instances the darkfield technique may detect vibrios when recognition by culture is prevented by overgrowth of other organisms.

The procedure can be simplified by testing with vibrio O group I antiserum to establish that the observed vibrios are V. cholerae; if desired, the vibrios can then be tested with the specific antisera. This sequence spares the more valuable antisera. For examination of antibiotic treated cases, if the direct examination is negative, enrichment should be done with a relatively large volume of enrichment broth. The availability of compact portable microscopes (McArthur, 1945) makes the technique practical even under the most primitive conditions. The simplicity of the prcedure places it within the grasp of any technician trained in basic microscopy, enabling him to detect vibrios literally within seconds. These attributes make the technique valuable in screening specimens for the presence of V. cholerae, minimizing delay in initiating epidemiologic or special clinical observations.

This procedure has advantages over fluorescent microscopy in that less costly equipment is required, the processing of the specimen is minimal, and less technical skill is necessary for achieving accurate results. Neither direct microscopic method provides material for confirmation or further studies; however, the rectal swabs in enrichment fluid need only be transported to a central laboratory for strain isolation, if desired (Monsur, 1963).

This diagnostic technique, based on equipment and media available in all general laboratories, makes diagnosis possible in minimal time, with an accuracy equal to that of the most selective vibrio culture system in cases of cholera suspect diarrhea.

Summary:

Darkfield examination of liquid stool or a rectal swab immersed in broth permits immediate recognition of the characteristic motility of vibrios. The addition of specific vibrio antisera results in total immobilization of the specific V. cholerae strains, providing specific diagnosis within five minutes in 79% of diarrhea cases culturally positive for V. cholerae.

In 71 V. Cholerae positive cases examined after enrichment, 94% were recognized by this method on admission, as compared to 96% recognized in the same material by culturing in highly selective media. Correlation of results on subsequent specimens from 57 positive cases remained high; 32% positives were recognized by darkfield against 29% by culture. Among 31 negative cases, no false positives were reported, although material from five of these patients contained non-cholera vibrios. The sensitivity of the method is such that approximately 10^5 vibrios per ml are necessary for recognition microscopically; with enrichment, the presence of very few vibrios is detected. In antibiotic treated cases, the enrichment broth should be of sufficient volume to permit dilution of any antibiotic substance.

RESULTS OF DARKFIELD AND CULTURE STUDY ON SPECIMENS FROM HOSPITALIZED DIARRHOEA PATIENTS

Material tested	Darkfield ('DF') and culture (Cult.) techniques							
	Observation and plating directly				Observation and plating after enrichment			
	DF + Cult.+	DF + Cult.-	DF - Cult.+	DF - Cult.-	DF + Cult.+	DF + Cult.-	DF - Cult.+	DF - Cult.-
V. cholerae-positive diarrhoea patients								
Specimens at admission from 107 patients.	49	0	13	0	66	1 [a]	2 [b]	2 [c]
Specimens after admission from 89 patients	30	3	12	70	39	7	3	95 [d]
V. cholerae-negative diarrhoea patients								
Specimens at admission from 32 patients	0	0	0	24 [e]	0	0	0	14 [e]
Specimens after admission from 22 patients.	0	0	0	24 [f]	0	0	0	27 [f]

a Reported as *V. cholerae* Ogawa by darkfield examination; reported as non cholera vibrio by culture. Agglutination showed rise in titre from < 1:40 with both antigens to 1:320 and 1:40 against Ogawa and Inaba antigens, respectively.

b In one case, vibrios were identified but were reported as non*cholera* vibrios.

c Both patients were positive in other specimens; one positive only on the second day.

d 26 tetracycline-treated patients were positive after routine enrichment technique in 15 ml broth.

e Two diagnosed as non cholera vibrios

f One diagnosed as " " "

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EFFECT OF MUKERJEE'S GROUP IV PHAGE ON EL TOR VIBRIOS *

by

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EFFECT OF MUKERJEE'S GROUP IV CHOLERA PHAGE ON EL TOR VIBRIOS

Introduction: Vibrios agglutinated by cholera "O" sera were originally classified as El Tor or as true cholera strains on the basis of their production of soluble haemolysin in the Greig test (Greig, 1914). Recently, other methods for differentiating these vibrios have been advocated (Gispen, 1938; Tanamal, 1948; Mukerjee, 1961; Han and Khie,1963). Mukerjee and his associates reported that the phage strain which they designated group IV phage would lyse all true cholera vibrios, but even when used undiluted, would not lyse any of the El Tor strains (Mukerjee, 1961; Mukerjee and Guha Roy, 1961). Recent observations made in our laboratory, presented in this communication, call for partial modification of this generalization.

MATERIAL AND METHODS

The phage and indicator strain. Group IV cholera phage was obtained from Dr. S. Mukerjee and stored in glass sealed ampules as seed virus. Vibrio cholerae Ogawa strain 154 (also provided by Dr. Mukerjee) was used for propagation and detection of the phage.

Test organisms. Thirty strains of El Tor cholera vibrios were tested. All were considered to be El Tor because they were haemolytic in tube or plate tests (Taylor, 1941; Feeley and Pittman, 1963), agglutinated chicken cells (Finkelstein and Mukerjee, 1963), and were resistant to low-titre group IV phage. Five strains isolated in Malacca in 1963 were furnished by Prof. M. B. Maitland. Five strains from Burma isolated in 1963, one from Indonesia, and one from Hong Kong were provided by Dr. S. Mukerjee. Ten isolated in Thailand in 1963 were provided by Dr. H. E. Noyes. Eight strains isolated in East Pakistan in November, 1963, were furnished by Dr. C. H. Yen. Of these thirty strains, six from Thailand were of Inaba serotype; the rest were Ogawa.

Culture media. As the fluid medium, broth containing trypticase (B.B.L.) 1.0% and sodium choloride 1.0% in distilled water was used. Bacto Agar (Difco) was added to give a concentration of 1.5% for use in normal agar plates, and of 0.7% for the preparation of soft agar. All media were autoclaved at 20 lbs. pressure for 20 mins; the pH was 7.1 and did not require any adjustment.

Phage propagation and titration. Phage with titre of approximately 10^{11} plaque forming units (p.f.u.) per ml. was obtained as follows:

Vibrio cholerae, strain 154, was grown for 5 hours under constant aeration at 37° C in Ehrlenmeyer Flasks (125 ml.) containing 50 ml. of tryticase broth. The total bacterial population in the flask at the end of this incubation period was about 10^{11}. Approx. 2×10^{11} p.f.u. of phage was then added to the flask to ensure infection of all bacteria and aeration was continued for an additional 3 hours incubation at 37°C.

Chloroform was added; after one hour, the fluid was centrifuged at low speed to sediment the bacterial bodies. The supernatant was used as the neat phage suspension. Phage plaques were counted by the agar-layer method (Adams, 1959).

Action of group IV phage on El Tor vibrios. The effect of group IV phage on El Tor vibrios was studied both on agar plates and in broth. Lawns of El Tor strains were seeded by streaking or by flooding the surface of agar plates with young broth culture and allowing the plates to dry. Serial 10-fold dilutions of the bacteriophage were spotted on the inoculated surface of the agar by drop or by loop. The phage dilutions were also tested on a lawn of V.cholerae strain 154. Chloroform-treated broth cultures of strain 154 were also spotted on lawns of El Tor to assure that the effect was not that of an adventitious agent in the phage propagating medium. The working phage was tested on lawns of salmonella, shigella, and Escherichia coli to rule out a non-specific anti-bacterial action. All plates were incubated overnight at 37°C and examined the following morning.

Broth dilutions of four El Tor cultures were separately mixed with phage to give various phage-to-bacteria ratios; after 15 mins at 37°C, appropriate dilutions were plated out to determine the viable bacterial count. Simultaneous titrations of the original bacterial cultures and the phage were done. All titrations were done in duplicate; plotted or reported data are arithmetic averages.

Mixtures of the same four El Tor vibrios and group IV phage were titrated at intervals for p.f.u. on strain 154 to determine the quantitative effect on the phage. These mixtures were also tested for phage activity on the homologous El Tor strain.

The effect of phage on El Tor vibrios was visually observed by mixing a young culture of the bacteria with high-titre phage and examining the wet preparation under dark-field and phase-contrast microscopy at 400 x magnification. To determine whether the phage caused lysis of the El Tor Vibrios 0'5 ml. of phage containing approx. 7×10^9 p.f.u. of phage was mixed with 4'5 ml. of bacterial suspension containing $1'4 \times 10^9$ viable bacteria to give a 5:1 phage-to-bacteria ratio. The optical density of this mixture was determined immediately and then at the end of 1 and 2 hours in a Coleman Jr. Spectrophotometer at 650 m (Herriot & Barlow, 1957). Full range of controls were included. Decrease in the optical density of such phage-bacteria mixtures at the end of 1 and 2 hours, is considered to be due to lysis of the bacteria (Herriot & Barlow, 1957).

RESULTS

The lawns of all 30 El Tor strains were clear where concentrated group IV phage had been applied. As the phage concentration dropped, clearing became less complete, but no discrete plaques were seen (Fig. 1). When phage dilutions were spotted on lawns prepared with serial 10-fold

dilutions of two different El Tor cultures, the clearing effect was seen to be dependant on the number of bacterial originally seeded on the lawn. Clearing was generally not perceptible, even with dilute broth culture, with a phage titer below 10^8 p.f.u. per ml. The chloroform-treated culture of strain 154 which had not been inoculated with phage did not produce any clearing. Concentrated phage had no effect on the lawns of salmonella, shigella, or E. coli.

Within 15 minutes after the El Tor vibrios were mixed with phage, a loss in viable bacterial count had occurred; the viable count fell as the phage-to-bacteria ratio increased (Fig. 2). Results with these four strains suggest differences in the interaction of individual El Tor strains with group IV phage.

Phage count progressively fell when vibrios were mixed with phage; after one hour, less than 10% of the original phage could be recovered. Over the same time interval with strain 154, the phage titre increased approximately thirty fold (Table 1). These incubated phage-El Tor mixtures had no effect on the homologous El Tor strain.

With dark ground or phase contrast microscopy, mixture of bacteria and phage exhibited a marked reduction in vibrio motility which was obvious in less than 10 minutes. No motility was evident at the highest phage concentration. The proportion of motile bacteria in the mixture increased as the concentration of phage was reduced.

Active phage resulted in reduction of optical density which was not less marked than that seen with strain 154 (Table II). No decrease in optical density occurred when the vibrios had been killed by heating.

DISCUSSION

These studies indicate that Mukerjee's group IV phage has a marked effect on El Tor vibrios, though the phenomenon may be different from that occurring with true cholera vibrios. At concentrations of 10^8 p.f.u. per ml. or higher, depending on the number of bacteria on the plate, this phage produces clearing on lawns of El Tor strains. In fluid media, the effect of phage on bacteria is soon evident; within 10 minutes, vibrio movement ceases; in 15 minutes, less than 10% of bacteria survive exposure to 100 p.f.u. per vibrio. With a ratio as low as 5 p.f.u. per vibrio, significant numbers of bacteria are lysed by the phage, as indicated by reduction in optical density (Herriott & Barlow, 1957).

Although group IV phage attacks El Tor vibrios, no indication of virus multiplication during this process has been found with either strain 154 or the homologous El Tor as indicator organism; in fact, a progressive fall in p.f.u. was noted. The failure to form plaques at terminal dilutions also argues against phage multiplication.

These results do not deprecate the value of phage IV as a toxonomic tool because the behavior of the phage towards true cholera and El Tor

vibrios is demonstrably different. Because the reported insusceptibility of El Tor strains to group IV phage is only relative, the concentration of phage used in the test can be critical. Such resistance or sensitivity should therefore be expressed in terms of plaque forming units of bacteriophage.

SYNOPSIS

El Tor vibrios are characterised by their insusceptibility to Mukerjee's group IV phage in routine test dilutions. This study shows that group IV in sufficient titre will produce clearing on lawns of El Tor vibrios resembling phage lysis. However, when the phage is mixed with El Tor cultures, a fall occurs in the viable count of both bacteria and phage. Dark-field microscopy shows that the bacteria lose their motility; measurement of the mixture's optical density indicates that bacterial lysis does occur.

Evidence to date provides no indication that group IV phage undergoes multiplication in El Tor vibrios. Because of the marked difference in its behavior towards them, phage IV is valuable in differentiating true cholera from El Tor vibrios.

SUMMARY

The studies have shown that Mukerjee's group IV phage, when present in sufficiently high concentration, will clear the lawns of the El Tor vibrios tested, all of which were isolated during several recent outbreaks of cholera. This clearing resembles phage lysis; however, on dilution, discrete plaques are not formed. A phage titer of 10^8 p.f.u. per ml or higher is necessary to observe this effect.

Within fifteen minutes after El Tor vibrios are mixed with Group IV phage, a loss in bacterial count occurs which is greater as the phage-to-bacteria ratio is increased; the phage count falls progressively so that after one hour less than 10% of the original phage can be recovered. Direct microscopy reveals that the phage immobilizes the vibrios; optical density studies indicate that the El Tor vibrios are actually lysed.

These observations do not deprecate the value of phage IV as a toxonomic tool; but indicate that the resistance or sensitivity of all types of vibrios to bacteriophage should be expressed in quantitative terms.

TABLE I

PHAGE COUNTS AFTER MIXING GROUP IV PHAGE WITH EL TOR VIBRIOS

Bacterial Strain	Phage p.f.u. per vibrio in original mixture	Phage Plaque Forming Units Recovered on Strain 154				
		Time in minutes				
		0	10	30	60	120
El Tor Vibrios						
CMS-1	0.2	1.5×10^7	6.5×10^6 (43)*	2.4×10^6 (16)	1.1×10^6 (7)	1.0×10^6 (7)
SE-52	0.25	"	9.4×10^6 (63)	1.1×10^6 (7)	1.0×10^6 (7)	7.9×10^5 (5)
HK-2	0.2	"	5.2×10^6 (35)	2.7×10^6 (18)	9.5×10^5 (6)	4.7×10^5 (3)
Ind-9	0.3	"	9.1×10^6 (61)	1.6×10^6 (11)	1.2×10^6 (8)	4.0×10^5 (3)
True Cholera						
Ogawa 154 (indicator strain)	0.4	"	1.5×10^7 (100)	1.9×10^7 (130)	4.7×10^8 (3100)	6.9×10^8 (4600)

* Percent of original plaque forming units.

TABLE II

LYSIS OF EL TOR VIBRIOS BY PHAGE IV

Bacterial Strain	Optical Density (in % of initial)							
	Live bacteria						Heat-killed bacteria*	
	Active phage		Heated phage*		No phage		Active phage	
	1 hr.	2 hr.	1 hr.	2 hr.	1 hr.	2hr.	1 hr.	2 hr.
El Tor Vibrios								
CMS-1	43	32	112	118	112	117	101	100
SE-52	63	46	105	110	108	114	102	103
HK-2	46	30	102	108	102	109	98	101
Ind-9	75	44	109	114	113	124	101	102
True Cholera								
Ogawa 154	67	51	104	108	106	112	102	100

* Bacteria were killed by heating at 56° for 1 hr; the phage was killed by immersion for 10 min. in a boiling water bath.

FIG. 1 Effect of group IV phage on El Tor vibrios (Ind-9) and V. Cholerae strain 154 (indicator organism for group IV phage) after application of log dilutions of group IV phage.

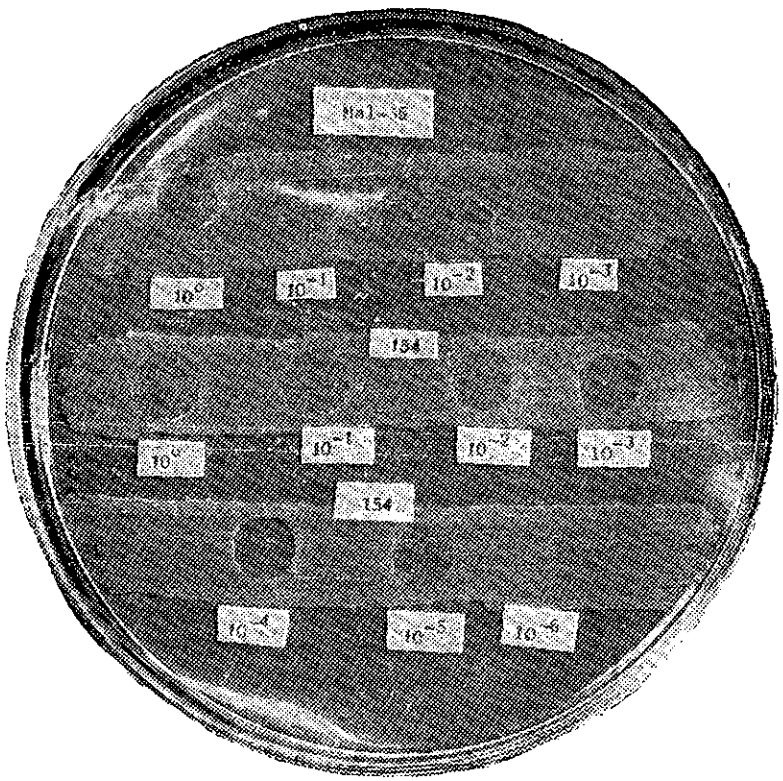
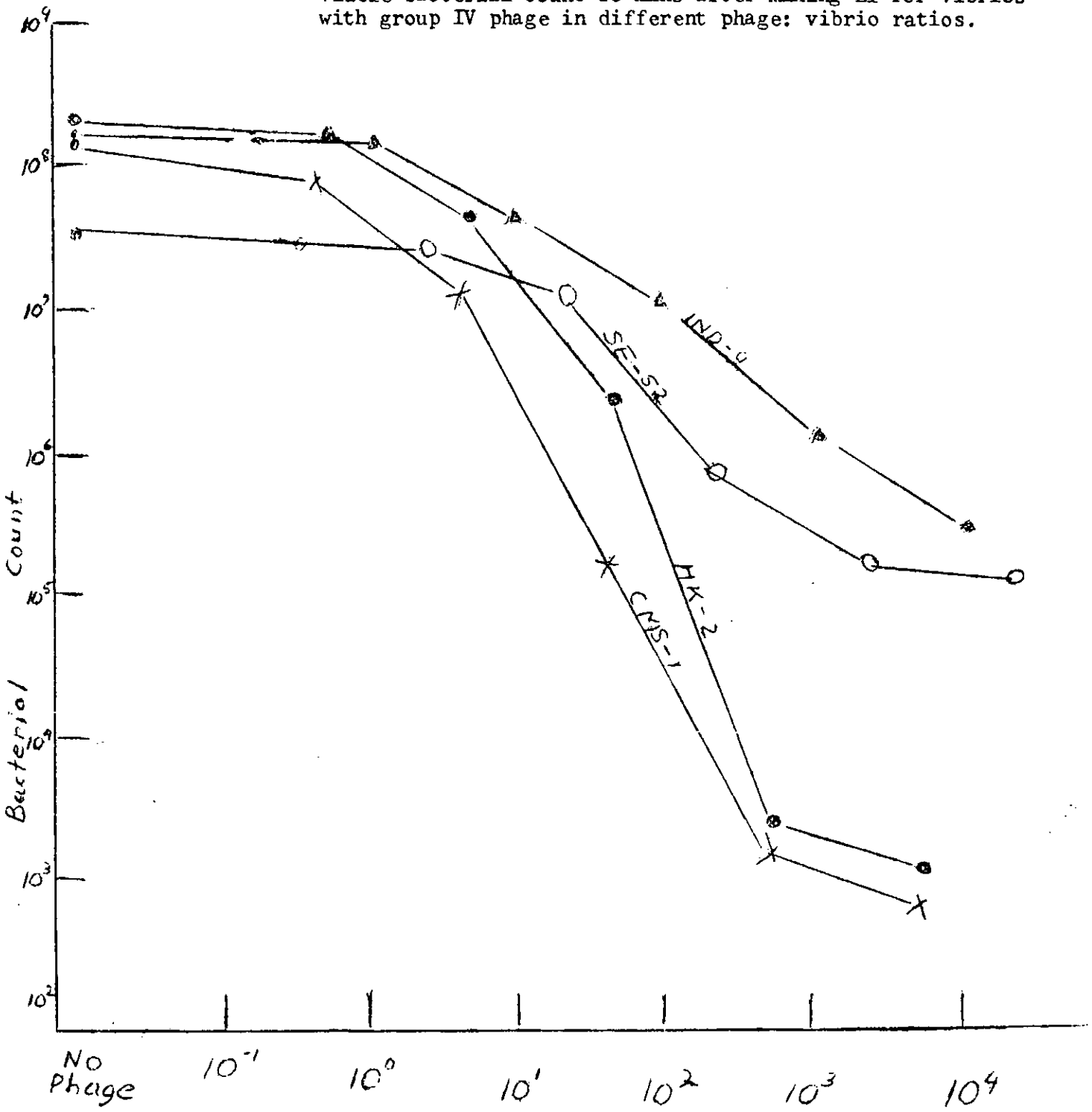

10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
154
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
154
10⁻⁴
10⁻⁵
10⁻⁶

FIGURE 2

Viable bacterial count 15 mins after mixing El Tor vibrios with group IV phage in different phage: vibrio ratios.



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CHOLERA IN EAST PAKISTAN FAMILIES, 1962-3

by

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S Y N O P S I S

The families of 85 cholera patients hospitalized during 1962-3 in Dacca District, East Pakistan, were investigated. Secondary cases were observed in 33 of the 85. Secondary attack rates were higher among children than adults. Spread of cholera within these families was suggested by the distribution of intervals between primary and subsequent cases, the relationship between lengthy home stay of index cases and increased numbers of secondary cases, and the effect of family structure on secondary attack rates.

CHOLERA IN EAST PAKISTAN
FAMILIES 1962-3

Introduction

The endemic and epidemic pattern of cholera in Bengal is well-known (Pollitzer, 1959); however, the precise manner in which cholera is spread and the disease perpetuated is not clear.

Since little is known about the behaviour of cholera in specific sub-groups of the population, such as families, studies of spread of infection in families of cholera patients in the Dacca District of East Pakistan were begun in 1962. This report describes the epidemiologic features of multiple cases occurring in a group of 85 families of patients hospitalized with bacteriologically confirmed cholera. Fifty-seven secondary cholera cases were observed in 33 of the 85 households. The present study provided an opportunity to examine the relationship between familial and individual characteristics and the likelihood of occurrence of multiple cases within households.

Method

Families located within a 10-mile radius of Dacca were selected for study usually within two days of hospitalization of a family member with bacteriologically proven cholera. Families were defined as units consisting of two or more individuals who lived and dined together. Members were not necessarily blood related. Cholera was defined as an acute diarrheal disease associated with recovery of V. cholerae (Inaba or Ogawa) organisms from faeces within five days of onset of symptoms. Six families were included although cultures were not obtained from index cases. These were rapidly fatal cases which occurred in families with one or more subsequent bacteriologically proven cases.

The families were visited at daily intervals, excluding weekends, for at least two weeks by teams comprised of physicians and sociologists. On the initial visits, questionnaires were completed to provide the following information about the families; location and type of housing, religious affiliation, travel away from home, recent contact with diarrhea and cholera cases and past cholera experience. Individual characteristics such as age, sex, marital status, and occupation were recorded. At each visit, inquiry was made concerning the health of every family member. Whenever diarrhea or vomiting was reported, a rectal swab was obtained for bacteriological investigation, and clinical details of the illness recorded. These were placed in bile peptone transport media and incubated overnight at 37°C. Subcultures were made on gelatin agar and Monsur's bile peptone tellurite medium and incubated overnight at 37°C. Suspicious colonies were tested for agglutination with specific antisera.

Results

The study was performed from late October 1962 through June 1963. Subsequent cases of cholera developed in 33 of the 85 families during the two-week observation periods. The frequency distribution of the number of affected individuals in families is shown in Table I.

The intervals between onset of illness in index cases and the appearance of the 57 subsequent cases in families are shown in Table 2. The bulk of secondary cases developed within 4 days. A few continued to appear each day thereafter. In 6 instances, intervals exceeded 7 days. The occurrence of secondary cases was not related to age or sex of index cases. Secondary attack rates are shown in Table 3. The number of affected persons below 15 years of age was significantly higher than that for adults, ($P = <.01$.) . There was no significant difference in secondary attack rates by sex.

The average family size was 6.1 individuals per family in the 52 households without subsequent cases, and 8.5 in the 33 multiple case families. The effect of family size and structure on the proportion of families with secondary cases and secondary attack rates is shown in Tables 4 and 5. Secondary attack rates increased with family size but were not significant at the 5% level. The attack rates increased significantly, however, when the families were classified by the number of children below 15 years of age ($P = <.05$).

The relationship between intervals from onset of illness in index cases to removal from the household, and the occurrence of multiple cases was examined(Table 6). Secondary attack rates were significantly higher in those families exposed to index cases longer than 12 hours than those exposed less than 12 hours. The effect of the length of exposure on the secondary attack rate was also demonstrable when family structure (the number of persons <15 years) was held constant ($P = <0.05$).

Discussion

In the present study, one or more additional cases of cholera were found among the families of 33 of 85 hospitalized cases. Thirteen contacts developed cholera on the same day symptoms appeared in the 85 index cases. These 13 cases, and possibly the 23 other illnesses occurring within 3 days of the onset of disease in index cases, may represent spread from a common source, infection from contact with primary cases, or a combination of both. "True secondary cases" cannot be identified precisely among multiple illnesses occurring in families since source of infection is difficult to prove and incubation periods in cholera may vary from a few hours to several days. Secondary cases as defined in this study provide, however, an estimate of risk among family members.

Several findings in the present study suggest that spread within families did occur. Cholera continued to appear in family contacts up to 10 days following primary cases. An increase in secondary illness was associated with prolonged household contact with index cases prior to

hospitalization. The relationship between secondary rates in families and the number of children below 15 years of age is consistent with intra-familial spread in a crowded setting. Multiple introductions cannot, however, be excluded.

The frequent occurrence of multiple cases of cholera in households was described during the last century in the United Kingdom by Snow(1855) and by Budd (1883). Recently, Morgan et al in Thailand (1960) and Dizon et al in the Philippines (1963) reported that multiple illnesses in the same family were uncommon. However, comparison of secondary attack rates in different studies is hazardous owing to variations in methods of morbidity data collection.

High incidence rates among children would be expected in a densely populated country in which cholera is a disease of continued high prevalence. Snow stated that "where a whole family live, sleep, cook, eat and wash in a single room, ... cholera has been found to spread when once introduced..."

Summary

This report describes the epidemiologic features of multiple cases of cholera occurring in the families of 85 hospitalized cholera patients. The relationship between familial and individual characteristics and the occurrence of multiple cases in households was examined. Fifty-seven secondary cases were observed in 33 of the 85 households. Most of the 57 cases appeared within four days after the onset of illness in index cases and a few cases occurred at daily intervals up to ten days. Secondary attack rates were higher among individuals below 15 years of age than among adults. The occurrence of secondary cases was not related to age or sex of index cases. When families were classified by the number of children below 15 years of age, secondary attack rates increased. Attack rates were higher among those exposed to index cases longer than 12 hours than in those less than 12 hours. These findings are consistent with intra-familial spread in a crowded setting.

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TABLE I

Number of cases per family.	Number of families
1	52
2	24
3	4
4	2
5	1
6	1
11	1
Total	85

Distribution of families by the number of cholera cases (index and secondary)

TABLE II.

	Intervals in days											
Subsequent cases	same	1	2	3	4	5	6	7	8	9	10	Total
Daily total	13	9	8	6	8	3	3	1	3	-	3	57
Cumulative total	13	22	30	36	44	47	50	51	54	54	57	57

Intervals between onset of illness in first cases and appearance of subsequent cases in families.

TABLE III

Age (years)	0 to 4		5 to 9		10 to 14		15+		Unknown	Total
Sex	M	F	M	F	M	F	M	F		
Index cases	18	9	3	14	7	4	8	19	3	85
Family members at risk	42	47	50	46	34	39	134	121	4	517
Secondary cases	10	8	9	8	6	5	6	5	-	57
Secondary Attack Rate	23.8	17.0	18.0	17.4	17.6	12.8	4.5	4.1	-	11.1

Age and sex distribution of index cases, secondary cases and population at risk in families.

TABLE IV.

No. of individuals in family	Proportion of families with secondary cases	No. of persons affected / No. of persons at risk			Secondary attack rates
		Age Groups (years)			
		<15	15+	Total	
2 - 5	8/37	7/48 (14.6%)	1/70 (1.4%)	8/118*	6.8%
6 - 9	14/32	15/102(14.7%)	7/98 (7.1%)	22/200	11.0%
10+	11/16	24/110(21.8%)	3/87 (3.4%)	27/197	13.7%
Totals	33/85	46/260(17.7%)	11/255(4.3%)	57/515	11.1%

* Ages of 2 persons unknown.

Family size and proportion of families with secondary cases and secondary attack rates in children and adults.

TABLE V

Number of persons under 15 years in families	Proportion of families with secondary cases	No. of persons affected / No. of persons at risk — Age Groups (years)			Secondary attack rates
		<15	15+	Total	
0	0/2	0/0 -	0/5 -	0/5	
1-2	8/28	7/29(24.1%)	1/66(1.5%)	8/95	8.4%
3-4	9/28	8/73(11.0%)	6/76(7.9%)	14/149	9.4%
5-6	7/15	11/69(15.9%)	1/55(1.8%)	12/124	9.7%
7+	9/11	20/89(22.5%)	3/53(5.7%)	23/142	16.2%
Totals	33/84*	46/260(17.7%)	11/255(4.3%)	57/515*	11.1%

* Age of 2 persons in 1 family unknown.

Family structure and proportion of families with secondary cases and secondary attack rates in families.

TABLE VI

No. of persons <15 years in families	Time Interval <12 hours	12+ hours	Total
0	0/1	0/4	0/5
1-2	1/40	7/55	8/95
3-4	1/77	7/62	8/139
5-6	10/72	2/52	12/124
7+	4/60	16/68	20/128
Total	16/250	32/241	48/491*

* 26/517 Persons at risk not classified due to unknown ages and/or intervals.

Number attacked/number at risk by

family structure and interval between onset

of illness in index cases to removal from household.

DIARRHEA CAUSED BY NON CHOLERA VIBRIOS*

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The nearly inevitable diarrhea which fells the traveler, often within hours of his arrival in certain parts of the world, is a problem which Kean and his associates[1] have described. The causes of this temporarily disabling illness are frequently not demonstrated even after careful search for bacterial and viral pathogens[2,3]. In this report is documented a diarrheal disease in both local residents of East Pakistan and U.S. personnel and their dependents, and from which the only likely pathogens isolated were non-cholera vibrios, (NCV). In 1954, Yajnik and Prasad[4] suggested that NCV were pathogenic in man after recovering them from pilgrims afflicted with gastroenteritis while attending the religious fair at Allahbad, India. Dutta, Panse, and Jhala[5] have reviewed the literature dealing with the possible pathogenic nature of these organisms and have reported the induction of a cholera-like disease in the infant rabbit with them.

Non-cholera vibrios fail to agglutinate in antiserum specific for Vibrio cholerae, the cause of epidemic cholera (hence the name "non-agglutinable" or "NAG" used by some writers) and usually differ from V. cholerae in fermentation or other biochemical tests. They can be distinguished readily from the microaerophilic vibrios which occasionally cause dysentery[6,7] as well as other human infections[8,9] by their abundant growth on simple media under aerobic conditions. The methods used in many enteric diagnostic bacteriology laboratories, however, might not permit recognition of these organisms. Because of world-wide distribution, NCV may possibly contribute to cases of undiagnosed diarrheal disease in areas far removed from the locality of our study.

The results given in the present paper provide additional evidence that some NCV are pathogenic for man. The organisms were isolated in abundance from cases of diarrhea, no other known pathogens were present and antibody titer rise directed against the infecting organism was observed during convalescence. In addition, these vibrios were found capable of inducing a cholera-like disease in infant rabbits.

METHODS

Rectal swabs from cases of acute diarrhea submitted to the wards of local hospitals, to the Pakistan-SEATO Cholera Research Laboratory (CRL) ward, or from U.S. personnel residing in Dacca, East Pakistan, were plated directly on taurocholate gelatin-agar[10] with or without added tellurite[11], for the detection of gelatinase producing vibrio colonies. They also were placed in alkaline peptone water or bile peptone fluid[12] and plated after incubation. Organisms showing gelatinase activities were confirmed as vibrios if the "string test"[13] was positive and a formalin fixed smear stained with crystal violet showed typical vibrio morphology. They were differentiated from V. cholerae by failure to agglutinate in vibrio O group I and "rough" antisera[14] by both slide and tube methods. Details of the bacteriological procedures used to characterize and distinguish NCV from other vibrios will be presented in a subsequent paper. If other pathogenic bacteria were found on MacConkey and SS agar plates

which also were inoculated directly with the swab or after enrichment in selenite broth, the patient was excluded from this study.

Blood was drawn from patients during the acute phase of the illness and during convalescence whenever possible. Serum was heated at 56°C for 30 minutes to inactivate complement. Agglutinins were measured by mixing 0.5 ml amounts of serial 0.45% saline dilutions of patient's serum and of an 18 hr. broth culture of the organism, isolated from the patient, diluted to a density of approximately one opacity unit (U.S. Opacity Standard); the tubes were held at 37°C for 1 hour and overnight at 4°C. The titer was designated as the highest final dilution in which agglutination was present.

Experimental NCV infection with strains isolated from three different patients (strains 696, 965, 1044) was induced in 10-day old rabbits using Dutta's technique.[15] The organisms had been maintained for six months by monthly serial transfers on 1% trypticase, 1% NaCl agar. Approximately 400 colony forming units (CFU) from a 4 to 5 hour heart infusion agar culture suspended in 1 ml of a 0.1% trypticase, 0.85% NaCl solution were injected at laparotomy into the small intestine under ether anesthesia. Five of 13 rabbits challenged with strain 696 received intraperitoneally 24 hrs. previously 1 ml of antiserum from rabbits immunized with living vibrios of the homologous type and having an agglutinin titer of 1:20,480. Control rabbits received either no intraintestinal injection, 1 ml of suspending fluid, or 490 CFU of an enteropathogenic Escherichia coli strain O111:B4.

After surgery the animals were observed closely for illness. They were sustained by gastric feeding of 5.0% glucose and 0.45% NaCl solution initially, followed by bovine whole milk. The time of onset of diarrhea and the time of death were noted. All animals dying or sacrificed either when death was imminent or showing no signs of illness at 120 hrs. (4 at 96 hrs) were autopsied and cultured.

RESULTS

PATIENT STUDIES

Nineteen patients with diarrheal disease from whom heavy growths of NCV were obtained on direct plating of rectal swabs were studied. Neither V. cholerae, Salmonella or Shigella were found in their stool cultures and no patient showed a definite rise in antibody titer against V. cholerae. Table 1 gives the clinical and laboratory features of the infection. The cases showed variation in the severity of their disease. Some had voluminous diarrhea lasting up to 50 hours and resembled true cholera. Others had a mild illness requiring no therapy and were only slightly disabled. The highest plasma protein, an index of dehydration, was 9.6 gm%. Only half of the patients were considered to require intravenous replacement of fluid losses. The disease occurred in both sexes. Fever of greater than 99° F was present in 7 of 16 patients on whom data are available. This fever was transient, usually disappearing before the end of diarrhea. The maximum stool volume was 8 liters and the longest

duration of diarrhea was 2.5 days. The median stool volume recorded after hospitalization was less than one liter, and the diarrhea ceased in less than 24 hours in one-half of the cases. Significant acidosis as measured by the CO_2 content of venous blood on admission was present in 5 of the 11 cases tested; the lowest value was 5.5 mEq/l. Seven cases received antibiotics. There was no striking difference between cases so treated and those not given antibiotics, although the rapid clearing of symptoms in case 19 is suggestive of therapeutic effect. The disease is naturally short and self-limited and the observed improvement could be fortuitous. In the 16 cases in whom satisfactory paired sera are available, six showed rises in agglutinin titers of four-fold or greater against the NCV strain isolated from them; two cases showed rises to a final titer of 1,280 or more.

The following case reports give a more detailed account of the character of the disease.

Case 1: CRL No. 160. A 30-year-old Hindu, Bengali wife of a fisherman, was transferred to the CRL from a local infectious diseases hospital, with a 10-hour history of purging diarrhea and vomiting. Her blood pressure on admission was 70/40 mm Hg, pulse 130/min and weak, her skin was hot and dry and fingers showed shriveling or "washer woman's" changes. Her temperature was 101° F, and her eyes appeared sunken. She was too weak to sit and cried for water. Her abdomen was slightly distended. Bowel sounds were normal. The remainder of the examination was within normal limits. She was given 5,250 ml of intravenous fluid in the first 24 hours. During this time she passed 5,655 ml watery stool. During the next 24 hours she passed only 710 ml stool and required no intravenous fluids. Laboratory studies on admission showed a WBC of 10,800/mm^3, plasma protein 8.6 gm%, Hct 47%, CO_2 15.2 mEq/l. A virtually pure culture of NCV was obtained from the rectal swab. Ova of both Ascaris lumbricoides and Trichuris trichiura also were present in her stool. Her hospital course was complicated by a right lower lobe pneumonia which responded to penicillin. Serum titer against her own NCV on tenth day was 8-fold greater than on second day. There was no rise in agglutinins against V. cholerae.

One year later this patient was treated at the CRL for typical severe cholera with V. cholerae, Inaba, present in nearly pure culture.

Case 2: CRL No. 243. A 19-year old Muslim, Bengali man was brought to the CRL six hours after the onset of purging diarrhea and vomiting. He had a palpable radial pulse and a BP of 85/45 mm Hg. His temperature was 100.4° F and he appeared mildly dehydrated. Laboratory studies on admission showed plasma protein 8.5 gm%, Hct 45%, WBC 20,500/mm^3, CO_2 13.9 mEq/l. He was given 2000 ml intravenous fluid in the first 24 hours and excreted slightly over 1000 ml of liquid stool. His temperature returned to normal and diarrhea ceased within 24 hours. Culture of his rectal swab yielded a nearly pure growth of NCV. Serum titer against his own NCV on the sixth day of disease was 32-fold greater than on first day. Direct platings of rectal swabs were positive for this NCV for a period of eight days.

Case 3: ASB, NCV No. 1035. After spending 36 hours in Calcutta, a 48-year-old American physician developed abdominal cramps at 9:00 a.m. followed by diarrheal bowel movements with considerable loss of fluid beginning three hours later. Culture of the stools on the second day of illness revealed NCV (No.1035). Diarrhea ceased on the second day only to return on the fourth day after the patient had ingested food in public eating places in Karachi and Rawalpindi. Copious fluid stools numbered four to five a day and were accompanied by minor diffuse abdominal cramps. The number of stools increased on the fifth day at which time a culture was taken which revealed again NCV (No.1044). Two "Polymagma" tablets representing a total of 300 mg of hydrostreptomycin and 100,000 units of Polymyxin B were taken late on the sixth day of illness. Complete cessation of the cramping and fluid stools followed. There was no fever during the illness. The patient had a rise in serum agglutinin titer of similar magnitude to the early and the late isolates which were also indistinguishable in their biochemical reactions.

ANIMAL STUDIES:

Table 2 shows that each of the three NCV strains tested was capable of inducing infection in 10-day old rabbits with as few as 300 - 480 CFU. Fifteen of the 17 rabbits developed enteritis manifested by large accumulation of fluid stool in both the small bowel and colon. Fluid accumulation however, was usually not as great as seen in infant rabbits infected with V. cholerae. Cultures of the fluids from the 15 rabbits were positive for NCV. Homologous hyperimmune serum administered intraperitoneally 24 hours prior to challenge provided protection to each of five rabbits against accumulation of intraintestinal fluid, and from two rabbits vibrios were not recovered. Nine control rabbits, including three challenged with a human enteropathogenic strain of E. coli, failed to develop characteristic pathological findings.

DISCUSSION

It has been noted previously[12] that occasional patients with cholera have been found to excrete NCV in addition to V. cholerae. We have observed this association at the CRL in the cultures of some patients with cholera, especially after enrichment of the rectal swab in selective media. These observations, coupled with the common occurrence of NCV in the surface water of the area have led some to doubt their pathogenicity.[16]

Since we have been able to demonstrate the presence of NCV in large numbers on direct platings of rectal swabs of patients ill with a diarrheal disease; have not found the organism on direct platings from healthy people; and have shown that these organisms produce a diarrheal disease in the infant rabbit similar to that produced by V. cholerae, we feel that the evidence that these NCV are pathogenic for man is strong. The serum agglutinating antibody response in NCV infection is less regular than that observed in infection with V. cholerae in which 91% of individuals have been shown to develop antibody rises.[17] However, the

development of antibody specific for the infecting NCV and the failure to develop antibody against V. cholerae further substantiate the specificity of the disease.

The relation of NCV to these enteric infections was recognized because they occurred in a cholera endemic area and were investigated in a laboratory studying vibrios. Since NCV are found in many parts of the world, they may well play an important part in the etiology of diarrheal disease in regions where classical cholera has not been present for many years. Schemes intended for isolation and identification of enteropathogenic members of the family Enterobacteriaceae are not entirely suitable for the demonstration of NCV, These organisms grow very poorly on the selective media usually employed and about two-thirds of them are late lactose fermenters. If isolated, it is likely that many bacteriologists would not recognize them as vibrios and would place them in the "paracolon" group, which, although the practice has been decried by Edwards and Ewing,[18] remains a "designation of convenience for organisms the exact nature of which was not determined or not understood." Application of the relatively simple techniques mentioned in this paper to the study of cases of gastroenteritis due to unknown causes will provide answers to the questions of how frequently and in what geographic areas NCV disease occurs.

SUMMARY

Patients with diarrheal illness occurring in Dacca, East Pakistan, from whom cultures showed heavy growths of non-cholera vibrios were studied. Cases occurred in both sexes and in both local residents and in U.S. personnel residing in Dacca. Unequivocal antibody titer rises against the infecting NCV were demonstrated in sera from certain cases. Survey of healthy individuals in the area failed to reveal any people with heavy infestations of NCV. Small numbers of organisms from the three strains tested were able to produce a diarrheal disease in infant rabbits and complete protection against diarrhea followed passive administration of hyperimmune serum. The demonstrated pathogenicity for man and the widespread distribution of NCV in surface waters of the world suggest that simple methods designed to identify these organisms should be included in the laboratory routines used in the study of diarrhea.

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TABLE 1

CLINICAL AND LABORATORY FEATURES OF NCV INFECTIONS

Case	Nationality	Sex	Body Weight Kg	Hypotensive	Adm. Plasma Prot. gm%	Adm. WBC/mm^3	Adm. CO_2 mEq/l	Temp. 99°F	Total IV Fluid ml.	Type and Volume of Stool ml	Length of D'hea Days	Antibiotic Rx	Serological Studies: Day of Disease	Agglutinating Titer V. cholerae Ogawa	Agglutinating Titer V. cholerae Inaba	Homologous NCV
1. NCV #696	Pak.	F	adult	yes	--	--	--	--	7,500	rice water	--	0	--	--	--	--
2. NCV #817	Pak.	F	adult	no	--	--	--	--	--	--	--	0	2	40	—	320
													21	20	—	2,660
3. CRL #2	Pak.	M	adult	yes	--	--		--	--	--	--	0	acute	320		160
													convalescent	320	--	1,280
4. CRL #126	Pak.	M	19	no	5.9	12,400	21.8	+	none	rice water 1,185	2 1/2	0	before illness	20	20	20
													1	20	20	20
													4	20	20	20
5. CRL #160	Pak.	F	32	yes	8.6	10,800	15.2	+	5,000	rice water 8,360	2 1/2	0	2	20	20	20
													10	20	20	160
													17	20	20	80
6. O.R.M. NCV #965	U.S.	M	70	no	--	--	--	+	0	brown water	1 1/2	Paromomycin	1	2,560	—	160
													7	5,120	--	160
7. CRL #185	Pak.	M	44	yes	8.3	18,000	17.7	0	1,000	rice water	1	Tetracycline	1	20	20	160
													1	40	20	80
													2	20	20	80
8. CRL #222	Pak.	M	20	no	6.6	10,800	--	0	0	no stool after admission	<1	0	2	40	40	20
													5	80	40	40
													7	80	40	160
													31	40	80	80
9. W.B.G. NCV #1073	U.S.	M	76	no	--	--	--	+	0	brown water	<1	0	28	640	—	320
													17	640	--	320
10. CRL #232	Pak.	F	39	no	7.2	--	--	0	1,000	no stool after adm.	<1	0	2	80	80	40
													3	160	160	40
													6	160	160	80

11. CRL #240	Pak.	M	10	no	7.6	--	--	0	0	no stool after admission	<1	0				
12. A.S.B. NCV* #1035 #1044	U.S.	M	72	no	--	--	--	0	0	brown water	2 & 4	0 1st polymyxin & hydros-treptomycin	-21 3 3 23	320 320 320 320	-- -- -- --	#1035 & #1044 20 20 20 80
13. CRL #243	Pak.	M	19	no	8.5	20,500 1,025	13.9	+	2,000	rice water	1	0	1 6	40 40	40 40	20 640
14. CRL #244	Pak	M	49	no	8.9	15,500	24.5	0	2,000	no stool after admission	<1	tetra-cycline	2 7	160 160	160 80	160 160
15. CRL #246	Pak	F	28	yes	6.7	--	12.2	0	2,300 2,105	green water	2	tetra-cycline	2 7 15	320 160 160	320 320 320	160 320 320
16. CRL #264	Pak	M	27	no	7.0	5,400	21.9	0	0	no stool after admission	<1	tetra-cycline	1 8	40 40	40 40	--
17. CRL #400	Pak.	M	42	no	7.8	13,700 1,540	19.5	+	3,600	rice water	1	tetra-cycline	0 6	40 40	40 40	40 40
18. CRL #712	Pak.	F	44	no	9.4	12,800	5.5	0	1,000	brown water	--	0	3 7 11 19	80 80 40 40	20 20 20 20	20 20 20 20
19. CRL #730	Pak.	M	54	no	9.6	8,700 210	19.8	+	2,000	brown water	<1	0	0 1 3 6	160 160 160 160	160 80 80 80	20 20 20 40

* Two separate infections or a relapse occurred four days apart.

TABLE 2.

Results of Experimental NCV Infection in Infant Rabbits.

Challenge strain	Dose CFU*	Number challenged	Number with diarrhea	Time of death or sacrifice (hours)	Autopsy Findings Number with Cholera	Autopsy Findings Number with Positive culture
965	320	3	3	66-96	3	3
	433	3	3	20-116	3	3
1044	270	3	2	51-96	2	2
696	480	3	3	38-96	3	3
	370	3	2	29-120	2	2
	300	2	2	40-47	2	2
696 and Antiserum IP	480	3	0	38-96	0	1
	300	2	0	120	0	2
Controls:						
Non-operated	0	3	0	120	0	0
Trypticase-Saline	0	3	0	120	0	0
E. coli O111:B4	490	3	0	120	0	ND**

* Colony forming units.
** Not examined specifically for challange strain.

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ISOLATION OF HEMAGGLUTINATIVE NON EL TOR VIBRIOS *

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During 1963, El Tor cholera spread through Thailand and Burma and finally appeared in the Chittagong area of East Pakistan in November (Yen et al 1964). With disease caused by this clearly marked serotype within 50 miles of areas under study by this laboratory, all vibrio isolates which agglutinated in specific sera were screened by testing for their ability to hemagglutinate chicken erythrocytes (Finkelstein & Mukerjee, 1963).

On 10 January, 1964, rectal swabs were received in alkaline tellurite bile peptone broth from 6 individuals suffering with the clinical syndrome of cholera. These had been obtained on 6 January by a government health visitor from a village adjacent to the vaccine study area of this laboratory. From all these cultures, Vibrio cholerae, Inaba, was isolated; agar surface growth of two of these agglutinated chicken cells in a slide agglutination test (Finkelstein & Mukerjee, 1963). During the ensuing six weeks, cholera vibrios were isolated from 384 other individuals. From 6 individuals, hemagglutinating organisms were obtained; in three of these, a non-hemagglutinating strain was isolated at the same time as the hemagglutinative strain. No hemagglutinative strains had been found among the cholera vibrios isolated from 750 individuals previously studied. Until 13 September, 1964, vibrios were isolated from an additional 160 individuals (17 Ogawa and 143 Inaba) but no hemagglutinative strains were isolated despite the search for such organisms. Sixty-six typical Ogawa El Tor strains received during this latter period from various other laboratories were all hemagglutinative.

Because typical El Tor organisms had been isolated in East Pakistan in November, 1963 (Yen et al 1964), these strains were believed to herald the appearance of El Tor cholera within our study area. Accordingly, the strains, together with the non-hemagglutinative strains isolated from the same individuals, were subjected to the classical tests designed to differentiate El Tor from classical vibrios. Susceptibility to Mukerjee's Group IV phage was determined quantitatively (Monsur et al, 1964). The presence of the hemolytic activity was examined by the method of Feeley and Pittman (1963) and by the plate hemolysis modification of Greig's test (Taylor, 1941). The following additional tests were also used: soda sublimate precipitation and soda serum agglutination (Tanamal, 1948); sensitivity to polymyxin B (Han & Khie, 1963); ability to grow in 0.9% trypsin (Han & Khie, 1963); Barritt's(1936) modification of the Voges-Proskauer reaction; the heat inactivation of specific serum agglutinability (Gispen, 1938); and the chloroform inactivation of specific serum agglutinability (Meyer, 1939). The results are presented in Table I. All strains tested gave the sugar fermentations of Heiberg Group I, all were motile, produced indol, and used nitrate as substrate. These hemagglutinative strains resemble classical vibrios in all respects except for their ability to agglutinate chicken red blood cells. The hemagglutinative and non-hemagglutinative strains isolated from the same individuals were recognized on the purity plate; the hemagglutinating and non-hemagglutinating colonies were sub-cultured and found to breed true on successive sub-cultures both on solid media and through serial passage in T_1N_1 (1% tryptic-case, 1% NaCl), 5% peptone water at pH 7.5, bile peptone water at pH 8.5

or trypticase soy broth. No difference in colonial morphology of the growth on gelatin agar or tellurite taurocholate gelatin agar plates was detected by microscopic examination with oblique illumination (Finkelstein & Gomez, 1963). The 8 individuals from whom these strains were isolated lived in 7 different villages within an area 30 miles in diameter, located in 2 districts of East Pakistan. With the exception of the first 2 cases, no inter-relationship could be established between cases. All but one of the cases suffered from diarrhea (Table II); one of the two first patients died. Of the 6 individuals who came under observation of CRL laboratory personnel, four were severely ill requiring intravenous therapy. A year-old male child had a mild diarrhea which required no therapy. The sixth was an asymptomatic contact of a 2-year old child with diarrhea from whom typical Inaba organisms (B 4048) were isolated (See Table I).

The chicken cell agglutinating property of Vibrio cholerae was correlated with the presence of hemolysin and resistance to attack by Mukerjee Group IV phage based on the examination of 287 classical strains and 349 El Tor strains. This finding of 8 hemagglutinative strains without resistance to Group IV phage or hemolytic properties, isolated during a limited period in East Pakistan, suggests that the chicken cell agglutinating property may represent a marker usually, but not always, associated with the other properties used to characterize the "El Tor vibrio."

Finkelstein & Mukerjee (1963) had isolated two hemagglutinative variants from one strain of V. cholerae after broth passage, and noted the appearance of hemagglutinative properties in tube tests when vibrios were cultivated in trypticase soy broth under forced aeration. The hemagglutinative property of the organisms reported herein was noted after a single passage through 1% trypticase, 1% sodium chloride broth, but this property was fixed on agar passage and was demonstrated with agar grown cultures in slide agglutination tests. Because all isolates in this laboratory are subjected to this procedure, these eight strains clearly differ from isolates found previously or since. Their occurrence once again suggests that the various properties taken to classify an organism as an El Tor strain merely represent a group of markers of the Vibrio cholerae which may be of primary epidemiological importance. The isolation of such intermediate strains is an additional argument against the establishment of El Tors as a separate taxonomic species.

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TABLE I

COMPARISON OF PROPERTIES OF CHICKEN-CELL AGGLUTINATING STRAINS WITH CLASSICAL AND EL TOR VIBRIOS

	Serotype	Mukerjee's Phage Type	Minimal Effective Dose to Group IV Phage	Chicken Cell Agglutination	Plate Haemolysis	Tube Haemolysis	Polymixin B Sensitivity	Growth in 0.9% Trypsin.	Voges Proskauer Reaction	Soda Serum Agglutination	Soda Sublimate Precipitation.	Gispen's Test.	Meyer's Test.	Date of Isolation.	Source of Strain.
EAST PAKISTAN CLASSICAL CHOLERA STRAINS:															
B 3302-A	IN	III	<10	-	-	-	+	Sl*	-	-	+	+	+	11.2.62	Comilla District
B 3802-A	IN	III	<10	-	-	-	+	Sl	-	-	+	+	+	17.2.64	Comilla District
B 4048	IN	III	<10	-	-	-	+	Sl	-	-	+	+	+	19.2.64	Comilla District
B 4273-A	IN	III	<10	-	-	-	+	Sl	-	-	+	+	+	20.2.64	Comilla District
B 6425	OG	III	<10	-	-	-	+	Sl	-	-	+	+	+	8.3.64	Dacca District
B 12789	IN	III	<10	-	-	-	+	Sl	-	-	+	+	+	21.4.64	Dacca District
B 12945	OG	III	<10	-	-	-	+	Sl	-	-	+	+	+	22.4.64	Dacca District
B 13296	OG	III	<10	-	-	-	+	Sl	-	-	+	+	+	26.4.64	Dacca District
EAST PAKISTAN CHICKEN CELL POSITIVE CHOLERA STRAINS:															
B 631	IN	III	<10	+	-	-	+	Sl	-	-	+	+	+	6.1.64	Comilla District
B 634	IN	III	<10	+	-	-	+	Sl	-	-	+	+	+	9.1.64	Comilla District
B 1716	IN	III	<10	+	-	-	+	Sl	-	-	+	+	+	26.1.64	Dacca District
B 1997	IN	III	<10	+	-	-	+	Sl	-	-	+	+	+	30.1.64	Dacca District
B 3302-B	IN	III	<10	+	-	-	+	Sl	-	-	+	+	+	11.2.64	Comilla District
B 3802-B	IN	III	<10	+	-	-	+	Sl	-	-	+	+	+	17.2.64	Comilla District
B 4273-B	IN	III	<10	+	-	-	+	Sl	-	-	+	+	+	20.2.64	Comilla District
B 4841	IN	III	<10	+	-	-	+	Sl	-	-	+	+	+	19.2.64	Comilla District
EL TOR STRAINS:															
Chittagong-1	OG	-	$>10^8$	+	+	-	-	+	+	+	-	-	-	16.11.63	Chittagong
Chittagong-12	OG	-	$>10^8$	+	+	-	-	+	+	+	-	-	-	16.11.63	Chittagong.
IND-9	OG	-	$>10^8$	+	+	+	-	+	+	+	-	-	-	1961	Indonesia
IND-40	OG	-	$>10^8$	+	+	+	-	+	+	+	-	-	-	1960	Indonesia
Thailand-SE,47	IN	-	$>10^8$	+	+	-	-	+	+	+	-	-	-	21.10.63	Bangkok
Malacca-3734	OG	-	$>10^8$	+	+	+	-	+	+	+	-	-	-	May 1963	Malacca
Hong Kong-2	OG	-	$>10^8$	+	+	+	-	+	+	+	-	-	-	16.8.63	Hong Kong
Hong Kong-50	OG	-	$>10^8$	+	+	+	-	+	+	+	-	-	-	29.8.63	Hong Kong

* SL = Slight turbidity

TABLE II

CLINICAL FEATURES OF INDIVIDUALS FROM WHOM HEMAGGLUTINATIVE STRAINS WERE ISOLATED.

Strain No.	Date of Isolation	Date of Onset	Patient's Identification	Age	Sex	Clinical Symptoms
B 631	6 Jan 1964	?	Monowara	16	F	Clinical cholera
B 634	6 Jan 1964	?	Mustafa	8	M	Fatal cholera
B 1716	26 Jan 1964	25 Jan	CRL 615	10	F	Hospitalized cholera
B 1997	30 Jan 1964	29 Jan	CRL 663	40	F	Hospitalized cholera
B 3302*	13 Feb 1964	12 Feb	Shaitnal 13	19	F	Clinical cholera
B 3802*	18 Feb 1964	17 Feb	E 95	1	M	Mild diarrhea
B 4273*	21 Feb 1964	None	W 1066	16	F	No symptoms
B 4841	19 Feb 1964	19 Feb	MH 115	10	M	Hospitalized cholera

* Both hemagglutinative and non-hemagglutinative strains isolated.

LIST OF PAKISTAN-SEATO CHOLERA
RESEARCH LABORATORY STAFF

November 1, 1964

DIRECTOR'S OFFICE

		Joined:
Dr. Abram S. Benenson	Director	28 June 1962
Mr. Robert E. Freise	Executive Officer	29 January 1964
	2 Steno/Secretaries	

CLINICAL RESEARCH SECTION:

Dr. W.B.Greenough, III	Chief, Clinical Res. Sec.	18 July 1962
Dr. John Lindenbaum	Assistant Chief	19 August 1963
Dr. Md. Rafiqul Islam	Senior Physician	13 Feb. 1963
Dr. Md. Matleb Ali	Junior Physician	22 March 1963
Dr. A.K.M. Jamiul Alam	Junior Physician	21 August 1963
Dr. Md. Shamsul Islam	Junior Physician	4 Sept. 1964
Miss Anisa Saad	Senior Res. Assistant	30 August 1962
	1 Junior Physician	
	4 Senior Res. Assistants	
	4 Senior Lab. Technicians	
	1 Senior Res. Nurse	
	1 Radiology Technician	
	1 Medical Record Secretary	
	1 Junior Lab. Assistant	
	5 Junior Lab. Technicians	
	1 Lab. Attendant	

CRL WARD:

Miss Dorothy G. Torrance	Supervisor	16 July 1963
	9 Senior Nurses	
	4 Junior Nurses	
	7 Aid Nurses	
	1 Ward Master	
	1 Chief House Keeper	
	2 Cooks	
	1 Cook's Helper	
	2 Ward Boys	
	8 Orderlies	
	2 Laundrymen	
	2 Glass Washers	

BACTERIOLOGY SECTION:

		Joined:
Dr. Saiyid Sibte Hasan Rizvi	Chief	27 August 1963
Dr. (Mrs.) Rezia Akbar	Research Physician	1 Feb. 1963
3	Senior Res. Assistants	
3	Bacteriological Assistants	
2	Lab. Technicians	
3	Lab. Assistants	
1	Junior Lab. Technician	
3	Media Makers	
1	Sample Taker	
7	Lab. Attendants(Glass Washers)	

EPIDEMIOLOGY SECTION:

Dr. Robert O. Oseasohn	Chief & Deputy Director	2 Sept. 1963
Dr. Moslem Uddin Khan	Deputy Chief	3 April 1964
Dr. (Mrs.) Shamsa Ahmed	Assistant Deputy Chief	1 May 1964
Dr. Robert M. Glasse	Anthropologist	18 Sept. 1963
Mrs. S.H. Glasse	Anthropologist	18 Sept. 1963
Dr. Md. Ashraful Islam	Physician	23 April 1963
Dr. A.S.M. Mizanur Rahman	Physician	29 April 1963
Dr. Ranjit Kumar Barui	Physician	11 Nov. 1963
3	Senior Res. Asstts.(Sociologist)	
1	Field Assistant	
2	Sanitary Inspectors	
2	Statistical Assistants	
7	Field Nurses	
2	Interpretors	

WATER STUDIES SECTION:

Mr. Syed Zafar Ahmed	Chief	1 October 1961
2	Senior Res. Assistants	
1	Junior Res. Assistant	
1	Senior Lab. Technician	
3	Sanitary Inspectors	
1	Junior Lab. Technician	
1	Glass Washer	
1	Lab. Attendant	

ADMINISTRATIVE SECTION:

Mr. Robert E. Freise	Chief	
	1	Property & Supply Officer
	1	Administrative Assistant
	1	Accountant
	4	Junior Administrative Assistants

GENERAL SERVICES SECTION:

Mr. N.A. Husain	Acting Chief	
	1	Maintenance Supervisor
	2	Maintenance Assistants
	1	Librarian
	2	Typist/Clerks
	1	Telephone Operator-Cum-Receptionist
	1	Dispatcher
	1	Mechanic
	1	Electrician
	14	Drivers
	5	Darwans
	2	Night Guards
	1	Veterinarian
	1	Animal Care Taker
	1	Animal Assistant
	1	Animal Handler
	1	Animal Cage Cleaner
	1	Gardener
	4	Sweepers
	2	Messengers
	1	Tailor

FOREIGN TRAINING PROGRAM:

Mr. M.R. Bashir	Administrative Officer	NIH, Bethesda, Maryland, U.S.A.
Mr. Imdadul Huq	Deputy Chief, Bact. Sec.	London School of Hygiene & Tropical Medicine
Mr. Syed Ahmed Khan	Sociologist	Western Reserve University, Cleveland, OHIO
Mr. S.I. Hasan	Research Assistant	Jefferson Medical College, Philadelphia

MATLAB VACCINE TRIAL:

EPIDEMIOLOGY SECTION:

		Joined:
Mr. S.O.Q. Haider Ali	Supervisor Personnel activities, Matlab	2 July 1962
Mr. Md. Shafiqul Islam	Supervisor, Project activities	2 July 1962
Mr. Abdul Momin Chowdhury	- do -	27 August 1962
Mr. Khwaja M. Ashraful Aziz	Census Supervisor	25 August 1964
11	Sanitary Inspectors	
29	Health Assistants	
34	Dais	

CLINICAL RESEARCH SECTION:

Dr. Zahedul Huq	Junior Physician	25 June 1964

CRL WARD:

1	Aid Nurse
3	Orderlies

GENERAL SERVICES SECTION:

2	Sarangs
1	Bargeman
5	Khalashies
2	Launch Drivers
2	Greasers
3	Boat Drivers

	PROFESSIONAL & EXECUTIVE	TECHNICAL	SUPPORTING	TOTAL
DIRECTOR'S OFFICE	2	-	2	4
CLINICAL RESEARCH SECTION	7	17	2	26
CRL WARD	1	20	19	40
BACTERIOLOGY SECTION	2	16	7	25
EPIDEMIOLOGY SECTION	8	13	4	25
WATER STUDIES SECTION	1	8	2	11
ADMINISTRATIVE SECTION	-	-	7	7
GENERAL SERVICES SECTION	-	-	44	44
MATLAB	5	75	18	98
FOREIGN TRAINING PROGRAM	3	1	-	4
GRAND TOTAL :	29	150	105	284

CONSULTANTS:

Dr. K.A. Monsur	Bacteriology Section
Dr. A.K.M. Abdul Wahed	Clinical Research Section
Dr. Md. Fahimuddin	Epidemiology Section