PROCEEDINGS

of the

SIXTH MEETING

of the

TECHNICAL COMMITTEE

of the

PAKISTAN-SEATO CHOLERA RESEARCH LABORATORY

Dacca, East Pakistan

December 9-10, 1968.

Second Edition

FOREWORD

The Pakistan-SEATO Cholera Research Laboratory is a part of the SEATO Cholera Research Program and is supported by the U. S. Agency for International Development, Department of State; the National Institutes of Health and the National Communicable Disease Center of the U. S. Department of Health, Education and Welfare; and by the Governments of Pakistan, United Kingdom and other SEATO nations. The NIH Cholera Advisory Committee coordinates the research program.

These studies were supported in part by Research Agreement No. 196802 between the National Institutes of Health, Bethesda, Maryland, U.S.A., and the Pakistan-SEATO Cholera Research Laboratory, Dacca, East Pakistan.

HONORS AND AWARDS

With justifiable pride the Pakistan-SEATO Cholera Research Laboratory wishes to mention the following Honors and Awards:

The January 4, 1969 issue of Nature reported that Dr. James W. Howie, Director of the Public Health Laboratory Service in London, had been knighted. Sir James has served as a member of the Technical Committee since 1967.

At a ceremonial luncheon given by the Albert and Mary Lasker Foundation, New York City, on November 9, 1967, Dr. Robert A. Phillips, Director of the Pakistan-SEATO Cholera Research Laboratory, received the Albert Lasker Award for Clinical Research. Dr. Phillips has served as Director of the Laboratory since 1965.

Editors Note

Volume I of the Proceedings includes this year certain excerpts of reports presented to the Directing Council which appear in Volume II. In particular, it is believed the "Director's Comments" regarding the administrative and scientific programs of the Laboratory, the text of the Administrative Report, and a compendium of PSCRL publications and papers given before medical conferences present the larger view of the scope of the activities of the Laboratory for those who receive copies of Volume I.

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I.

LIST OF PERSONS ATTENDING MEETINGS and TECHNICAL COMMITTEE REPORT

Pakistan-SEATO Cholera Research Laboratory
Dacca, East Pakistan

REPORT OF THE SIXTH MEETING OF THE TECHNICAL COMMITTEE

December 9-10, 1968

The Technical Committee met in joint session with the Directing Council, five members of the Clinical Investigation Committee and six Consultants to the PSCRL on December 9 and 10, 1968.

In attendance at the meetings were the following :

Members of the Technical Committee

Dr. Colin MacLeod
Chairman of the Technical Committee
(Vice President for Medical Affairs
The Commonwealth Fund
New York, New York, U.S.A.)

Dr. Ziaur Rahman
(Deputy Director General of Health
Government of Pakistan
Islamabad, West Pakistan)

Dr. James W. Howie
(Director, Public Health Laboratory Service
London, England)

In addition to the members of the Technical Committee, the following persons were in attendance at the sessions on both days :

Member-designates of and representatives to the Directing Council

Professor Robert Cruickshank
Chairman of the Directing Council
(University of Edinburgh
Edinburgh, Scotland)

Dr. Mohammed Asiruddin
Vice-Chairman of the Directing Council
(Director of Health Services
Government of East Pakistan
Dacca, East Pakistan)

Dr. Jean F. Rogier
(Chief Public Health Advisor
U. S. Agency for International Development, Dacca
Dacca, East Pakistan)

Dr. John R. Seal
(Director of Intramural Research and
Chairman of the NIH Cholera Advisory Committee
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland, U.S.A.)

Mr. David A. Wraight
(Deputy Secretary General
South-East Asia Treaty Organization
Bangkok, Thailand)

Dr. Robert A. Phillips, Director
Pakistan-SEATO Cholera Research Laboratory

Dr. W. Kendrick Hare, Deputy Director
Pakistan-SEATO Cholera Research Laboratory

Members of the Clinical Investigation Committee

Dr. Nazir Ahmed
(Joint Secretary of Health
Government of West Pakistan
Lahore, West Pakistan)

Dr. A. K. Shamsuddin Ahmed
(Director, Institute of Diseases of the
Chest and Hospital
Government of East Pakistan
Dacca, East Pakistan)

Dr. Graham Bull
(Director, Clinical Research Centre of the
Medical Research Council
London, England)

Prof. D. A. K. Black
(Chairman, Department of Medicine
University of Manchester
Manchester, England)

Dr. Stanley E. Bradley
(Director, Medical Service
Columbia University College of Physicians and Surgeons
Presbyterian Hospital
New York, New York, U.S.A.)

Consultants

Dr. K. A. Monsur
(Director, Institute of Public Health
Government of East Pakistan
Dacca, East Pakistan)

Prof. W. E. Van Heyningen
(St. Cross College, University of Oxford
Oxford, England)

Prof. Joel Mandelstam
(Dept. of Biochemistry, University of Oxford
Oxford, England)

Dr. A. L. Furniss
(Public Health Laboratory
Kent, England)

Dr. A. H. G. Love
(Senior Lecturer in Medicine
Queen's University of Belfast
Institute of Clinical Science
Northern Ireland)

Dr. Alexander Langmuir
(Chief, Epidemiology Section
National Communicable Disease Center
Atlanta, Georgia, U.S.A.)

The Technical Committee is grateful for the assistance of all these men and for their continuing interest in the work of the Laboratory. The excellent cooperation of the Director and his staff made our review both pleasant and profitable.

We wish to compliment the Section Chiefs and their associates on their reports and presentations, as well as the conciseness and clarity of the protocols they submitted. We should also note that their resources are severely strained at this time because of the major epidemic outbursts both in Dacca and in the Matlab Bazaar area.

It is the consensus of the Technical Committee that a joint session has many advantages as compared to separate meetings of the three Committees held at different times. It is recommended, therefore, that this format be followed for the next annual meeting of the Technical Committee, the Directing Council and the Clinical Investigation Committee.

Since the meeting of the Technical Committee one year ago there has been gratifying development in most aspects of the work of the Laboratory. While there has been improvement in some of the physical facilities, certain major deficiencies continue to hamper development of the research program. Of these, the most important pertain to inadequate housing of essential research components, the inadequate and inconstant supply of electric power which has been noted frequently in the past, and the lack of a central supply of hot water, also noted repeatedly in previous reports.

We recommend that early attention be given to the inadequacy of the ward space which is grossly insufficient to accommodate the large number of patients who must be cared for. In addition, the laundry is too close to the ward and should be moved elsewhere so that this space can be set aside for a clinical study area. Laboratory space in all areas fails by far to meet the current needs and the need for expanded library stack space is acute. There has been some improvement in the Animal House but it still falls far short of the requirements of the present research program and prevents extension of research into areas the Committee believes are important for more rapid and effective development of knowledge of cholera.

Compounding the problems caused by insufficient and inadequate space are those created by the deficiencies in the electric power supply. It is essential for effective research work in this climate that working spaces, including the animal quarters, be ventilated and cooled. There is also the requirement for the operation of Laboratory equipment that a stable source of electric power is available. Surveys of the needs have been made over the past few years on more than one occasion, but little has been done in a constructive way. It is recommended that provision of an efficient and stable power supply be given a high priority in the plans for the immediate future.

Similarly, provision of an adequate supply of hot water is long overdue. We recommend, therefore, that this be given immediate attention.

There are other problems of facilities which affect the research program that require early attention, such as a waste disposal system, especially an incinerator for the Animal House, and better transport for patients and supplies, both in Dacca and Matlab and between these places. However, the Technical Committee agrees with the Director and the Deputy Director that the first three sets of items (housing requirements, electric power and hot water) in that order, should receive priority.

Despite the curtailment of research potential caused by inadequate facilities, excellent progress has been made. In large measure this has been due to the high morale of the staff and is a tribute to Dr. Phillips and Dr. Hare.

One of the most significant scientific and public health achievements has been the demonstration of the effectiveness of oral therapy of cholera, both in the ward at PSCRL and subsequently at the cottage hospital in the Matlab Rural Health Center. This development augurs well for the wide extension of effective therapy through areas of this and other countries afflicted by cholera and at a much more modest cost in terms of personnel and material than present methods which rely almost exclusively on parenteral administration of water and electrolytes.

It is recommended that training in clinical management of cholera be offered to about 100 Pakistani doctors and nurses during the next year. They should attend for instruction in groups of two or three, or in whatever numbers may be convenient.

In the difficult task of evaluating antibacterial vaccines in natural conditions, the early results obtained during the present severe epidemic on the protective effect of the purified Inaba lipopolysaccharide antigen and the monovalent Inaba whole cell bacterial vaccine are encouraging. It seems likely that research has now reached the point where purified antigens can be employed, hopefully in adjuvant-containing preparations, so that the immune response can be heightened and prolonged.

The Technical Committee is impressed with the increasing evidence for the role of an exotoxin of the cholera vibrios in the production of the severe diarrhea which is the chief manifestation of this disease. The Committee recommends that work on the diarrhea-producing toxin be pushed forward with all possible vigor both in PSCRL and in laboratories in the United States and elsewhere. The aims are to elucidate the action of the toxin in changing the permeability of the gut and to determine whether antitoxic immunity will prevent these manifestations of the disease. If the latter is the case, which seems likely, the use of a toxoid, alone or in combination with bacterial antigens, should be considered for human immunization.

In our report FY 67, the Technical Committee noted that future planning for PSCRL should be undertaken on a long term basis of at least ten years. We see no reason to modify this estimate at this time, although we recognize that the pace of scientific advance is increasing rapidly with each passing year. There is, however, an inevitable time lag between discovery and application. It is to be hoped that this lag can be shortened, especially on the immunological side.

EPIDEMIOLOGY SECTION

1. Vaccine Field Evaluations

During the 1967-68 season the incidence of cholera in the vaccine field trial area in Matlab Thana was too low to permit a definitive test. That such "lean" years might be expected has long been recognised and justifies the necessity for planning the development and evaluation of cholera vaccine over a 10-year period or longer.

During the 1968-69 season, cholera has become epidemic. The incidence in the expanded study populations is already sufficient to ensure statistically significant results. In this new test populations it is highly probable that definitive answers will be provided regarding the effectiveness of monovalent whole cell Inaba vaccine and the purified Inaba vaccine. It is also likely that the presence or absence of heterologous cross immunity of monovalent Ogawa whole cell vaccine will be tested. Unless the incidence of Ogawa cholera increases, there will be no opportunity to test protection against this serotype. In the old test areas an excellent evaluation of the duration of effectiveness of standard commercial vaccine with annual booster inoculations can be anticipated.

During the 1969-70 season, it is recommended that consideration should be given to booster inoculations of homologous monovalent whole bacterial vaccines be given to the two test groups that received these vaccines in the fall of 1968. The group which received the purified Inaba vaccine should be observed without additional antigenic stimulus in order to determine duration of immunity. The control group should be continued.

A special problem is presented by the old test area which has now been under observation for three consecutive years. It will not be a favourable area in which to test a new vaccine in 1969-70, but by the season of 1970-71, it is estimated that approximately 20,000 children under 5 years of age will have been added into the population through new births. This susceptible group will be an ideal one for a controlled study. Certain types of new vaccines, for example, a toxoid, might be tested on all age groups.

It is recommended, therefore, that immediate steps be taken to ensure the availability of new vaccines for a major field test during the season of 1970-71. The two types of vaccine which now seem most promising are :

1) A bivalent vaccine containing purified Ogawa and Inaba bacterial antigens possibly with and appropriate adjuvant.

2) A toxoid.

2. Serological Studies

The microtiter vibriocidal test continues to be a most valuable tool for epidemiological studies. During the 1967-68 season the low incidence of cholera served to verify the specificity of this technique. Essentially no antibody responses were detected in the extensive population studies conducted in Dacca and Matlab indicating that nonspecific stimuli of vibriocidal antibody were not occuring .

In accordance with the recommendations of the Technical Committee, December 1967, plans have been developed for " a series of carefully selected immunity surveys in representative areas of Pakistan, other SEATO countries, the Middle East, and elsewhere". In view of the heavy demands on the serological laboratory resulting from the present cholera epidemic, it is recommended that these planned serological surveys be postponed until a later date.

Quantitative studies of the neutralization of various lots of cholera toxin have been undertaken using rabbit loops and the skin test. Some evidence supports the view that these two assay systems measure the same antigen-antibody reaction. If further experiments confirm this conclusion, the rabbit skin test will become a valuable tool for further research study, both for characterizing and purifying the cholera toxin and for studying the prevalence of antitoxin in the population. It is recommended that these studies be pursued with high priority, both in the laboratory and the field.

3. Field Epidemiology

The studies of inapparent infection with the cholera vibrio have been greatly expanded in the Matlab study areas. Monthly blood specimens were collected from a systematic sample of 1,000 children in the study area during the "lean" season of 1967-68. During the present epidemic, monthly blood specimens, plus biweekly rectal swabs are being collected from a fresh sample of 1,000 children. These studies should clearly define the frequency with which inapparent infection occurs and the relative value of rectal swabs and repeated serum antibody tests for detecting such infections. It is recommended that these epidemiological tools continue to be used and sharpened for appropriate future field studies.

The recognition of El Tor Cholera in Chittagong during the summer of 1968 led to a number of limited field studies which revealed the much more widespread occurrence of this infection than had been realized. In the fall of 1968 classical cholera also appeared in the city while the field studies were underway. Thus a unique opportunity has been presented for a descriptive field study of these two biotypes of cholera occurring in the same city concurrently. The co-operation of the District Health authorities and the faculty of the Chittagong Medical College has been excellent.

A study of past records in Chittagong suggests that cholera reoccurs annually with a peak incidence in the late winter and spring months - more compatible with the pattern of Calcutta than of Dacca. It is recommended that the limited field studies in Chittagong be continued to their logical conclusion commensurate with other priorities and obligations of PSCRL. It is quite possible that Chittagong may provide a source of cases for clinical and epidemiological study at times when such cases may not be accessible in Dacca or Matlab.

4. Demographic Studies

The vaccine study population in Matlab has grown to include a total of 230,000 persons who are kept under close scrutiny in order to ensure that no cases of cholera occur without receiving the benefit of modern treatment. As a pure by-product of the vaccine trials, the population data are of considerable demographic interest such as :

1) Descriptive demography. The birth and death rates and in and out migration rates are determined with precision. A marked seasonal variation in the birth rate has been observed. Age specific fatality rates can be extracted by simple machine tabulation from the already punched vaccine records.

2) Maternal mortality. The PSCRL collaborated with the East Pakistan Research and Evaluation Center by making accessible records of all deaths among women in the child-bearing ages. These were subjected to a follow-up field investigation by an obstetrician assigned from EPREC. A maternal mortality of 7 per 1,000 was determined. This was considerably lower than comparable rates based on hospitalized patients. Eclampsia was the commonest cause of death among primiparas, but rarely occurred among multiparas.

It is recommended, provided the studies have the full support of the Government of Pakistan and AID/Pakistan, that the PSCRL continue to collaborate with appropriate agencies in the conduct of studies related to demography and reproductive physiology and provided also that they in no way limit or restrict the primary mission of the Laboratory.

5. Organization

In the Technical Committee Report of December 1967, a recommendation was made that " at least one additional epidemiologist" be added to the staff of PSCRL. In view of the highly productive epidemiology program and the many opportunities for effective contributions to the control of cholera that must be passed up for want of sufficient staff, it is recommended that, if possible, this additional epidemiologist be made available by the summer of 1969.

ANIMAL RESOURCES SECTION

Animal House

Although considerably improved in the last two years, the facilities are still poor and cannot fulfil the need for a reasonable supply of animals of high quality for the many experimental programs. As was stated in our report for FY 67, "the present building, already overstrained, needs improvement in lighting, ventilation, vermin-proofing, sterilization, arrangements for preparing animal food, and the supply of cages for animals." To this list, we should add hot water for washing cages, air conditioning and an incinerator for dead animals.

When the godown to house supplies and maintenance is completed, some space in the Animal House will be released. When this occurs, steps should be taken promptly to put the Animal House in order. Failure to do this will continue the large waste of money and the time of scientists long known to be caused by poor animals in short supply.

The animal house provides all the mice that are needed, and an adequate supply of rats is on hand. There is a shortage of rabbits, because of a shortage of cages and racks. The rabbits are affected by coccidiosis, which presumably can be got rid of only by disposing of the entire rabbit stock and providing rust-free racks and cages which could be properly cleaned, and by rendering the building vermin-proof. Food preparation would also have to be controlled.

Cage washing is a serious problem without hot water and unless cages can be well cleaned it is impossible to establish the needed hygienic conditions.

Purification of Enterotoxin

It is strongly recommended that plans to purify the toxin from stools be abandoned. This is an exceptionally difficult project and in any case the Committee is not convinced of its desirability.

Preservation of Toxin-Containing Stools

This is desirable but because the properties decline on standing, perhaps due to the presence of proteolytic enzymes, freeze-drying on a fairly large scale should be considered.

The Toxin-Inhibiting Factor From Rat Intestinal Washes

This is a potentially important phenomenon and needs confirmation. It is also important to confirm the presence of an inhibitor in normal serum, to survey several species of animals, to determine whether the inhibitor in intestinal washings and serum is the same and to compare the relative potencies.

Assay of Toxin

The first priority is the confirmation and extension of the work reported by Dr. Mosley in which it was shown by titrations of different samples of toxin against different samples of antisera that the same end points were obtained whether the intestinal loop or the skin test was used as an indicator of the presence of toxin. If the skin toxin and the "loop" toxin are the same, the use of the loop assay can be reduced for toxin assay purposes. All toxin assays should be done by titrating toxin against antitoxin, using the reaction in the loop or the skin (we hope the latter) as a qualitative indication of toxin excess. Recent studies appear to show that Evans blue may be capable of "neutralising" cholera toxin in the gut assay system (Animal Resources).

Studies on cholera toxins, particularly that active in the intestine (fluid accumulating factor, loop toxin) need to be put on a rational basis at an early date if progress is to be speeded. We recommend that a provisional Antitoxin Unit should be set up with the view to reaching international agreement as soon as possible. Test doses of toxin could then be established and communication between different workers made more coherent. It is not recommended, however, that this be the responsibility of PSCRL.

CLINICAL INVESTIGATION SECTION

In last year's report the long term objectives of this section were listed and it is convenient to consider the progress and the forward program under the same three headings.

1. "The development of an oral regime of therapy which will be at least as effective as the present intravenous one..."

It is a pleasure to note that substantial progress has been made in this direction and that ongoing studies promise still further improvement.

During the current year it has been shown (CI/P4) that the oral regime studied earlier under close laboratory control can be used in the field for treating mild cases without intravenous therapy and when used in conjunction with intravenous therapy can reduce substantially the volumes of intravenous fluid required.

Studies presently in progress which were reported verbally (CI/P4.2) suggest that the addition of glycine to the oral regime may further enhance sodium absorption and this, together with some reduction in Na concentration in the mixture, may reduce stooling rate.

Other studies only very recently commenced, which do not appear in protocols, indicate still further possible-lines of progress. In the first study preliminary observations suggest that kaolin administration can reduce stooling rate in patients and prevent fluid accumulation in animal gut loops challenged with toxin.

2. "The development of a form of treatment which will specifically reverse the disturbance .... (which) would be based on an understanding of the mode of action of cholera toxin"

There has been progress in this field and in particular the year has brought further evidence (CI/P1 and 2) reinforcing earlier studies from this Laboratory which suggest that the principal disturbance is one of transport of Na from the gut lumen to plasma.

This is at variance with animal experiments carried out in other laboratories by different methods which suggest that the primary defect is one of increased movement of Na into the gut lumen.

These differences may be due to non-concordance of the animal model with the disease in man or to various methodological factors.

The matter must still be regarded as sub judice but proposed work shown in CI/P2 (revised) and CI/P28 should resolve certain issues. However, the present approach based as it is on the study of short segments of intestine will have to be supplemented by studies of the whole gastro-intestinal tract if a clear picture is to be obtained.

It has been observed (CI/P5) that the choleric stool in children has a lower content of sodium (90 mEq/L compared with 140 mEq/L). This confirms previous observations and has the practical implication that replacement solutions based on the composition of the adult cholera stool may be inappropriate for use in children. Hypokalemia has indeed been observed in 7 children during treatment with 5:4:1 solution.

Studies are in progress (CI/P31) on the age at which this pattern of stool composition merges into the adult pattern. Preliminary results suggest that the change takes place around puberty but the age factor has not yet been identified as the sole correlate of the change which might equally be related to nutritional status or body size.

With recovery, the electrolyte composition of the stool in children approaches but does not reach the adult composition. This suggests that rate of stooling may be a factor. Studies were proposed (CI/P30) to explore this factor by observing the stooling rate and electrolyte composition of stools in children developing cholera while under observation as family contacts of established cases. It was also proposed (CI/P5) to study the effect of dehydration on gut volume and stool composition by allowing children with cholera, after initial rehydration, to become once more dehydrated to a limited extent (specific gravity of plasma approaching 1.032) while observations were made. This proposal was criticised and it was suggested that similar information might be obtained by other means such as:

a. Bulk infusion into the caecum of volunteers.
b. Further analysis of existing data (CI/P5).

A further approach under consideration is the use of spironolactone to see whether the high K/Na ratio of the child's stool in cholera is related to aldosterone activity.

Other studies are in progress on gastric acidity in patients with cholera and in family contacts (CI/P37). Regulation of the osmolality of intestinal contents in the dog (CI/P29) is also under investigation.

3. "Information on the mechanisms of immunity..."

The role of humoral immunity in cholera is far from clear. Nevertheless it may be assumed to be involved in some way, for the current vaccine trials have shown significant albeit short-lived protection.

Further evidence on immunity is being sought in the intestinal contents and in particular the presence of immunoglobulins in this site (CI/P9-13 ((1967)) now incorporated in CI/P8 ((1968))). Despite the difficulties arising from proteolysis in the intestine it has been shown that IgA is regularly present in cholera stool and jejunal aspirates. Moreover, although with more difficulty, IgG and IgM have also been demonstrated.

Ongoing studies in the 1968 protocols using more refined immunological methods should throw more light on this difficult problem.

4. Miscellaneous

Other studies designed to elucidate specific problems include:

CI P20. Studies of Na acetate as a replacement for bicarbonate or lactate in repair solutions have been carried out and a short publication already submitted. Considerable further data have been and are being collected during this cholera season which will have to be analysed. It would seem, however, that the material is safe and may well have the advantage of producing less overswing to alkalosis when given over prolonged periods than Na bicarbonate-containing solutions.

CI/P36.is a bacteriological study designed to elucidate certain problems in connection with non-cholera diarrhoeas. It came under criticism on technical grounds and it is recommended that it should not be given high priority.

CI/P23 is an important study continued this year in which massive doses of phage were administered to cholera patients. It has shown that phage is capable of massive lowering of vibrio counts in stool and of reducing stooling rate. However it often proved possible to culture vibrio from mucus. Also cessation of phage administration was often followed by a remultiplication of vibrio and a return of diarrhoea. It is now clear that this is not a practicable form of treatment but it may prove to be a useful tool for the study of some aspects of cholera.

The possibility of mounting a long term follow up of recovered cholera patients was discussed.

BACTERIOLOGY SECTION

The Bacteriology Laboratory provides support and services but does not initiate investigations. Unless an experienced investigator is found who would be willing to give two or more years' service in the Laboratory, this position will have to be accepted, but if the following five points were also accepted it would enable and encourage the Laboratory to provide better support and service.

1. Physical alterations of structure and layout to provide a better work flow should continue to proceed according to plans drawn up with the advice of Dr. Furniss, a visiting Consultant from the Public Health Laboratory Service, Maidstone Kent, who has been at the Laboratory September to December 1968. This work would need to be put under the personal care and responsibility of some senior person - either a member of staff or visiting consultant. This will implement recommendations in the Report of the Technical Committee of November 1967.

2. Procedures for examining and reporting routine specimens should be adopted according to the scheme outlined by Dr. Furniss in agreement with Dr. Phillips and with the staff of the Laboratory and those who use it. This will eliminate some work of little value, upgrade much other work, save time, improve the sense of reality, and remove the temptation to reduce standards in order to meet excessive requirements.

3. Investigations and projects requiring Laboratory support should not be initiated without discussion and agreement among all concerned, including Dr. Phillips and the Laboratory staff. The Laboratory staff need to be protected from their own desire to acquiesce with all requests made to them. They cannot meet all the requests and maintain standards. When requests need critical assessment, visiting consultants should be asked for advice. The present work load is running at 1,400 specimens a day. This about twice the normal work load (which is still very great) and about half the work load to be expected at the height of an epidemic.

4. Visiting consultants should be encouraged to maintain contacts with the Laboratory by periodic visits, by receiving workers from Dacca for acquisition of particular techniques, and by exchanges of material and ideas.

5. The Laboratory staff, with the advice and support of their visiting consultants, should develop certain aspects of their routine to a level where it makes a contribution to new knowledge of cholera. For example:

a. Are freshly isolated cholera vibrios equally toxigenic ? The strain can be characterized biologically at PSCRL and sent to the U.S. or England to measure toxigenicity.

b. What patterns may be identified among vibrios similar to those identified among other organisms by colicines and pyocines ?

c. What shigellas, salmonellas and enteropathogenic *E. coli* are found in non-vibrio cholera ?

d. What patterns of antibiotic resistance are seen among cholera vibrio and other enterobacteria involved in causing urinary tract and other infections in Dacca ?

Such work would raise morale and give the Laboratory staff a much-needed sense of contributing more personally to the investigative effort.

The Committee wishes to commend the staff of the Bacteriology laboratory on its performance under very demanding conditions.

CHEMISTRY SECTION

Although the Chemistry Section has been in existence only a little more than two years, it has grown from a group of a few research and water chemistry technicians into a substantial corps of well-trained men of first class technical ability.

The chemical analyses which support the various clinical investigators are of very high quality. The group is supervised by two men who have the ability to set up new techniques, train a new technicians and critically evaluate results. During the cholera season it becomes difficult to cover both emergency and experimental analyses round-the-clock. The heavy load is part of the excitement and challenge of the cholera season and it is not considered desirable to increase the number of technicians unless the work-load is greatly increased. Fortunately, the preparation of intravenous fluids has now been moved from this section.

The work performed by the Laboratory, outside of the ordinary clinical analyses, depends largely on the demand of the clinical investigators.

The equipment available is adequate. The needs are largely maintenance and spare parts. New laboratory furniture, which was needed, is being installed. The worst defect in the laboratories is the impossibility of keeping them clean during the dry season and dry during the wet season. Correction of these annoyances depends on sealing windows and doors, repairing roofs, and doing something to the rough cement floor and walls (such as tiling).

In summary, the laboratory is doing its job well and has no major needs for the coming year except physical rehabilitation. We wish to congratulate Dr. Ruth Hare on the remarkable improvements that have been made in all aspects of the Chemistry Section's work during the past two years.

SIGNED by members of the
Technical Commitee:

(Signed)

(Dr. Colin M. Macleod)
Chairman

(Signed)

(Dr. Ziaur Rahman

(Signed)

(Dr. James W. Howie)

on 11th December 1968 in Dacca, East Pakistan.

II.

DIRECTOR'S COMMENTS

on

Administrative Program

and

Scientific Program

Director's Comments

on

Administrative Program

While there have been many and marked improvements in the Laboratory administration in FY 1968, much needs to be accomplished. The addition of a trained Maintenance Officer has enabled the Laboratory to set up a preventive maintenance program. The decision by NIAID to assist in setting up a proper supply system and the near completion of new godown which will house Maintenance and Supply are important accomplishments.

However, the existing utilities, as reported to you by the Deputy Director, are still very inadequate and the development of proper housing and breeding colonies for the Animal Resources facility has been much slower than had been hoped.

DIRECTOR'S COMMENTS ON SCIENTIFIC PROGRAM

The Scientific progress of the Laboratory has been, in the view of the Director, showing a most satisfactory growth. This is documented, in part, by the following :

Table A

Publications and Medical Meeting Presentations

Fiscal Year	Publications			Presentations at Scientific Meetings	Total
	Published	In Press	Accepted		
1961	2	-	-	-	2
1962	2	-	-	-	2
1963	5	-	-	-	5
1964	6	-	-	1	7
1965	20	-	-	9	29
1966	10	-	-	9	19
1967	13*	-	-	38	51+
1968	23*	13	5	17	58+

* This figure excludes the later publication of papers presented at meetings if copies are not received and/or verified.

Among the factors responsible for the developing scientific ability of the Laboratory is the increased sophistication of the vaccine trial program. This program now has nearly a quarter million people in rural Pakistan under daily house surveillance and is also supporting clinical investigation studies in their treatment centers, epidemiological studies in the vaccine trial population and some of the best demographic information available today on rural populations.

The achievements of the Epidemiology Section are due in part to the presence of a Section Chief who has now been in the Laboratory for nearly four years and the increased support which has been given the program during the epidemic seasons by the National Communicable Diseases Center and NIAID.

The Laboratory also has shown its capability by conducting studies on cholera patients in the Cox's Bazar area, some 300 miles South of Dacca during the FY 1968 season when cholera patients were not available on the Dacca ward.

A further factor has been the continued excellence of the Laboratory techniques by the two support laboratories, Bacteriology and Chemistry. This excellence has enabled them to provide accurate analyses despite excessive demands on these two laboratories.

The advice provided by our consultants, both official and non-official, has been most helpful, particularly in developing programs.

Finally should be mentioned not only the ability but the loyalty and devotion of the entire Laboratory staff.

III.

SECTION CHIEFS' and CONSULTANTS' REPORTS

and

PROTOCOLS

ADMINISTRATIVE REPORT

Fiscal Year 1968

(July 1, 1967 - June 30, 1968)

Introduction:

The Administrative Report for the fiscal year 1968 will stress the importance of providing proper administrative support to the research of the Laboratory. This "theme" was also the focal point in the Administrative Report for fiscal year 1967 in which it was stated that "whether or not the objectives of the research programs of this Laboratory will be met on schedule will, to a considerable degree, be dependent on the quality of the administrative support available to those programs". However, despite the fact that the Laboratory continued to expand during fiscal year 1968 in terms of personnel and research activities, the Laboratory also continued to do with inadequate supportive staff. Specifically, what is needed in terms of staff in administration, are properly qualified NIH-assigned administrative specialists atleast in the areas of personnel, finance and supply. These three areas are vital to the operation of the Laboratory. At the present time one NIH-assigned staff is allocated to not only administer these three areas but also transportation and general services, and in addition has to provide staff support to the Director. Not only must this individual deal with all of the above in day-to-day operations, but also find time to decide problems, some of which are unique, that invariably crop up in an organization of this size operating under adverse conditions.

In other words, if the objective of this Laboratory is first class research then first class support should and must be provided not only in terms of supportive administrative personnel but also in terms of adequate space and proper facilities and utilities.

The rest of this report will deal more specifically with the various areas of administration and items of special interest.

Personnel :

Total staff increased from 576 on July 1,1967, to 616 on June 30,1968 - a smaller increase than during the previous year. Please refer to Table A for a break-down of the staff by section. During fiscal year 1968 two new sections were created - Maintenance and Nursing & Hospital Services. Staff from the Administrative Section were transferred to Maintenance Section and, similarly, staff from Clinical Investigation Section were transferred to Nursing & Hospital Services Section.

It should be noted that of the total staff of 616 as of June 30, 1968, 418 were regularly appointed staff while 198 were of a temporary or seasonal type, such as the dais at Matlab. For comparison, the respective figures as of July 1, 1967, were 380 and 196. Therefore, the main increase in staff actually occurred at headquarters. For example, Animal Resources Section increased its staff from 9 to 23 as a result of increased demand for experimental animals. Administrative Section also increased somewhat even though the new Maintenance Section was transferred from the former. Extra drivers were hired when weekly duty hours of drivers were reduced from 60 to 48; extra positions were also created in Supply.

Table B is a list of Laboratory staff either sent on foreign training during fiscal year 1968 or staff who have not yet returned and who were sent for training prior to fiscal year 1968. Table C is a list of key personnel changes for FY 68.

Physical Facilities :

As mentioned in last year's report, adequate space is still an acute problem. While the Laboratory has taken on new staff, actual space available to the Laboratory has not increased, and furthermore, the probability of acquiring more space in the near future is not hopeful. No progress has been made in implementing the planned new four-storey building since last year's report. No extra space has been made available to the Institutes of Public Health and this Laboratory's combined Library.

However, the Laboratory has been fortunate to plan and finance a one-storey 100' x 80' structure to house both the Maintenance Section workshops and the storage of supplied. As of the date of t is report (November 1,1968) this building is expected to be completed by the first of the new year. As a footnote to the acute shortage of space, the Laboratory was forced to lease storage in the industrial area of Dacca city to store bulk supplies and equipment.

Utilities :

The Laboratory is still lacking in proper utilities. The Laboratory has no central hot water supply and gas for laboratory use. Regarding the improvement of electrical power to the Laboratory, a report is in the process of being prepared by a Dacca consulting firm and is expected to be ready by the end of November, after which bids will be let. The 250 Kw standby electrical generator arrived this summer that will be incorporated in a new substation, as yet incomplete, which will service both the Institute of Public Health and the Laboratory to provide a constant and dependable source of electrical power.

Maintenance :

Since the arrival of the Maintenance Officer from NIH in August, 1967, the quality and scope of maintenance support to the Laboratory has greatly improved despite a paucity of proper maintenance facilities and tools. Proper tools and maintenance and replacement supplies have been ordered from NIH, and some of these have been received. However, the Maintenance Section will not be functioning at optimum level until it moves into its new quarters when the new one-storey maintenance and supply building is completed and when additional requested supplies and shop equipment have been received. Additional staff will be necessary when the move to the new quarters is made.

Aside from routine maintenance and preventive maintenance, the Maintenance Officer and his staff have undertaken or have supervised contractors' work for the following projects :

1. Major repairs and renovations of existing facilities.
2. Renovation of animal quarters.
3. Supervision of plans and construction for the new Maintenance and Supply Branch.
4. Designed, contracted and supervised the glass enclosure of all verandas.
5. Assisted in design and supervised complete renovation of Bacteriology Section.
6. Installed two walk-in cold rooms and one walk-in incubator obtained from the National Institutes of Health on surplus.
7. Assisted local electric contractors in planning modern electrical power supply for the Laboratory.

Transportation:

The Laboratory is currently operating 22 vehicles including the Director's and Deputy Director's personal vehicles on loan to the Laboratory. The Laboratory received three new jeeps donated by the Australian Government during fiscal year 1968 in September of this year. A Microbus ambulance is expected shortly from the same source.

Despite these welcomed additions to the Laboratory fleet of vehicles, a glance at Table D will reveal that the majority of vehicles have very high mileage, and as a result 5 to 7

vehicles will be out of commission at any one time due to breakdowns.

Obviously, most of these vehicles need replacing. It is strongly suggested that a request be made to the Australian Government to make a similar contribution of vehicles for fiscal year 1969 as they did in 1968.

Due to the increased area of surveillance in Matlab four new Boston Whaler outboards and ten new outboard engines (including replacements) were ordered in fiscal year 1968 and have now been received and put into service.

Communications:

As of November 1, 1968, the Laboratory has not received the wireless set from Australia, although the Laboratory did finally receive the license to operate the set effective December 1967, from the Government of Pakistan. The wireless set was originally offered to improve communications between headquarters in Dacca and the Matlab field station.

Supplies and Equipment:

The Laboratory was very fortunate to receive from the Central Board of Revenue, Government of Pakistan, an order exempting it from all custom and import duties on all supplies and equipment received from donating countries. Furthermore, the exemption was made retroactive to the inception of the Laboratory. This action overcame a problem that for a long time had been troubling the administrative staff.

Improvements are being made to streamline supply operations by initiating stock control on expendable supplies and setting up appropriate re-order levels.

TABLE 'A'

BREAKDOWN OF PSCRL PERSONNEL BY SECTION

	A.S.	A.R.S.	B.S.	C.I.S. R.	C.I.S. CRL W.	C.S.	D.O.	E.S. HQ	E.S. M VTS	M.S.	N&H S.S.	C.	Total
July 1, 1967													
Local	92	9	32	33	45	23	2	37	286	-	-	3	562
Foreign	-	-	1	3	1	1	4	4	-	-	-	-	14
June 30, 1968													
Local	95	23	35	37	-**	32	2	38	279	17*	42**	3	603
Foreign	-	-	-	3	-**	1	4	3	-	1	1**	-	13

* The Maintenance Section was organized on October 2, 1967 with staff transferred from the Administrative Section.

** The former CRL Ward under Clinical Investigation Section was organized on Feb. 21, 1968 as Nursing & Hospital Services Section.

Key:

A.S. Administrative Section
A.R.S. Animal Resources Section
B.S. Bacteriology Section
C.I.S. Clinical Investigation Section
R. Research
CRL W. CRL Ward
C.S. Chemistry Section
D.O. Director's Office
E.S. Epidemiology Section
M.S. Maintenance Section
N&HS.S. Nursing & Hospital Services Section
C. Consultant
HQ Headquarters
M.VTS Matlab Vaccine Trial Surveillance

TABLE 'B'

FOREIGN TRAINING DURING FISCAL YEAR 1967 - 1968

	Name & Designation	Date Departed	Date Returned or Expected Date of Return	Purpose of Training	Place of Training	Remarks
1.	Dr. A.K.M. Jamiul Alam Physician	Jan. 7, 1966	Sept. 9,1968	Higher training in medicine and gastroenterology	Birmingham General Hospital(British Post-Graduate Medical Federation), Birmingham, U.K.	
2.	Dr. (Mrs.) K.M. Ally Physician	June 1966	-	Higher training at Bellevue Hospital, Univ. of Columbia New York, N.Y., U.S.A. and various hospitals in U.K.	Dept. of Medicine, College of Physicians and Surgeons, Univ. of Columbia New York, N.Y., U.S.A. and U.K.	Staying on her own expense. Released from PSCRL on Feb. 18,1968.
3.	Dr. (Mrs.) Rezia Akbar Physician	Feb. 18, 1967	-	Post-graduate Student in Public Health, Univ. of Calif., Los Angeles, Calif., U.S.A.	Univ. of California, Los Angeles, Calif., U.S.A.	Continuing on her own. Released from PSCRL on Feb. 18,1968

TABLE 'B' (CONTD)

	Name & Designation	Date Departed	Date Returned or Expected Date of Return	Purpose of Training	Place of Training	Remarks
4.	Mr. Yousuf A. Saeed Sr. Research Asstt.	Oct. 12, 1966	Nov. 1968	Training in Bacterial Genetics	Dept. of Biology, Univ.of Calgary, Alberta, Canada	Training period extended at his own expense for one year from Nov. 22, 1967
5.	Dr. R. K. Barui Physician	June 21, 1968	Oct.1,1968	Epidemic Intelligence Service Officer's Training course in Epid.	National Communicable Diseases Centre, Epid. Program, Atlanta, Georgia 30333, U.S.A.	
6.	Mr. M. Sobhani Electronic Specialist	May 13, 1968	June 26,1968	Training on Xerox Copier	Rank Xerox Central Training School, London	

TABLE 'C'

KEY PERSONNEL CHANGES DURING FY 68

	Name & Designation	Section	Date of Joining	Date of Departure
1.	Dr. James O. Taylor Chief	Clinical Inv.	July 12, 1965	May 15, 1968
2.	Dr. W. McCormack Research Physician	Epidemiology	June 10,1966	May 22, 1968
3.	Miss. Marian A. Smith Private Secretary	Director's Office	June 24, 1966	March 1, 1968
4.	Dr. Joseph L. Kinzie Research Physician	Clinical Inv.	Sept. 18, 1966	May 1, 1968
5.	Dr. Albert R. Martin Research Physician	Epidemiology	Dec. 25, 1966	May 1, 1968
6.	Mr. Nuran Nabi Administrative Officer	Administrative	July 1, 1967	-
7.	Mr. Mark P. Tucker Chief	Maintenance	Aug. 8, 1967	-
8.	Dr. David R. Nalin Research Physician	Clinical Inv.	Aug. 13, 1967	-
9.	Dr. Richard A. Cash Research Physician	Clinical Inv.	Aug. 17, 1967	-

TABLE 'C' (CONTD)

	Name & Designation	Section	Date of Joining	Date of Departure
10.	Mrs. Constance G. Miccio Private Secretary	Director's Office	April 13, 1968	-
11.	Dr. William E. Woodward Research Physician	Epidemiology	May 2, 1968	-
12.	Dr. Kenneth J. Bart Research Physician	Epidemiology	June 1, 1968	-
13.	Dr. Jon E. Rohde Research Physician	Clinical Inv.	Aug. 15, 1968	-

TABLE 'D'

STATUS OF P-SCRL VEHICLES

November 1, 1968

	MAKE	MODEL	LOCAL REGISTRATION NUMBER	MILEAGE AS OF DECEMBER 1, 1967	MILEAGE AS OF NOVEMBER 1, 1968
1.	Plymouth	1964	7156	61,006	80,368
2.	Wagoneer	1967	7449	9,000	34,570
3.	Wagoneer	1967	7450	8,447	30,433*
4.	Austin 850	1963	6033	81,312	99,699
5.	Austin 850	1963	6034	60,009	78,114**
6.	Austin 850	1964	7026	70,480	85,360**
7.	Austin 850	1964	7027	60,150	70,544*
8.	Austin 1100	1963	6032	64,065	88,216**
9.	Austin 1100	1966	4677	50,726	65,918**
10.	Austin 1100	1966	4678	- ***	68,820
11.	Austin 1100	1967	7198	18,475	51,017
12.	Austin 1100	1967	7199	21,125	59,904

TABLE 'D' (CONTD.)

MAKE	MODEL	LOCAL REGISTRATION NUMBER	MILEAGE AS OF DECEMBER 1, 1967	MILEAGE AS OF NOVEMBER 1, 1968
13. Jeep Station Wagon	1964	8721	81,071	100,307
14. Dodge Ambulance	1962	2003	41,256	60,000*
15. Land Rover	1962	2781	67,523	76,523*
16. Land Rover	1962	2899	45,858	60,229**
17. Chevrolet Carryall	1961	8978	60,148	79,453*
18. Australian Willys Jeep	1968	474	-	263**
19. Australian Willys Jeep	1968	475	-	1,974
20. Australian Willys Jeep	1968	482	-	1,551
21. Mercedes Benz	1962	5569	87,500	104,934 Km**
22. Dodge Sedan	1964	3423	30,500	41,079

*Estimated mileage; speedometer out of order.
**Under repair.
***Speedometer out of order.

TABLE 'E'

STATUS OF P-SCRL BOATS & OUTBOARD MOTORS

NOVEMBER 1, 1968

BOATS:

	TYPE & LENGTH	QUANTITY	SOURCE	YEAR PURCHASED	CONDITION
*	Boston Whaler 13½'	3	U.S.	1966	Good
*	Boston Whaler 13½'	4	U.S.	1968	New
**	Sears Boat 14'	1	U.S.	1965	Poor
**	Sears Boat 14'	1	U.S.	1964	Poor
**	Sears Boat 10'	1	U.S.	1964	Good
**	Sears Boat 10'	1	U.S.	1963	Good
*	Dowty Jet Boat 14½'	1	U.K.	1966	Poor
*	Pakbay Boat 12¾'	1	Local	1963	Poor
*	Pakbay Boat 12¾'	2	Local	1964	Poor
*	Pakbay Boat 12¾'	1	Local	1966	Poor
	TOTAL:	16			

OUTBOARD MOTORS:

MAKE & HP				
Johnson, 5½	1	Local	1963	Poor
Johnson, 7½	2	U.S.	1964	Poor
Johnson, 9½	4	U.S.	1968	Good
West Bend, 18	1	Local	1963	Poor
Johnson, 28	1	U.S.	1964	Poor
Johnson, 28	1	Local	1964	Poor
Johnson, 33	1	U.S.	1964	Good
Johnson, 33	9	U.S.	1966	Good
Johnson, 33	8	U.S.	1968	Good
Evinrude, 40	1	Local	1966	Good
Total:	29			

*Fiberglas

**Aluminum

ANIMAL RESOURCES SECTION REPORT FY'68

Animal Resources became a separate section in May 1967. The research staff which has Dr. K.M.S. Aziz as a Guest Investigator remained in the Clinical Research Section for some time. In January 1968 the research staff was transferred to the Animal Resources Section. The section is now comprised of a staff of 26 persons.

Research with experimental animals increased in FY'68. Animals are needed for antitoxin titration of samples obtained from humans. Aside from this aspect of epidemiological studies, many immunological studies require experimental animals. There are inadequacies in the described techniques of bidirectional flux studies in humans; such techniques can and should be perfected in experimental animals. Succus entericus from the rat intestine has been found, in this Laboratory, to inhibit toxin activity; animals are required for the continuation of these studies. Since there is no cholera during 6 months or more of the year, the use of animal models will make the Laboratory much more productive.

The following report is divided into two parts - Part I concerns Laboratory animal caretaking and Part II concerns research.

Part I. Laboratory Animal Caretaking:

Over the period of last year research with Laboratory animals has greatly increased. There has been general improvement of facilities in the animal house.

The following is a breakdown of staff involved in Laboratory animal caretaking.

2 veterinarians
1 animal caretaker
3 assistant animal caretakers
9 animal handlers

Out of 12,464 animals 6,314 animals have been used by various sections (Table I). The total number of animals have been doubled over FY'67 and have increased ten-fold over FY'66.

New research projects have been started on dogs and rats by Drs. Hare, Cash, Nalin, Rohde and Aziz. Major expansion has been planned for dog and rat colonies. There is a growing demand for rabbits, rats, mice and dogs. To meet the investigators' needs for quality Laboratory animals, controlled environment is necessary. Sterilizing facilities and oven are needed for food preparation and sterilization of equipments. Better quality cages and racks are needed for improved caretaking.

Part II - Research:

There are 11 members of the technical staff of 26 involved in research, one of which is engaged full time for Drs. Nalin and Cash. Several others are engaged part-time in animal caretaking.

1 research associate
1 veterinarian
1 research assistant
1 research technician
1 animal caretaker
1 assistant animal caretaker
2 technicians
2 Laboratory attendants

Dr. R.A. Phillips and Dr. W.K. Hare have taken an active part in the research program of this section. Dr. W.E. van Heyningen visited this Laboratory in February 1967 to review the research carried out in this section and later made several recommendations.

The following is a report of studies completed in 1967-68:

It was determined that the fluid accumulation factor (FAF) was produced by Vibrio cholerae Inaba in peptone culture broth as early as 4 hours. Total amount of toxin produced at 8 hours was not significantly different from 18 hours, in a shake culture method at 30°C ± 1°C (Table II). This study was carried out according to recommendations made by Dr. van Heyningen.

An attempt was made to determine the number bluing doses (expressed as BD_7 for units of permeability factor) that would be needed for the production of positive ileal loops in adult rabbits (Table III). On the one hand 100 BD_7 and 1.5 BD_7 of culture 569B were enough to produce positive ileal loops; on the other hand 60 BD_7 and 200 BD_7 of culture 35A failed to produce any positive loops. Similar observations made with cholera stool indicate that may not be any direct correlation between the permeability factor and the fluid accumulation factor.

A glycerinated standard preparation containing the fluid accumulation factor was prepared according to the recommendations made by Dr. van Heyningen. For this purpose 12-hour culture filtrates of Vibrio cholerae Inaba 569B were centrifuged, pooled and millipore sterilized. The crude filtrate thus obtained was concentrated 100-fold by ultrafiltration through dialysing tubes. Glycerine was added to this concentrated product in a 2:1 ratio. The FAF contained in this preparation was stored in the refrigerator at 4°C for four months without measurable loss of potency.

The possibility of developing the rat as a model for the detection of cholera toxin was studied. The initial results of rat intestinal loop experiments were erratic until it was noted that both succus entericus and feces would nullify FAF activity. Finally the following procedure was developed. Forty-day-old rats were given water and Nabisco biscuits for 72 hours. During the next 72 hours they were given a 5% solution of glucose in water ad libitum. Laparotomy was then performed under ether anaesthesia and the lumen of the small intestine was lavaged throughly with 20 ml. of normal saline. Two loops measuring approximately 10 cm. were then prepared, starting about 2 cm. above the caecum, with a 1-2 cm. interloop between the study loops. The diluted glycerinated filtrate or the diluent was then injected into one end of the loop in 0.5 ml. volume and the injected site ligated. The intestine was then put back into the peritoneal cavity, the wound was closed with sutures and the animal allowed to recover from the anaesthetic. Water was supplied ad libitum during the post operative period. After eight hours the animal was sacrificed by excessive ether anaesthsia. The peritoneal cavity was opened, the loops were examined, the fluid accumulated in the loops and the loop lengths were measured. It can be seen (Table IV) that of 96 loops in 48 rats only one loop recorded a false positive and there were no false negatives. An average of 3 ml. fluid accumulated in an average loop length of 12.8 cm. which had received the equivalent of 0.05 ml. of the original unconcentrated filtrate.

At present we are carrying out experiments in five loops in the small intestine of 3-month-old rats. Plans are in progress to develop the rat model to carry out reliable assay of cholera toxin.

It was established that rat feces or saline washings of the small bowel for the rat (RIW) would render filtrates containing FAF inactive. Studies were made in rabbit ileal loops using the technique of Burrows and Musteikis except that the loops were harvested at eight instead of eighteen hours. One ml. of a suitable dilution of the conventional glycerinated filtrate in buffer was mixed with either 1 ml. of unheated RIW or 1 ml. of RIW which had been heated for thirty minutes at 56°C. It can be seen (Table V) that both heated and unheated RIW are effective in nullifying FAF activity. Table VI shows that inhibitory activity is retained by RIW in the filtrate after millipore filtration, freezing and thawing.

In a few preliminary experiments rat serum was found to be able to neutralize FAF. When .01 ml. of rat serum was mixed with .01 ml. of Wyeth toxin, the FAF in Wyeth toxin was neutralized.

Germ-free rats were found to contain the FAF inactivating factor. This indicates that FAF inactivating factor in rat intestine is not produced by the micro-organisms that reside in the

intestine. This study was carried out in collaboration with Dr. Carl Miller and Dr. John Feeley at the National Institutes of Health, Bethesda, Maryland (U.S.A.).

In a preliminary study suggested by Dr. van Heyningen it was observed that RIW was capable in inhibiting the action of the toxin even when the toxin was introduced in ligated ileal loops of adult rabbits 3 minutes before RIW was injected in the same loops.

The discovery of a neutralizing factor which can inactivate the fluid accumulation factor even when the rat intestinal wash is added 3 minutes after the toxin administra ion, in a ligated ileal loop in rabbit, may add significantly in the progress of cholera research.

TABLE I

NUMBER OF DIFFERENT ANIMALS PRODUCED AND USED IN FY'68

Species	No. of animals produced	No. of animals used	Remarks
Rabbit	1,976	1,248	
Guinea pig	347	103	
Mice	8,711	4,096	
Rat	1,311	771	
Sheep	28	6	
Dog	55	48	Procured locally
Monkey	23	23	" "
Goat	9	9	" "
Poultry	10	10	" "
TOTAL	12,464	6,314	

TABLE II

TOXIN PRODUCTION AT DIFFERENT HOURS

Ligated ileal loop assay in adult rabbits.
Average of 3 rabbits per sample.

Hours of Culture	Vibrio count Direct	Volume to length ratio with			
		1 ml.	10^{-1} ml	10^{-2} ml	10^{-3} ml
4	6.2×10^{7}	0.31	0	0	-
6	3.2×10^{8}	1.39	0.99	0.27	-
8	5.3×10^{8}	-	1.51	1.06	0
10	7.2×10^{8}	-	1.52	0.97	0.55
12	8.5×10^{9}	-	1.64	0.82	0
14	1.0×10^{10}	-	1.72	1.09	0.26
16	1.1×10^{10}	-	1.44	0.86	0.43
18	1.2×10^{10}	-	1.58	1.21	0.27

TABLE III

A COMPARISON OF SKIN PERMEABILITY FACTOR AND FLUID ACCUMULATION FACTOR

Volume / Length Average of 4 loops in 4 rabbits (ileal loop assay)

BD_7 Average of 4 spots in 2 rabbits (intradermal assay)

Batch No.	Vibrio cholerae strain	BD_7 in the amount injected in loops	Volume to length ratio
87/3	569B	100	1.08
87/3	35A	60	0
88/3	569B	1.5	0.67
88/3	35A	220	0

TABLE IV

RAT MODEL FOR THE ASSAY OF CHOLERA TOXIN

0.05 ml. toxin injected in 0.5 ml total volume

Average of 8 rats in each set.

	Buffer		Toxin 0.05 ml. in 0.5 ml total volume	
Rat No.	Average Loop Length cm.	Average Fl. Volume ml.	Average Loop Length cm	Average Fluid Volume ml.
1 to 8	9.8	0	11.8	2.8
9 - 16	10.0	0.1	11.5	2.5
17 - 24	10.4	0	13.7	3.1
25 - 32	9.5	0	14.0	3.0
33 - 40	10.5	0	11.6	2.8
41 - 48	10.5	0	14.2	3.9
Average of 48	10.1	0	12.8	3.0

TABLE V

TOXIN NEUTRALIZATION BY RAT INTESTINAL WASH FLUID

Ligated ileal loop assay in adult rabbits
Volume to length ratio average of 4 loops in 4 rabbits

		Volume to length ratio			
Rabbit No.	Toxin 0.02 ml.	Toxin 0.02 ml.	Unheated + RIWM	Toxin 0.02 ml.	*Heated + RIWM → Buffer
1	1.37		0		0
2	1.00		0.29		0.1
3	0.68		0		0
4	0.54		0		0

*Heated for 56°C for 30 minutes.

RIWM = Millipore sterilized rat intestinal wash.

TABLE VI

NEUTRALIZATION OF TOXIN BY CENTRIFUGED AND MILLIPORE STERILIZED RAT INTESTINAL WASH FLUID

Ligated ileal loop assay in adult rabbits. Volume to length ratio average of 4 loops in 4 rabbits.

	Volume to length ratio			
Rabbit No.	Toxin 0.02 ml.	Toxin 0.02 ml. + RIWM	Toxin 0.02 ml. RIWM Frozen + and Thawed	Buffer
1	0.99	0	0	0
2	1.3	0	0	0
3	1.0	0	0	0
4	1.08	0	0	0

RIWM = Millipore sterilized rat intestinal wash.

P R O T O C O L

PURIFICATION AND PROPERTIES OF TOXIN IN CHOLERA STOOL

by

K.M.S. Aziz, W.K. Hare and R.A. Phillips

Introduction and Specific Aims

Finkelstein and several other investigators (1-6) are working on cholera toxin obtained from pure culture suspension of Vibrio cholerae. Currently little work is being done on the toxin obtained from cholera stool. However, Craig (7-8) worked on the cholera stool and culture filtrates in studying the permeability factor.

The Pakistan-SEATO Cholera Research Laboratory, Dacca is a unique place to study cholera patients and their rice water stools. The identification of the causative agent of the cholera syndrome should obviously begin with the rice water stool. Studies of intestinal lumen aspirates are also planned.

The specific aims of this project are to purify the cholera toxin obtained from cholera stool and determine its properties. It is desirable to know how much toxin is produced in the intestine of cholera patients and what is the pattern of toxin production from the beginning of the disease to the convalescent stage. It is also necessary to know the relationships between the net plasma to lumen flow, vibrio count and toxicity. The effect of vaccination, various antibiotics and enzyme poisons on the toxin product(s) should also be investigated. Ultimately this may lead to better treatment of cholera patients and the development of a more effective vaccine.

Methods of Procedure

Cholera stool and vibrio culture suspension will both be used for the study of the toxin. However, emphasis will be on the study of the toxin obtained from cholera stool. Pure culture of vibrios will be made by strains isolated from the stool under study.

Collection of cholera stool will be made by rectal catheter from cholera patients passing stool at a minimum rate of 300 ml. per hour. The first 2 ml. of the stool will be taken out by a syringe and 1 ml. will be filtered through millipore syringe filter immediately. The other 1 ml. of stool will be sent for immediate viable count. From the agar plates Vibrio cholerae will be

cultured and maintained in pure culture. The rest of the sample will be collected in a 500 ml. Erlenmeyer flask kept in an ice bath. The flask will be marked at 330 ml. and the sample removed when the stool level exceeds 330 ml. 320 ml. of this will be immediately centrifuged at 10,000 r.p.m. for one hour at 0°C. Following centrifugation the supernatant will be filtered through 450 m millipore filter. Prefiltration through microfiber glass will be done to facilitate quick filtration. The filtrate will be stored at - 20°C. Three more such collections of 330 ml. stool will be made from the same patient and processed as described above. All the samples will then be pooled together, out of which one liter will be concentrated by dialysing. Out of the 230 ml. left, 50 ml. will be used for intradermal skin test in rabbits (7), ligated ileal loops in rabbits (9,10) and ligated ileal loops in rats (11). The remainder of the 230 ml. will again be stored at -20°C for future reference. The dialysing will be carried out under vacuum in a special apparatus. A specially made funnel will be fitted in a rubber bung in the mouth of conical sidearm flask. At the lower end of the funnel an 8 mm. diameter dialysing tube, 1-3 meters long, will be attached. The lower end of the dialysing tube will be sealed off and the supernatant will be poured into the funnel. Suction will then be applied and dialysis carried out until 1 liter is reduced to about 10 ml.

This viscous fluid will be tested in all the three animal models for toxicity. 5 ml. of the fluid will be lyophilized and stored at -20°C. The remainder will be passed through DEAE-Cellulose chromatography column. The fractions will be collected in ten test tubes. Each of these will then be tested for toxicity in all three animal models mentioned above. The active fractions will be pooled and dialysed to make 1-2 ml. and molecular sieve filtration (12) will be done by Sephadex G 100 column chromatography to obtain further fractions. These fractions will again be pooled in ten screw-capped tubes and tested for toxicity by the three animal assay techniques. The active fraction(s) will be further purified by disc electrophoresis. This purified active fraction (toxin) will be used to immunize animals. The antisera will be tested in the three animal assay techniques and by Ouchterlony double diffusion agar gel. The ultraviolet absorption spectrum will be determined. The purified product will be concentrated by dialysis, lyophilized and stored at -20°C.

After the cholera epidemic is over the isolated vibrios from the patients' stool will be passed through rabbits. The culture suspension of the rabbit passaged strain will be processed as for rice water stool and experiments will be performed in the same sequence as above.

The above experiments will be carried out on the rice water stools of at least six cholera patients.

After the toxin is purified, its nature and properties may be determined. Molecular weight of the toxin may be determined by molecular sieve filtration (13,14). The molecular weight may also be determined by density gradient separation by ultracentrifugation and the results compared.

After a 95% or more purification of the toxin is achieved, some peptide mapping and structural work may be carried out in this Laboratory. Peptide mapping will be done after tryptic and other enzymatic digestion. The sequence of amino acids will be studied in each peptide, hopefully so that the amino acid sequence of the toxin molecule may be established.

Following Professor van Heyningen's suggestion, cholera culture filtrates were concentrated a hundredfold and two volumes of glycerin were added to one volume of the concentrated filtrate. As indicated above, the fluid accumulation factor appears to be stabilized by this procedure. Studies will be undertaken to determine if the fluid accumulation factor in the cholera stool of man can also be stabilized by this procedure.

Significance of this Research

This research may lead to the discovery of the toxic component(s) in the cholera stool that causes the cholera syndrome. If curative and prophylactic measures are to be improved we must know the *in vivo* vibrio product(s) that cause the disease. Knowledge of the properties of the toxin(s), both physical and chemical, will enable us to carry out further meaningful research.

Facilities Available

The Laboratory is presently being set up. Most chemicals and equipment have been procured. Some equipment is still on order. The animal house of the Pakistan-SEATO Cholera Research Laboratory is presently undergoing improvement to meet the standards which animal experimentation needs. About 600 adult rabbits and 1,200 adult rats will be needed. The following is a list of items of permanent equipment:

Equipment Available in the Laboratory

1. International centrifuge
2. Chromatography column
3. Refrigerator
4. 30°C Incubator
5. Vacuum pump
6. Lyophilizing machine
7. Beckman pH meter
8. Millipore filters
9. Spectrophotometer
10. Model L International ultracentrifuge
11. Fraction collector
12. Disc electrophoresis unit
13. Temperature controlled room (0°-10°C)
14. Temperature controlled room (30°C)

Equipment Needed

1. Deep freeze - 20°C (on order)
2. Paper chromotography apparatus
3. Protein sequenator

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PROTOCOL

STUDIES ON THE INTESTINAL WASH CONTENTS IN VARIOUS ANIMALS AND MAN

K.M.S. Aziz, W.K. Hare, and R.A. Phillips

Introduction and Specific Aims:

It has been observed that rat feces or rat intestinal wash is capable of inactivating the fluid accumulation factor (FAF) in culture filtrates of Vibrio cholerae (1). The aim of this work is to determine whether feces or intestinal contents in several other animals and man contains any toxin inactivating factor.

Methods of Procedure:

Initially only studies with fresh feces of animals and man will be carried out. Feces from sheep, goats, cows, horses, dogs, monkeys and humans will be collected. 10 gms. of the feces of the particular animal will be mixed in 10 ml. of normal saline. This will be centrifuged at 10,000 r.p.m. for 30 minutes and the supernatant poured in an Erlenmeyer flask. 10 ml. more of normal saline would then be added to the sediment, thoroughly mixed and centrifuged again. The supernatant will be added to the supernatant obtained from the first centrifugation. The pH of this fluid will be determined at this time, one ml of this fluid will be mixed with 1 ml of buffer containing 0.25 mg. of the Wyeth toxin. This mixture will then be tested in ligated small intestine loops of adult rabbits by a previously established procedure (1). Further studies with any particular animal will be pursued only if the toxin is found to be inactivated in the experiments above.

In case of positive results, intestinal wash contents will be obtained by operating or immediate washing of small intestine available in the slaughter house in Dacca in the case of animals. In the cases of human beings ileal and jejunal aspirates obtained by other investigators from patients not treated with any antibiotics will be taken for study. The centrifuged supernatant will then be mixed with Wyeth toxin and assayed. It may be necessary to concentrate the supernatant by ultrafiltration if unconcentrated washings are ineffective.

Significance of this Research:

Rat intestine contents inactivate cholera toxin but it will take an enormous number of rats to obtain materials to inactivate any appreciable amount of toxin (say 100 ml. of toxin). If gut contents of the large animals studied are able to inactivate the toxin a

large supply of the toxin inactivating factor can be made readily available. Further, variation in the toxin inactivating substance in human intestine may explain if only in part the differential susceptibility of human beings to cholera.

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P R O T O C O L

STUDIES OF THE NATURE AND PROPERTIES OF THE TOXIN INACTIVATING FACTOR IN THE RAT INTESTINE

K.M.S. Aziz, W.K. Hare, and R.A. Phillips

Introduction and Specific Aims:

It has been observed that rat intestinal wash (RIW) contents are able to inactivate cholera toxin, i.e., the fluid accumulation factor in the culture filtrate of Vibrio cholerae (1). This toxin inactivating substance (contained in RIW) is filterable, nondializable and stable at 56°C for 30 minutes. It can be stored in either deep freeze or at about 4°C. The aims of this research are to find out the nature and properties of this toxin inactivating substance and also to find out whether this substance is capable of reversing the action of toxin in the small intestine. A separate study of the immunological aspects of the rat intestinal wash is also being carried out (2).

Methods of Procedure:

(1) Stability at different temperatures: RIW will be heated at 70°C and 90°C for 30 minutes and 1 hour. These heated preparations will be tested for toxin inactivation.

(2) Hydrolysis and enzymatic digestion: HCl, trypsin and pepsin treated RIW will be tested for toxin inactivation.

(3) Rat serum will be titrated for antitoxin activity (3). A correlation will be attempted between the antitoxin titers of rat serum and RIW.

(4) Reversal of toxin action in ligated ileal loops of adult rabbits (4) by RIW: For this purpose .01 ml Wyeth toxin will be administered in five loops. In the first series of eight rabbits horse antiserum from Swiss Serum Institute, normal rat serum and RIW will be administered five minutes after the challenge of the toxin. The other two loops will be used for positive and negative controls. If RIW is found to be reversing the effect of the toxin, RIW will be administered in the loops 10 minutes, 30 minutes and 1 hour after the loops are exposed to the toxin (5). Again 8 rabbits will be used for each experiment.

(5) Purification of RIW

If RIW is established as a protein and if it proves to be important enough, fractionation will be carried out by molecular

sieve filtration, ion exchange chromatrography and disc electrophoresis.

Significance of this Research:

The discovery of a substance capable of reversing the action of the toxin in ligated small intestine of rabbits may be of great significance. It is important to know the nature and properties of such a substance.

References:

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P R O T O C O L

TITRATION OF CHOLERA TOXIN IN RATS AND MICE

K.M.S. Aziz, W.K. Hare, and R.A. Phillips

Introduction and Specific Aims:

Assay of cholera toxin has been carried out in various animal models (1-2). Only in the past few months has it been found that the rat can serve as a cholera model (3). The aim of this research will be to attempt to develop techniques of titration of cholera toxins in ligated small intestine loops of adult rats and mice. The term cholera toxin will be used for the Fluid Accumulation Factor contained in crude culture filtrate or in cholera stool.

Methods of Procedure:

1. Three-month-old rats weighing 100 gms $\pm$ 10 gms will be used for the ligated intestinal loop technique. Rats will be biscuit-fed for 72 hours and then glucose-fed for 72 hours. Laparotomy will be performed under ether anaesthesia. The small intestine will then be thoroughly washed (3). Five ligated loops, about 6 cm long will be made with 1-2 cm interloops in between. The ligatures will be made by silk thread and blood vessels will be carefully avoided. The loop closest to the duodenum, to be known as loop 1 will receive a standard dose of toxin. Loop 2 will be used for a negative control. The three remaining loops will be used for titration of the toxin. Dilution and coding will be carried out and recorded. Each dilution of the toxin used will be put in each of these three sites in three different rats. After the operation is over the animals will be kept in separate cages and water supplied ad libitum.

2. Eight hours after the operation is finished the rat will be sacrificed by excessive ether anaesthesia and the peritoneal cavity opened. Starting from loop No. 1 (the loop nearest to the duodenum) the loops will be carefully cut and removed and placed one by one in previously numbered and tared glass or plastic dishes with covers. Precaution will be taken to cut as close to the loop as possible.

3. Determination of W, W_1, W_2, and W_3 (4).

(a) The weight of the container with the loop before draining off fluids minus the tare, W, is the wet weight of the loop, W_1.

(b) The fluid, if any, will be drained from the loop, first 5 ml (or less if there is less fluid produced) being collected by a syringe and transferred to a numbered tube containing liquid paraffin. The emptied loop and the container will be blotted off to remove the excess fluid. The container with this drained and blotted loop, will be weighed. W, will then be subtracted from the weight of the loop without fluid, W_2 will be determined.

(c) After the determination of W_2 the container with the loop will be transferred to an oven and kept at 95°C overnight. Next morning the container with the dried loop will be transferred to a dessicator for about 2 hours. The container with the dried loop will then be weighed immediately after taking it out of the dessicator. From this weight, W, will be subtracted; thus the dry weight of the loop W_3 will be noted.

(d) All details of the weight of the container, weight of the lid and other weights taken will be registered in a notebook maintained by the person who will be taking the weights. The time and date of taking both the wet weights and dry weights for every set of 5 loops will be recorded.

(e) From the data above the ratio of the loop with fluid and without fluid and also the ratio of the wet weight and dry weight will be determined.

4. Determination of electrolyte and CO_2 composition (mEq/liter) of the loop fluids.

(a) Fluid as collected in step 3b above will be analysed in the Chemistry Section for Na, K, Mg, Cl, CO_2 and pH.

(b) Once the pattern of the electrolyte composition of the loop fluids are established this step (step 4) will no longer be done routinely.

5. Similar methods as described above will be followed for titration of cholera toxin in mice. Three ligated small intestine loops will be made in mice, instead of five, as proposed in rats.

Significance of this Research:

Procurement and/or breeding of large number of rabbits is expensive. Rats are easy to breed in large numbers. Hence, the assay of cholera toxin in rats may remove the bottleneck in research caused by shortage of animals.

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BACTERIOLOGY SECTION REPORT FOR FY'68
PERIOD JULY 1, 1967 TO JUNE 30, 1968

During the year ending June 30, 1968 the diagnostic unit of the Bacteriology Section received a total of 47,129 specimens. Like all other previous years all these specimens were in the form of rectal swabs or stools from the patients of Pakistan-SEATO Cholera Research Laboratory Ward and people under surveillance by the Epidemiology Section at Dacca and suburbs and also Matlab Bazar. Out of these total specimens 14,047 were processed in the field laboratory at Matlab. Like last year (1966-67), we were also asked this year by the Government of Pakistan to culture all the Hajis going for pilgrimage to Saudi Arabia; a total of 5908 people were swabbed.

During the year under review the following groups of pathogenic organisms were isolated.

Vibrio cholerae Inaba	–	1070
Vibrio cholerae Ogawa	–	6
El Tor Inaba	–	0
El Tor Ogawa	–	13
Non-cholera vibrios	–	87
Salmonella typhi	–	14
Salmonella group A	–	2
Salmonella group B	–	12
Salmonella group C_1	–	10
Salmonella group C_2	–	0
Salmonella group D	–	2
Salmonella group E	–	11
Shigella group A	–	51
Shigella group B	–	275
Shigella group C	–	14
Shigella group D	–	64

As can be seen from the list, like last year, this year also we had an epidemic of Inaba biotype. All the 13 El Tor Ogawa isolations were made in June 1968 from Chittagong and Dacca.

The visit of Dr. K. P. Carpenter from Central Public Health Laboratory, Colindale, London, and Dr. W.J. Martin from National Communicable Diseases Center, Atlanta, Georgia, U.S.A. during January and February 1968 helped us in streamlining existing diagnostic procedures and including a few new tests in the routine work.

The diagnostic unit is also supporting studies on all research projects which require bacteriological examinations.

Report of the Research Unit

A brief outline of the specific studies carried out by the Bacteriology Section is as follows:

1. A Previously Undescribed Group of Vibrios Isolated from Stool Samples

During the course of routine isolation of vibrios from stool samples collected during night-soil sampling we came across a group of gram-negative curved organisms which look like vibrios on routine solid media and grew well on them. They did not agglutinate with Venkatraman's O Group I antiserum. On routine biochemical fermentation tests the organisms fell into Heiberg Group II but failed to produce indole and reduce nitrate. It was suspected that this may form a different type in the genus vibrio.

Cultural, morphological and biochemical characteristics were looked into utilising standard procedures. Indole production, nitrate reduction, gelatin liquifaction, methyl red and Voges-Proskauer tests, cholera-red reaction, Hugh and Liefson test, cytochrome oxidase test, catalase test, H_2S production, urease production, breakdown of different sugars in peptone water sugar media, growth in presence of 8% to 10% sodium chloride, decarboxylase reactions and action of 0129 pteridine compound were tested using standard techniques. The findings of our Laboratory, supported by the findings of other foreign laboratories, suggest that these organisms may form an unidentified type within the genus vibrios.

2. The Efficacy of Enrichment Media in the Diagnosis of Carriers and Cases of Inapparent Infection.

Since 1887 when alkaline peptone water was introduced for the enrichment of Vibrio cholerae many studies have been done on the efficacy of enrichment media for vibrio isolations. Pal et al (1967) compared the efficiency of three different enrichment madia: (a) alkaline peptone water (PW), taurocholate tellurite peptone water (TTP) and alkaline peptone water with tellurite (PWT). They observed that TTP and PWT gave almost identical results, whereas PW was found to be inferior in respect of isolation of vibrios. Monsur et al (1965) also compared TTP and PW and found 72% positive with TTP in contrast to 54% with PW. As TTP medium is used extensively in our Laboratory it was decided to use this medium in this study.

101 rectal swabs and stool cultures examined bacteriologically indicated the following results: 22% of the positive cases indicated no Vibrio cholerae on direct plate but gave positive growth on enrichment; this was in contrast to 2% of cases where Vibrio cholerae was isolated from direct plate but were missed after 6 hours enrichment.

In 9% of the positive cases direct plate indicated only a few colonies of vibrios whereas on enrichment there was confluent growth; this was in contrast to 2% of cases where direct plates indicated confluent growth and enrichment after 6 hours indicated only a few colonies of vibrios.

3. Search for a Single Enrichment Medium for Vibrio, Salmonella and Shigella.

Many different enrichment media have been described in the literature for the isolation of vibrios, salmonella and shigella, but no single medium has been effective in the isolation of all three together. It is for this reason that it was decided to look for a single enrichment medium for all three groups of organisms.

It has been found that though selenite broth is good for salmonella typhi and a few other salmonella, it does not support the growth of shigella excepting a few shigella sonnei. Tetrathionate broth is not good for shigellae or vibrios. T_1N_1 broth enriches all three groups but as the medium is non-selective it grows all other organisms such as escherichia coli, klebsiella species, proteus species etc., and thus makes identification very difficult. The results obtained with salmonella-shigella agar filtrate without sodium citrate and also modified TTP medium (Monsur's TTP substituting brilliant green for tellurite and reducing pH to 7.5) showed that both of them might be suitable as enrichment media for members of all three genera but, compared to the modified TTP medium, modified salmonella-shigella broth showed poor growth on all plates. Modified salmonella-shigella broth showed poor growth on all plates. Modified TTP medium also enriches vibrios, shigella and salmonella in presence of normal stool flora. The only disadvantages observed in modified TTP medium were that isolation of some shigella becomes difficult due to changed colonial character.

4. Intestinal Bacterial Flora in Normal Healthy Adults of East Pakistan

The purpose of this study was to find out the bacterial population of the gut of normal healthy adults in East Pakistan.

Two groups of people were included in this study. 850 people were selected arbitrarily from 5908 people and swabbed before their departure to Saudi Arabia for pilgrimage.

Of the 850 rectal swabs examined aerobically 560 indicated pure growth of Escherichia coli and the remaining 290 growth of Escherichia coli, klebsiella and non-lactose-fermenting organisms. Among the pathogenic organisms isolated from these 290 case, shigella were indicated in 2% of individuals in contrast to salmonella indicated in only 0.24%. Enteropathogenic Escherichia coli were indicated in only 1.6% of the individuals. Among the gram-negative non-pathogenic organisms, Escherichia coli (one type or other) appeared in 100% of the individuals swabbed, followed by proteus 10%, plesiomonas shigelloides (C_27)- 3.5%, klebsiella - 5.4%, enterobacter 3.4%, providence and citrobacter 1.5% each and so on. Among gram-positive organisms alpha-haemolytic streptococci appeared in 58% followed by beta-haemolytic streptococci 34%, Staphylococcus albus 33%, Staphylococcus aureus 10% and clostridium 19%. We did not look for bacteroides. Though the bacteriological finding of non-pathogenic organisms is not significant, the results obtained with pathogenic organisms are of importance. Considering the growing worldwide interest in the study of human gut flora, this may serve as baseline data for normal individuals of East Pakistan.

5. 'Sulfa' Sensitivity in the Differentiation of Vibrio Comma and Vibrio El Tor.

In Vitro studies done in our laboratory have shown that 'Sulfa' drugs (e.g. sulfadiazine, sulfathiazole, sulfamerazine and sulfaguanidine) are active against Vibrio El Tor at very low concentrations (MIC 0.1 mg/ml), in contrastto classical Vibrio cholerae (MIC 10 mg/ml). Thinking that this differential drug sensitivity may serve as a useful tool in differentiating true cholera and El Tor vibrios, we followed the procedure mentioned by Han, G. K. (1962) in his polymixin B differentiation test of classical Vibrio cholerae and El Tors.

The results obtained in our experiment with 30 strains of Vibrio El Tor and 83 strains of classical Vibrio cholerae indicated that within 8 hours of incubation sulfadiazine and sulfaguanidine indicated a clear zone of inhibition (measuring 12-14 mm) around the El Tor strains whereas during the same time classical strains did not show any inhibition of growth. Excepting 2 El Tor strains (6.6%) and two Ogawa classical strains (2.4%), all the cultures in both groups indicated consistent results. The results obtained from overnight incubation of plates were confusing, as in many cases growth occurs in the place of inhibition in cases of El Tor..

From the results obtained so far it seems that action of discs containing 'Sulfa' drug may serve as a useful tool in the differentiation of Vibrio El Tor and classical Vibrio cholerae. 'Sulfa' discs have an advantage over polymixin discs in that they are easily available, cheap and easy to make. Results can be obtained within the same day, the zone of inhibition is greater than in polymixin B, and thus are easy to read. Best and consistent results can be obtained from freshly isolated cultures as has been indicated in our Laboratory with recently isolated strains from Chittagong.

Copy of

REPORT ON THE BACTERIOLOGY LABORATORY

Dr. A. L. Furniss
Public Health Laboratory Service
Maidstone, Kent

September-December 1968

As a routine diagnostic laboratory the Bacteriology Section is doing excellent work under difficult conditions. Details of workload, space and staff are given in Appendix 1. Much, however, could be done to improve the efficiency of the work. Alterations that could be made in the Laboratory to provide a more economic use of space with a better work-flow are appended as a separate report. ("Outline Report on Suggested Laboratory Alterations.")

The routine work of the Laboratory suffers from unregulated demands and pressures from all users of the Laboratory. The work-load has recently increased greatly and, although there is no evidence that this has happened, there is a danger that the quality of the work might suffer for the sake of quantity. There is often considerable pressure to undertake investigations which are foolish or unnecessary - for example to identify individual species in what is simply a commensal flora. This wastes time, and it may lead to bad reporting in the sense that some of the "identifications" are based on insufficient characterization. The bacteriology laboratory badly needs protection from the demands of its users and from its own willingness to please everyone by doing just as it is asked. This protection is necessary in order that it may give the best service.

Methods of reporting have already been discussed and the Laboratory should be encouraged to keep to such a standard scheme as in Appendix 2.

No research project involving bacteriological work should be commenced without open discussion involving not only the staff of the bacteriology laboratory but also all investigators whose projects depend in any way on the work of the bacteriology staff. Although most of the time of the Laboratory staff is occupied with supporting work, there is no reason why they should be discouraged from original bacteriological work. In fact, applied investigations of Laboratory procedures is essential for both efficiency and morale. If a medically-qualified bacteriologist could be found who was keen to undertake bacteriological research work in Dacca for a substantial period - say one year at least - he should come for a preliminary period of a few weeks to see the situation and let himself be seen. For the present, however, I recommend that I should return for short periods of a week or two which could enable me to help in the alterations to the

Laboratory and provide a better basis for liaison with the Public Health Laboratory Service and with Colindale and Maidstone in particular. For domestic reasons, I cannot undertake a two or three-year stay in Dacca.

APPENDIX I

Copy of

P-SCRL BACTERIOLOGY LABORATORY WORK AND RESOURCES REPORT

December 1968

1. WORK

Currently at the rate of about 1,400 specimens a day from nine sources:

(i) Chittagong El Tor Project (Dr. Kenneth J. Bart)
Latrine surveillance 375-400 specimens a day for cholera only.

(ii) Matlab Surveillance - Vaccine Trial (Dr. W. H. Mosley)
A variable number but an average of about 100 rectal swabs a day for cholera only.

(iii) Matlab - Study of Inapparent Infection
560 rectal swabs a day for cholera only.

(iv) Matlab - Routine Hospital Specimens
Stool and rectal swabs mainly, for salmonella, shigella, and other specific intestinal pathogens, 50-60 a day.

(v) Rayer Bazar
Community study and latrine surveillance, 170-200 a day for cholera only - stopped a week before date of this report, on December 6, 1968.

(vi) Specimens (rectal swabs) from P-SCRL Ward for All Pathogens
Now about 50 patients in ward - build up expected to be rapid from now. Other specimens of all kinds average about 25-30 a day.

(vii) Purgation Study - P-SCRL Ward
20-40 rectal swabs a day for cholera only.

(viii) Study of Jejunal Flora - Intubation of patients with Diarrhea (Dr. R. S. Northrup)
Six specimens a day at present. Requests made for increase. If less than 5 specimens are received they are examined quantitatively (a serial dilution in broth and plating on selective media) for E. coli, klebsiellas, streptococci, micrococci, and other organisms, including anaerobes. If more than 5 specimens are received they are examined for cholera only.

(ix) Family study in Dacca
About 20-25 rectal swabs a day for the presence of vibrios only.

2. SPACE

	Sq. ft.
Laboratory 1	400
" 2	400
" 3	200
Media making, plate pouring and media storage	400
Wash-up room	400
	1800

Autoclaves, hot-air ovens and large incubator in corridors outside.

3. STAFF

1. Mr. Imdadul Huq, M.Sc., Dip. Bact.
   Section Chief
2. Mr. Chittaranjan Dey, M.Sc., Dip. Bact.
   Senior Research Assistant
3. Mr. A. F. Raisur Rahman, M.Sc.
   Senior Research Assistant
4. Mrs. Akhtari Alam, M.Sc.
   Senior Research Assistant
5. Mr. Ahmadul Huq, M.Sc.
   Research Assistant
6. Mr. P. K. Bose Neogi, B.Sc.
   Senior Research Assistant (on loan to Dr. Northrup)

7-11. Five Research Technicians

12-27. Sixteen laboratory technicians

28-29. Twelve laboratory attendants

(Of these, 4 are employed at Matlab and 3 at Chittagong)

A shift system. (3 shifts) operates from 7:30 a.m. to 10 p.m.

6 December, 1968

J. W. Howie

APPENDIX II

SCHEME FOR BACTERIOLOGY REPORTS

Blood Cultures:

Positive cultures will be reported as soon as possible. A final negative report will be sent after 15 days' incubation.

Probable contaminants such as micrococci, ''achromobacter' and Staph albus will not be investigated in detail; it will be assumed that further repeated cultures will be sent to the Laboratory if septicaemia is still suspected.

Throat Swabs:

Group A Beta-haemolytic streptococci will be reported as Strep pyogenes, other Beta-haemolytic streptococci will not be grouped, but will be reported as such.

Antibiotic sensitivities will not be reported. All such streptococci are penicillin-sensitive.

Sputum:

Sputum will be examined by direct film but will not be cultured for mycobacteria.

Strept, pneumoniae and Haemophilus influenzae will be reported if present, but without antibiotic sensitivities.

A heavy and predominant growth of Staph. aureus will be reported with sensitivities. The occasional colony of Staph. aureus or Candida albicans will not be considered significant.

Enterobacteraceae will not be reported because of their doubtful aetiological significance.

Otherwise the reported will be: commensal flora only.

Urines:

It is impossible to provide a satisfactory bacteriological report on a specimen of urine unless it has been correctly collected with aseptic technique into a sterile container and sent immediately to the laboratory. Any delay may make bacteriological examination useless because of the rapid multiplication of any contaminating organisms.

Any mixed growth will be reported as contaminants. It will be assumed that further clean specimens will be sent to the Laboratory.

No antibiotic sensitivity tests will be carried out on organisms not considered aetiologically significant. For this purpose the recognised standard of a count of 10^5 organisms per ml. urine will be used. Below this figure the report will be in the form: No significant growth.

Counts will not be reported.

Faeces and Rectal Swabs:

Apart from vibrios, salmonellae and shigellae will be the only organisms routinely reported.

Antibiotic Sensitivities:

An organisms will be reported as 'sensitive' or 'resistant' to the appropriate antibiotic. Minimal inhibitory concentrations of antibiotics will not be reported.

Copy of

OUTLINE REPORT ON SUGGESTED LABORATORY ALTERATIONS*

1. "Dirty" autoclave. The autoclave for sterilizing all discarded cultures, etc. should be situated at the end of the verandah outside the latrines and kept for this purpose.

2. Washing-up room (117-8). The present sinks are too deep and clumsy. Two sets of 3 sinks, one in the middle of the wall next to the latrines and one in the centre under the windows, should be installed. They need to be only 9"-12" deep. 'Draining board' benches should be on either side of the set of sinks. This room becomes terribly hot and adequate ventilation is essential.

3. Media Room (115-6). To complete the improvements the following are still needed: laminated plastic top to bench along the wall of 116 - this bench to run right up to the window. Laminated plastic topped bench down the centre. Cooling mechanism for plate pouring room.

4. Cold Room. To be constructed between 115 and 114 in space now prepared with shelves parallel with wall.

5. Laminated plastic tops for benches in rooms 113 and 114.

6. Door or archway to be constructed between 113 and 112 next to the verandah.

7. Laminated plastic tops for benches in room 112 and 111.

8. Construction of centre bench from window with laminated plastic top fitted with gas and electric points.

9. Door or archway to be constructed between 111 and 110 next to the verandah.

10. Partition between 109 and 110 completely separating them into two rooms.

11. Construction of bench along this partition.

12. Laminated plastic tops for benches in 110.

13. Angle-poise lamps to be fitted to all working benches.

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*Full details and sketch plan will be left with the Deputy Director (Dr. Kendrick Hare). The numbers refer to the rooms so identified in the sketch plan.

CLEANING

It is also the aim that the Laboratory should be more easily cleanable and less liable to accumulations of dusts, so that dust-collecting surfaces should be avoided as far as possible, and all walls should be washable. There should be enough cupboard space so that nothing is kept on the floor. Regular cleaning by the Laboratory staff can then more easily be carried out.

CHEMISTRY SECTION REPORT FOR FY 68

As usual, the Chemistry Section has supported research in the patho-physiology of cholera. As the activities have expanded so has the supporting personnel. The ever-increasing work load has necessitated the reorganization of the Section. A proposal for this reorganization has been submitted.

Preparation of intravenous fluid (5:4:1) is still the responsibility of this Section. This solution has now replaced all imported preparations in the CRL Ward and in Matlab Field Hospital.

The work done in the Fiscal Year is shown in the tables. Table (1) and (2) show the work done under routine Chemistry.

Tables 3 to 11 show the work done under different research protocols of the Research Physicians and Investigators.

The Chemistry Section has been receiving more and more requests from other medical and research institutions for analysis of blood and other biological fluids. The requests have mostly been limited to determinations we perform regularly, but there have been requests for other biochemical determinations. We have helped as much as we could in order to keep up the good will and high tradition of the Laboratory.

A review of the work load as given in Tables 1 to 12 clearly shows that this Laboratory has been overloaded. This only the analytical side of it. The other supporting activity the Section is performing is the preparation of lavage and oral therapy solution. Sometimes it becomes very difficult to cope with the situation. At a time studies like oral therapy flux study, pediatric balance studies are started and each study requires lavage solutions of 10-50 liters for each study. Thus the Section has to prepare 100-150 liters of solution of varying composition in the course of 24 or 36 hours of study duration. This consumes huge amounts of distilled water; the production of intravenous solution also runs in full swing.

Further, the study is started any time the patient qualifies for different protocol. This necessitates the coverage of the Laboratory round the clock and analysis of certain constituents immediately after sampling creates problem for us to odd working hours. For example, 3 different studies are started and each study requires immediately analysis of certain factors. This multiples to 10 to 12 analyses at a time and, at night, with 1 or 2 technicians it becomes very, very difficult to cope. On top of this, some investigators press for the result soon after they send the samples. This amounts to asking for almost "impossibles" to be made possible.

This is besides the emergency clinical specimen from the other patients in the Ward which is regular all the time. The present arrangement of "Call Duty Personnel" for emergency work and "Night Shift" personnel (two at a time) for study patient becomes inadequate due to the pressure from different studies running side by side.

The Tables referred to in this report are not being made a part of the official record for the Technical Committee. Copies of the Tables are available from the Director's Office on request.

APPENDIX 'A'

ROUTINE CHEMISTRY

Unit Supervisor - Grade proposed E-D

In addition to this he has also the responsibility of deputing the Section Chief in administrative activities.

Supporting Personnel:

Sr. Research Assistant	-	1
Research Assistant	-	1
Research Technician	-	2
Laboratory Technician	-	3

Position Occupied:

Sr. Research Assistant	-	1
Research Technician	-	1
Laboratory Technician	-	2

Position Required (new):

Research Assistant	-	1
Laboratory Technician	-	2

SPECIAL CHEMISTRY

Unit Supervisor - Grade Proposed E-D

Supporting Personnel:

Sr. Research Assistant	-	1
Research Assistant	-	5
Research Technician	-	4
Laboratory Technician	-	2

Position Occupied:

Research Assistant	-	5
Research Technicians	-	3
Laboratory Technician	-	2

Position Required (new):

Sr. Research Assistant	-	1
Research Technician	-	1

I. V. FLUID

Unit-in-Charge - Proposed Grade I-H

Supporting Personnel:

Laboratory Technician	-	1
Laboratory Attendants	-	4

Dr. Hare will advise on expansion or reorganization of this Unit.

GLASS WASHING

Unit-in-charge - Proposed Grade I-H

Supporting Personnel:

Laboratory Technician	-	1
Laboratory Attendants	-	4

Position Required (new)

Unit-in-charge		1
Laboratory Attendant	-	1

CLINICAL INVESTIGATION SECTION REPORT

FOR FY 68

Introduction

FY 68 provided sufficient clinical material for the completion of a number of studies by the Clinical Investigation Section, and for significant progress to be made in other studies despite the reduced number of cholera cases admitted to the Dacca ward. In late November, 1967 the Memorial Christian Hospital, located in Malumghat, between Cox's Bazar and Chittagong, appealed for help in the management of an outbreak of cholera in their area. The response to this call led eventually to the establishment of a large-scale field treatment center on the hospital grounds, staffed completely by CRL personnel. Three hundred and fourteen patients were admitted, of whom two hundred and eleven had demonstrated cholera; one hundred and seventy-four outpatients were treated. Laboratory and study facilities were also established. Thus, during the three-month operation of this unit, when cases in Dacca were absent or few in number, research activities were able to continue.

As activities in Malumghat ceased, cholera cases began to appear in Dacca and studies begun in Malumghat were continued in Dacca through June, 1968.

Drs. David Nalin and Richard Cash joined the staff in July, 1967. They have established a program of animal experimentation, as well as carrying out clinical research projects.

Dr. Richard Wenzel, followed by Dr. Stanley Music, from the University of Maryland, was in charge of routine patient management in the Malumghat and Dacca wards during the most active months, thus allowing the permanent staff to concentrate on their research projects.

Dr. Joseph Kinzie and Dr. James Taylor returned to the U.S. upon completion of their assignments in May, 1968. Dr. Jon Rohde joined the staff in August after training at Oak Ridge in radioisotope techniques, and will continue the intestinal flux studies (CI/P2) in collaboration with Dr. Ruth Hare. Dr. Taylor returned to Dacca on October 24 for a two-month stay and will participate in these studies during that time. Dr. A. H. G. Love from Belfast will join this team, also on a temporary basis, for several weeks.

Dr. Robert Northrup, Chief of the Section, is continuing his work on the immunology of the gastrointestinal tract in cholera (CI/P8-13). Dr. M. M. Rahaman, Ph.D., guest investigator in the section, continues his studies on pathophysiological and nutritional aspects of pediatric cholera cases. Dr. Kenneth Bart, who

joined the Epidemiology Section in June, 1968, has completed his residency in pediatrics in the U.S. and, besides his activities in the Epidemiology Section, is participating actively in the care and study of the pediatric cases on the ward.

Dr. Jamiul Alam returned in September from two and a half years' study in the U.K. and is expected to take an active role in the research activities of the ward. The other full-time ward physicians, headed by Dr. Rafiqul Islam, Chief Resident, are Drs. Z. Hoque, Toaha, Majid Molla, Saha, Waliur Rahman, L. Khondaker and Roy. Dr. S. Music and Dr. R. Wenzel are expected to return from the University of Maryland to aid in running the ward during the cholera season.

CLINICAL INVESTIGATION STUDIES

COMPLETED IN 1967-68

CI/P1 & P2 Unidirectional Sodium Flux Study in Acute and Convalescent Cholera

This study is described in detail in protocols CI/P1 and CI/P2 of the 1967 Technical Committee Report. The study was carried out in Malumghat (see above), but all analyses were done at the P-SCRL in Dacca.

Seven patients were studied in the acute phase of cholera and again one month after recovery. Of these, three were also studied in early convalescence. Three patients were studied for the first time in early convalescence and again one month later. One patient was studied only in the acute phase and another only in early convalescence. Total studies were eight in acute, seven in early, and ten in later, convalescence. Only jejunal segments were studied.

Analysis of the results indicated that the volume of the segment is not as constant as expected at the flow rates used even though the flow rate was relatively constant. Volume of the segment was larger and more variable in acute cholera than in late convalescence. Volume in early convalescence was intermediate between the two. The calculated sodium pool varied in exactly the same way.

The rate constant for sodium transfer out of the segments was significantly less during acute cholera as compared to the early or late convalescents. Since this value is independent of the volume of sodium pool determinations, this was felt to be the most reliable parameter investigated.

The effect of glucose is to increase the sodium transfer rate to exactly the same extent in all three groups. Outflux, net flux and influx all are increased. It is apparent that the sodium flux activated by glucose is unimpaired in acute cholera.

The numerical results are shown graphically.

If only the paired data on the seven patients studied during acute cholera and in late convalescence are considered, one would conclude that change in the size of the pool exactly compensates for the rate constant change, so that flux out of the segment is unchanged, while flux in is greater in the acute state. However, if the early convalescent is considered (data can no longer be paired), the rate constant has risen faster than the volume has fallen, and we now see an increase in sodium outflux with no change in influx. Average values for the three patients studied three times show the same.

It was concluded, therefore, that cessation of diarrhea is associated with, or probably caused by, an increased transfer rate of sodium and an increased outflux of sodium with no change in sodium influx. One month later the rate constant remains the same, but both influx and outflux are lower because the pool is smaller.

CI/P4 Clinical Trial of Oral Glucose and Electrolyte Solution in Adult Cholera.

This study, utilizing the information obtained from the oral glucose study of Hirschhorn carried out during the previous season, employed glucose and electrolyte solutions in a controlled clinical trial. The method proved to be both practical and effective. Nineteen patients were treated with the oral solution. Intravenous requirements in this group were reduced by 80% compared to the control group. It was noted that most of the I.V. fluid administered was during the initial rehydration phase, before any oral solution had been given. Patients initially were given the solution by oro-gastric tube, but oral administration by drinking subsequently proved equally effective and free of complications.

CI/P5 Electrolyte and Fluid Balance in Pediatric Cholera

Twenty-eight children under four years of age were studied for periods ranging from 8 to 72 hours. Mean admission stool sodium was 90 meq/L and during the course of diarrhea rose to 110 meq/L. The mean potassium content of the stool was 40 meq/L on admission, and it gradually fell to 25 meq/L as the patients' stooling rates decreased. Mean stool bicarbonate was 33 meq/L on admission and did not change appreciably during the study.

These values for stool electrolytes are markedly different from the Dacca 5:4:1 solution which was being used to maintain fluid balance in these children during the study (Na = 133 meq/L, K = 13 meq/L, HCO_3 = 47 meq/L). Seven cases did develop hypokalemia requiring additional potassium administration.

The study confirms the study of Griffiths (1968) that the ideal intravenous replacement solution for young pediatric cholera cases should contain less sodium and more potassium than the solution currently in use. There were, however, no serious complications in the group treated with the Dacca solution.

CI/P8-13 Intestinal Immunology in Cholera

A soluble antigen obtained from vibrios by ultrasonication was effectively labelled with I^{125}. Using this labelled antigen, radioimmunodiffusion studies of convalescent sera having high vibriocidal and vibrio agglutination titers were carried out in

agar gel. These showed good antibody activity in IgA and IgM but no IgG activity. Since this was in contrast to other serum fractionation studies, the reasons for failure to demonstrate IgG antibody were sought. It was found that prolonged washing of the initial IgG precipitate led to its dissolution. When the period of washing was reduced, antibody activity by this technique was found in all Ig classes in some sera, but the majority continued to show absence of activity in IgG, and presence of IgA and IgM activity. Acute sera obtained from the same cases as the positive convalescent sera were usually negative, but occasionally showed IgM antibody activity. The serum of an immunized American showed IgG activity, but no IgA or IgM activity.

Initial studies of stools of cholera patients by the radio-immunodiffusion technique were encouraging and accordingly 84 samples were collected from cholera patients at Malumghat, both during their acute illness and during convalescence. The convalescent stools were collected by mannitol purge.

Most of these samples have been precipitated and concentrated and are being analyzed at present. No final conclusions can be drawn yet but IgA antibody is frequently present in the convalescent stool specimens. IgG antibody is present less often.. IgM itself is usually not detected and thus antibody activity is not demonstrated.

Immunochemical studies were carried out in Louvain (Belgium), Buffalo (U.S.A.) and Dacca, of the proteins of cholera stool. Sucrose density gradient ultracentrifugation studies showed that stool IgA sedimented at a rate characteristic of serum IgM and faster than serum IgA, suggesting that its molecular weight was greater than that of serum IgA. IgM from cholera stool, on the other hand, sedimented at a slower rate than serum IgM, suggesting that its molecular weight was less. Sephadex chromatography of stool proteins suggested a reduction in the size of the IgG molecule, and also showed that IgA antigenic determinants were found in portions of the eluate corresponding to a wide range of molecular or molecular fragment size.

Antigenic analysis of the stool IgM showed that it appeared to be present as a molecular fragment consisting of a light chain and an IgM heavy chain joined by a disulphide bond.

These evidences of proteolytic breakdown of the immunoglobulins during the period of time between secretion into the intestine and excretion as stool may account for the inconsistent results of the coproantibody radioimmunodiffusion studies. In order to reduce this problem, jejunal aspirates will be used in 1969.

CI/P17 Dog Chronic Thiry-Vella Loop Studies

Chronic Thiry-Vella loops were prepared in a number of dogs. The effect of cholera diarrheogenic toxin on these loops was neutralized when the toxin was incubated with a variety of agents before instilling it into the loop. These agents included sera having a high titer of antitoxin (as measured in rabbit loop tests), charcoal, and kaolin. These agents had no neutralizing effect if added to the loops after the toxin.

Two loops were prepared in some dogs. Toxin placed in one loop did not cause diarrhea in the other loop. Furthermore, the response to toxin in the second loop was not changed when toxin was given first to one loop, then to the other. Thus diarrheogenic toxin does not appear to be absorbed in amounts sufficient to cause diarrhea systemically, even though the toxin, as shown by Carpenter, can cause diarrhea in a loop if injected into the loop's arterial blood supply.

CI/P18 Effect of Acetazolamide on Fluid and Electrolyte Losses in Cholera

Norris, in an unpublished observation, reported that acetazolamide reduced cholera toxin-induced fluid losses into a rabbit ileal loop preparation. Taylor, using large intravenous doses in purging cholera patients, and Carpenter, in chronic dog Thiry-Vella loop studies, failed to confirm this observation.

Four moderately active adult male cholera patients were given 30-80 mg./kg. of acetazolamide orally in three liters of 5:4:1 over an 8 to 16-hour period. Net stooling rate did not decrease, and one patient's stooling rate, after acetazolamide administration had ceased, actually increased. Urinary output increased or remained constant, thus requiring additional intravenous fluid administration.

Although these studies cannot be considered conclusive, it appears very unlikely that acetazolamide has any place in the clinical therapy of cholera.

CI/P20 Acetate in the Treatment of Base Deficit Acidosis of Diarrhea

Acetate was substituted for bicarbonate in the intravenous solution used to treat both acute dehydration and also longer-term losses of base and fluid from cholera and other diarrheas. Acetate proved as effective as bicarbonate in correcting acute acidosis. The slight delay in pH correction with acetate-containing solutions, a matter of minutes, was not felt to be of importance in any but the most severely acidotic and dehydrated patients. Acetate also was found not to have the "alkaline over-shoot" of pH correction

seen with bicarbonate. No complications thought to be associated with acetate were noted in patients treated with acetate solutions for maintenance of fluid and electrolyte balance during the total course of their diarrhea. Acetate, in comparison with bicarbonate and lactate, is cheap, can be autoclaved, and has a long shelf life. This study allows us to recommend its substitution for bicarbonate or lactate in intravenous solutions used to treat cholera and other diarrheas.

CI/P23 Treatment of Cholera by Massive Doses of Phage

Techniques for the preparation of phage at the Institute of Public Health were improved such that large quantities of phage preparations of titer greater than 2.5×10^{12} pfu/ml. could be produced. This preparation was administered during the past year to four actively purging cholera patients in doses designed to ensure the presence of at least 300 phage particles for each vibrio in the gut, and a titer of over 10^{11} pfu/ml. in the stool. A loading dose was given, after which subsequent doses were given until recovery of the patient, the amount depending on the stooling rate of the patient and his size.

The vibrio count was markedly reduced in all patients. The stooling rate in two patients was reduced to each an extent as to be comparable to that produced by tetracycline, although the other two patients did not show a reduction in stooling enough to be significantly different from controls. This work will continue during the 1968-69 cholera season.

CLINICAL INVESTIGATION STUDIES
PLANNED FOR 1968-69, OR IN PROGRESS

Studies completed during 1967-68 provided the basis for a number of investigations planned for 1968-69. Many of these are extensions of the previous work utilizing alterations of methods suggested by the 1967-68 results. Other studies have arisen de novo.

CI/P2 Studies of Water and Electrolyte Fluxes in Human Cholera

Using the multi-lumen tube bolus technique, unidirectional chloride fluxes will be studied, as well as sodium and water. Intestinal permeability studies, not feasible last year, will be performed. Modification of perfusion rates will be made in an attempt to control the volume of the study segment. Balloons added to the tube will be used to isolate an intestinal segment, thus allowing complete collection of the segmental contents, and the avoidance of bolus technique sampling errors.

CI/P4 Oral Therapy of Cholera

Amino acids have been shown to increase sodium movement out of the intestinal lumen in a manner similar to that of glucose. Glycine, one of the actively transported amino acids having this property, will be compared with glucose in patients treated by oral therapy. Evidence suggests that the two compounds are absorbed by different pathways. Initial results of glycine solutions in five patients suggest that they are absorbed well enough to be of practical therapeutic use. If subsequent results confirm this, glycine will be combined with glucose in an oral solution. It is expected that the combined glycine-glucose solution will be significantly more effective than the single substrate solution.

Sucrose is much less expensive and more readily available than glucose on the local market. It too will be substituted for glucose in the oral therapeutic solution and the results in patients assessed.

A field trial in the Matlab vaccine trial area hospital will be carried out using the previously-studied oral glucose solution to determine:

1. Whether adults with mild cholera (without shock) can be treated with oral therapy alone,

2. Whether oral therapy can be used effectively and economically under field hospital ward conditions.

Initial results, using a simplified treatment scheme, are extremely encouraging. Two physicians from the Epidemic Intelligence Service in Atlanta are expected to join Drs. Cash and Nalin in the supervision of the trial.

CI/P5 Effects of Dehydration and Hydration on Stool Composition and Transit Time in Pediatric Cholera.

The alteration of the composition of intestinal secretions from an adult pattern in the upper intestine to the pattern seen in pediatric stool has been found to take place in the colon, according to Mahalanabis. It is possible that the colon in the child absorbs sodium and secretes potassium more actively than that of the adult. This exchange may be related to transit time through the colon, which may in turn be related to the degree of hydration and the stooling rate. A study to investigate these interrelationships in different states of hydration is planned.

CI/P8 Intestinal Antibody in Cholera

A continuation of the previous year's studies is planned, utilizing jejunal aspirates instead of stool. This approach will reduce the amount of proteolysis which takes place before the specimen is collected and is expected to make the results of cholera intestinal immunologic studies more consistent with those reported in other diseases and in animals.

The radioimmunodiffusion technique will continue to be employed. In addition, coating of vibrios by intestinal antibody will be studied using a fluorescent antibody technique, and also using a technique in which rabbits injected with the coated vibrios serve to detect the antibody.

Rat intestinal washings, demonstrated by Dr. K.M.S. Aziz to inhibit toxin activity, will be investigated immunochemically using standard techniques.

CI/P23 Bacteriophage in Cholera Therapy and Prophylaxis

With the improved methods of phage production, larger amounts of phage will be utilized, with the goal of keeping the concentration of phage in the stool to over 10^{11} pfu/ml. In order to avoid possible effects of acid gastric secretions, the material will be infused by tube below the pylorus.

The prolonged transit time in persons without diarrhea may make phage prophylaxis against cholera based on the principle of lysogenesis, a possibility. If circumstances permit, a trial of phage prophylaxis in family contacts will be carried out.

CI/P28,29 Studies Utilizing Dog Thiry-Vella Loops

These studies will be pursued further by a number of investigators. Many of the variables and difficulties in the human intestinal flux studies can be controlled or avoided in the animal model. Volume and distension, collection techniques, sampling errors, bidirectional fluxes and segment isolation, as well as pore size and tracer transport kinetics will be investigated (Drs. Rohde and R. Hare).

Loops prepared acutely will be used to study the adjustment of the osmolarity of fluids by the small intestine (Drs. K. Hare and Nalin).

Studies of toxin-inhibiting or neutralizing substances will be continued with the aim of arriving at a technique which might have therapeutic use in human cholera (Drs. Cash and Nalin).

CI/P31 Age Specific Ionic Composition of Cholera Stool

This ionic composition of pediatric stool may change gradually to the adult pattern as the child matures, or perhaps may change rapidly under the influences of the hormonal changes occurring at puberty. Studies of pediatric and young adult patients are planned to investigate this point in order to provide a further basis for specific pediatric therapy recommendations.

CI/P30,34,37 Combined Jejunal Intubation and Purging Study in Family contacts of Cholera Patients

Many studies now attest to the fact that inapparent infection rates in a cholera-affected area are vastly higher than was previously thought, as many as 100 for each clinical case. The factors related to the subsequent development of disease are theoretically many in number. Intestinal antibody is presumably the most important, but other factors, including gastric acidity, the numbers and presence or absence of vibrios or other organisms in the upper intestine, and the concentration of various nutrients or nonimmunologic inhibitors (such as aromatic amino acids) may play a role. These factors will be studied in a collaborative study of family contacts of cholera cases, at least 27% of whom can be expected to have demonstrable infection. The reliability of jejunal aspirates to show cholera infection, as compared to purging and rectal swabs, will also be studied. Those patients who do develop diarrhea can be studied for stool composition and electrolyte losses during the initial stages of cholera.

CI/P2
Revised

P R O T O C O L

STUDIES OF

WATER AND ELECTROLYTE FLUXES IN HUMAN CHOLERA

by

Jon E. Rohde, M.D. and
R. S. Hare, Ph.D.

Study CI/P1 and CI/P3 have been incorporated into CI/P2 and the protocol revised as follows.

The bolus technique described in CI/P2 of Pakistan-SEATO Cholera Research Laboratory Technical Committee Report 1967 offers a method of studying unidirectional movement of electrolytes and water as well as other solutes. This technique may be utilized to measure unidirectional fluxes (CI/P1) or permeability characteristics (CI/P3) of the intestinal wall. The present proposal is to continue such studies with modifications and additions, but without a basic change in over-all technique. It is also hoped that an isolated segment can be studied in a way exactly analagous to the Thiry Vella loop in dogs (refer to protocol).

Patients selected for the study will be adults with severe cholera. They will be studied once during the acute phage of the disease and again when diarrhea has stopped. Thus, each patient serves as his own control decreasing the influence of the large patient-to-patient variability on interpretation of the results.

1. Studies of the unidirectional fluxes of sodium and water will be continued, in addition, the unidirectional flux of chloride will be investigated, since increased movement of chloride into the gut lumen could account for a greater fluid influx. Effects of alteration of perfusion rates and of the composition of the infusion solution on these various fluxes will be investigated, as well as the effects of cholera itself.

2. The measurement of intestinal permeability as described in Protocol CI/P3 1967 was not done last season but will be carried out this year.

3. An attempt will be made to control the volume of the study segment. Last year's studies indicated that while volume was poorly measured, it was larger in acute cholera than in convalescence. It is hoped that perfusion rates can be modified in such a way as to make the volume comparable in both studies.

Utilizing a six lumen tube with two balloons, a twenty centimeter segment of gut may be isolated for study eliminating effects of flow rate and variable volume. Markers above and within the segment would detect leakage during the 5-10 minute study period for each flux calculation. As complete collection avoids the sampling error inherent in the bolus technique, it could be most desirable to use both methods in the same patient to further compare the flux measurement.

In summary, unidirectional flux studies of water, sodium and chloride will be investigated as well as the permeability of the mucosa to molecules of different sizes. It is hoped that not only the bolus technique but also the isolated segment will be useful.

P R O T O C O L

WARD AND FIELD HOSPITAL TRIAL OF AN ORAL SOLUTION OF GLUCOSE AND ELECTROLYTES FOR MAINTENANCE THERAPY FOR CHOLERA IN ADULTS

by

Cash, R. A., M.D., Nalin, D. R., M.D.,
Rahman, A.S.M. Mijanur, M.B.B.S.
Reller, R., M.D., and Rochat, R., M.D.

Purpose

During the past year it has been demonstrated that an oral glucose-electrolyte solution can reduce the intravenous fluid requirements of adult cholera patients by 80%[1]. This form of treatment has not yet been used in ward conditions to treat large numbers of patients. The purpose of this study is two-fold:

1. To determine whether adults with mild cholera (without shock for hypotension) can be treated with oral therapy alone.

2. To determine whether oral therapy can be used effectively and economically by medical and paramedical personnel in the treatment of cholera patients in ward conditions.

Materials and Methods

Physicians, nurses and paramedical personnel participating in this study will be trained in the use of oral therapy at Dacca and at Matlab. Patients will be those with no complicating illness and who are not in another study. Darkfield will not be required. The composition of the oral solution will be as follows:

Meq. per Liter of Water*

Na	120	Citrate	15	Cl	72
K	15	HCO_3	48	Glucose	111 (mm/l)

The ingredients will be purchased from the local market and each batch will be chemically analyzed before weighing.

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1 "Oral Therapy for Cholera in Adults" Nalin, D.R., M.D., Cash, R.A., M.D., Islam, R., M.B.B.S., Molla, M., M.B.B.S., and Phillips, R.A., M.D., The Lancet, August 17, 1968, pp. 370-374.

* One gram of tetracycline will be added per fifteen liters for bacteriostasis.

On admission, alert patients without shock or hypotension will receive oral therapy alone. Patients with shock or hypotension will receive intravenous fluid until shock and hypotension are ameliorated. Oral therapy will be begun as soon as the patient is alert and may overlap with intravenous therapy in some cases. The oral solution will be given at the rate of 500-750 ml. per hour during the first four hours. If stool losses during the first four hours exceed the estimated amount, then intake will be increased up to 1500 ml. to match output. After the first four hours the oral therapy given for the next eight hours will be twice the volume of stool and vomitus lost during the first four hours of therapy. Subsequently, additional eight-hour oral intake will equal previous eight-hour output volumes, excluding urine. Tetracycline capsules (syrup in children), 250 mg. every six hours will be given to all patients. The oral solution will be kept at 40-45°C. Patients' vital signs and intake and output will be checked regularly and intake and output records will be recorded during the first four hours and thereafter at eight-hour intervals. Patients will be transferred to the convalescent ward when their stooling rate is 300 cc. or less during any four-hour period.

Discussed October 23, 1968
Revised October 24, 1968

P R O T O C O L

USE OF AMINO ACIDS AND SUCROSE IN ORAL THERAPY SOLUTIONS
CONTINUATION OF WORK ON ORAL THERAPY

by

D. R. Nalin, M.D. and R. A. Cash, M.D.

A. It has been demonstrated at this laboratory that an oral solution containing electrolytes and glucose can reduce the intravenous fluid requirement by 80% (1). It has long been known that amino acids increase intestinal sodium flux (2, 3, 4). This study is designed to determine whether glycine, one of the actively transported amino acids, is absorbed by cholera patients and whether a solution of glycine and electrolytes is useful as oral therapy for cholera. Glycine is readily available and inexpensive.

Patients admitted to the P-SCRL ward with acidosis and dehydration and a positive darkfield examination for cholera will be admitted to the study. Intravenous fluid (5:4:1) will be given until the blood pressure is normal and plasma specific gravity less than 1.030. The same oral therapy procedure will be followed as in the oral glucose study. Serum, stool and urine electrolytes and serum urea, creatinine and specific gravity will be analyzed every four hours. Results will be compared to the control patients (intravenous fluid alone), oral glucose patients and patients receiving a plasma electrolyte solution (no glucose or glycine added).

B. If glycine proves to be effective, it will be combined with glucose in an oral solution. Evidence suggests that each is absorbed by a different mechanism so they may prove to be additive.

C. Sucrose is much less expensive than glucose and more readily available on the local market. Sucrose will be substituted in an oral therapy solution and these results will be compared with those of the glucose oral solution.

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P R O T O C O L

EFFECT OF DEHYDRATION AND HYDRATION ON GUT VOLUME, AND CONCENTRATIONS OF ELECTROLYTES IN THE STOOL OF PEDIATRIC CHOLERA PATIENTS

by

Rahaman, M. M., M.B.B.S., M.Sc., Ph.D.,
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Twenty-eight cholera-positive children below five years of age were studied for fluid and electrolyte balance at the Pakistan-SEATO Cholera Research Laboratory during the year 1967-68. It was found that the children's stools on admission were relatively low in sodium (mean 90 meq/L) and high in potassium (K = 40 meq/L) compared to the published values for adults (Na = 140 meq/L, K = 15 meq/L). Similar findings were reported from the Naval Medical Research Unit No.2 in Taipei. Dr. Mahalanabis has shown that the change in Na and K takes place in the colon (personal communication). It is possible that the colon of the small child absorbs sodium and secretes potassium more actively than that of the adult. This exchange may be related to the transit time through the colon.

A study was conducted last year on one child who was allowed to purge under close observation without intravenous fluid replacement. Stool potassium rose and sodium fell slightly after six hours of dehydration. At this time plasma specific gravity was 1.030. The stooling rate was decreased. Measurement of transit time and gut volume through the small intestine and colon during active purging in a well-hydrated child may yield valuable information for comparison with the similar findings during the stage of decreased stool formation, i.e., when the child is allowed to dehydrate under observation.

The following is a revision of the protocol based on last year's results. Protocols CI/P6 and CI/P7 have been incorporated into CI/P5 since they are in essence controls for CI/P5.

Materials and Methods

Cholera-positive male children aged between five and ten years will be studied. As soon as a cholera suspected child comes into the hospital, he will be taken into the study room and after brief physical examination will be weighed and a blood and stool sample collected in the usual manner and analyzed for Na, K and other electrolytes. He will be hydrated with the Dacca solution, plus 1% glucose and maintained in fluid balance until the study

begins. No antibiotics will be given and only water allowed by mouth. Immediately after hydration, or as soon as the child is able to cooperate, a single lumen polythylene tube will be passed per os. As soon as the tip reaches the duodenum, BSP or PEG will be injected via the tube and subsequent stools will be collected and carefully timed until the bolus has passed. An estimate of mean flow rate may be made if input plus output is divided by two. Nothing will be given per os during the balance study.

From mean transit time and mean flow rate, volume can be calculated. Throughout the period of observation, except during measurement of transit time, hourly stool samples will be obtained by rectal catheter and analyzed for Na and K.

After the bolus has passed, the administration of fluid will be stopped, the child will be weighed and blood, stool and urine samples collected for electrolyte analyses. As soon as the specific gravity of the plasma becomes approximately 1.032, the measurement of transit time will be repeated. If time permits a second measurement of the transit time will be made. The maximum allowable limit of dehydration will be a plasma specific gravity of 1.034.

The child will be reweighed at the end of this period of dehydration and blood and stool samples obtained for Na, K and other electrolyte analyses. Intravenous fluid administration will then be started.

After the child is completely rehydrated, transit time will again be measured. During the period of rehydration, hourly stool samples will be collected for Na and K analysis. It is hoped that the duration of the study will be 24 hours or less. At the end of the study, the child will be weighed and a final blood sample collected and the tube removed. He may then be returned to the ward on a full diet and antibiotics.

Selected cases which are still purging a large volume of stool at the end of the study outlined above may be kept in the study and allowed food but given no antibiotics. The tube will be left in place and allowed to migrate to the terminal ileum as determined by X-ray. Transit time through the colon may then be measured during hydration and dehydration as described above.

P R O T O C O L

DETECTION OF INTESTINAL IMMUNOGLOBULIN RESPONSE TO CHOLERA INFECTION BY FLUORESCENT ANTIBODY METHOD

by

Northrup, R.S., M. D.

CI/P9-13 have been incorporated into CI/P8 since there has been found to be sufficient overlap in design and interpretation of results as to make individual projects of little meaning at this time and CI/P8 has been rewritten.

Previous work by a variety of investigators and investigating techniques have not been successful in consistently detecting the presence of antibody activity in the stool of cholera patients. Studies of cholera stool immunoglobulins have shown that a substantial degree of proteolysis has occurred by the time the proteins are excreted. This may be the reason for failure to demonstrate antibody activity. On the other hand, it may be that antibody activity assay techniques previous employed have been incorrect. IgA in other systems, for instance, has not bound complement: thus a vibriocidal test which requires complement binding would not be likely to demonstrate the presence of IgA antibody activity.

The most basic antibody activity is that of binding to the antigen. An assay system which will detect the occurrence of this process, without requiring the subsequent occurrence of a second process in order to detect the presence of the first, is thus the most likely to show the presence of antibody activity.

With the above considerations in mind, the indirect fluorescent antibody method, applied to jejunal aspirates, seems ideal. It avoids proteolysis by obtaining the secretions at a point nearest to their origin, and requires only the presence of an antigen-antibody complex for a positive result. The vibrios already present in the jejunum of cholera patients can serve as the antigen.

Plan

Jejunal aspirates obtained from cholera patients during the course of their disease (days 1, 2, 3, 4, 5, 6, 7, 10, 14, 21) and convalescence, will be examined under the Darkfield microscope for the presence of vibrios and cultured quantitatively for all organisms. The aspirates will be heated to 56° for 30 minutes to eliminate enzymatic activity, after which they will be either examined immediately, or frozen until use.

Specimens containing vibrios will be handled as follows. The specimen will be spun at 10,000 rpm (212,000g) x 30 min. and the sediment will be resuspended in a minimum of saline (the supernate will be treated as below); 2-3 drops will be placed on three slides and the slides air-dried. Two of the slides will be utilized for fluorescent staining, the other for gram-staining.

All slides for fluorescent staining will be incubated with fluorescent-labelled goat antiserum against IgA, IGM and IgG in a humid chamber for 30 minutes, after which they will be washed for 5 minutes in each of three changes of phospate buffered saline pH 7.5 and one change of 1/2 molar glycine in saline, covered with a coverslip and examined with the fluorescent microscope. The second will be stained first with unlabelled goat antiserum as above, washed in Phosphate-buffered saline, then stained with fluorescein-labelled rabbit antiserum against goat serum, washed with saline and glycerol, mounted and examined. The third slide will be examined by light microscope after gram staining.

Those specimens not containing vibrios on Darkfield examination will be spun, as above, and the sediment discarded. The supernates from the vibrio-containing specimens will also be handled as follows.

The sediment from a chloroformed overnight culture of vibrios (569 B strain) will be suspended in a minimum of 5% BSA in saline as with the above sediment, a few drops placed on a slide and the slide air-dried. After drying, the slides will be placed in a 60° oven for 15 minutes, to fix the vibrios to the slide. After this a few drops of the specimen will be added and the slide incubated for 60 minutes at 37°. Following this it will be washed three times in phosphate-buffered saline and glycerine.

From this point, the slides will be treated with labelled and unlabelled goat and rabbit antiserum as described above and examined under the fluorescent microscope. Controls will be:

1 - Specimens from non-cholera diarrheas.

2 - Use of blocking antisera.

All antisera will be examined for non-specificity both of antibody activity and staining activity, and absorbed if necessary. Commercial antisera will be used and labelled by us.

Stools from the patients will be collected at the same time as the jejunal aspirates, heated and stored frozen. When positive findings are obtained in the jejunal specimen, the stools will be examined for purposes of comparison. Also, dilutions of positive specimens will be prepared and examined similarly, for semi-quantitation of antibody activity.

P R O T O C O L

INVESTIGATION OF EXPERIMENTAL CHOLERA IN THE CANINE THIRY VELLA LOOP

Rohde, Jon E., M.D., and Hare, R. S., Ph. D

The demonstration by Sack et al. (2) that the canine intestine is susceptible to the diarrheagenic effect of cholera vibrio toxin and the subsequent finding of fluid production in the chronic Thiry Vella loop provide a large and convenient experimental model which can serve as its own control for the investigation of various pathophysiological aspects of cholera. A great deal has been written about the normal handling of H_2O and electrolytes in the Thiry Vella loop as well as the effects of various hormones, drugs and non electrolyte solutes and there is a large body of information about the normal function of the system. The availability of an isolated segment of normally functioning intestine to study removes many of the problems and criticisms attendant upon the use of the open ended system currently in investigational use in the intact patient. Thus, in the calculation of fluxes, instilled and recovered volumes can be accurately measured and luminal contents can be more easily controlled.

Preliminary studies indicate that with the use of non-absorbable marker and radioisotopes of H, Na and Cl, unidirectional fluxes can be accurately assessed in normal and infected dog loops, each dog serving as its own control, since there may be large differences in fluxes in different dogs. The same preliminary studies have also shown that BSP is not suitable marker in this preparation, since up to about 20% of it is apparently bound rapidly to the mucosa. PEG is currently in use and it is planned to investigate PSP.

The following experimental outline summarizes the studies envisioned.

1. Since the entire contents of the loop can be recovered we will do perfusion studies and fluxes by the bolus technique as used in patients (1967 CI/P2) collecting the total effluent rather than just port 2 sampling thereby enabling enabling us to verify the validity of the open ended bolus technique. Furthermore, validation of the bidirectional flux calculations using only one isotopic marker will be done through the use of both Na^{22} and Na^{24} for simultaneous measurement of both fluxes.

2. Although the production of frank luminal fluid in the cholera-affected loop does not begin for about 2 hours after exposure, it has been shown that the toxins are fixed almost immediately. Love (1) in the rabbit, has shown the first changes of cholera to be an

increased Na flux into the lumen. We will study simultaneous bidirectional fluxes of Na, Cl, and water at intervals over the entire course of cholera from exposure through maximal response to full recovery thereby delineating the sequence of changes in undirectional fluxes during experimental cholera.

3. The closed loop offers an ideal model to test the effect of luminal volume and distension on fluxes. We will test, in random order, an individual loop over a wide range of volumes both in the normal and the cholera-affected state. A positive correlation of volume to transfer rate would indicate a strong need for more uniform volume in our open ended perfusion system, while a negative correlation would further strengthen the conclusions obtained in the patient studies in which calculated segment volumes vary greatly.

4. Love (1) has recently described aberrations in the permeability characteristics of the cholera-infected rabbit ileum. Utilizing the methods of diffusion ratio described in 1967 TC protocol P3 we will investigate the effective pore size in the dog loop. As we will employ C^{14} tracer compounds in this study, we use small osmotically insignificant amounts, thereby reducing pressure gradients and solvent drag effects from water movement. This will also perform a useful trial for the proposed human study and should do a great deal to settle the persistent controversy over the role of intestinal permeability in cholera.

5. All our tracer studies measure the appearance or disappearance of a moiety in the gut lumen but do not indicate the kinetics or routing of its transport into the plasma. By cannulating the vein of a single arcade of isolated gut segment and sampling luminal contents simultaneously with effluent blood flow, we will determine both the flux from the lumen and appearance in plasma. If the functional intestinal wall acts as a unit membrane the curves should be complementary. Compartmentalization wherein concentrations are actively established leading to local diffusion gradients would cause a characteristic delay in the appearance of the isotope in plasma. All animals, facilities and equipment needed for this study are available at PSCRL.

In conclusion, the dog model offers an always available experimental system for investigating the pathophysiology of cholera and perfecting the techniques that will be used in human cholera patients.

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P R O T O C O L

OSMOLAR REGULATION OF INTESTINAL FLUIDS

by

Hare, W.K., M.D., and Nalin, D.R., M.D

The regulation of the osmolarity of extracellular fluid is heavily documented for many species of mammals. A change in the solute concentration of extracellular fluid initiates, through osmoreceptors, a reflex release of hormones that compensate for the aberration. Intestinal luminal fluids are not a part of the extracellular fluid but they are maintained at a solute concentration equal to that of extracellular fluid. If a hypertonic solution is introduced into the lumen of the small intestine its osmolarity falls until it is at the plasma level; if the solution is hypotonic its concentration increases. These changes are accomplished in spite of the nature of the solute, which may be nonabsorbable, absorbed by active transport or other means or be either organic or electrolytic.

The adjustment of the osmolarity of fluids in the small intestine of the dog will be observed in acute experiments in which the pattern of the venous and arterial arcades will determine the site and length of the intestinal loop chosen for study. A Southey-type plastic tube will be passed throughout the intestinal loop for introduction, mixing and sampling of the luminal fluid. Solutions of different strengths of sodium chloride, mannitol, urea and glucose will be placed into the loop. Cannulation of the venous return and a femoral artery will permit calculation of A-V differences and loop blood flow.

Total osmolarity, sodium, potassium, bicarbonate, chloride, urea and glucose will be determined on serial venous and arterial plasmas and on luminal fluid. The temporal course of the changes can be determined from curves representing the osmolarity of samples obtained during the study period.

P R O T O C O L

LOSS OF ELECTROLYTES THROUGH STOOLING AND CHANGES IN THE COMPOSITION OF STOOL IN THE EARLY STAGES OF PEDIATRIC CHOLERA

by

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Rahman, Waliur, M.B.B.S.

Introduction

A full balance study of diarrhea involves measuring the output and input of fluid and electrolytes during the course of the disease. Most of these studies have been carried out in patients who were admitted sometime after the onset of diarrhea. Balances were calculated on the basis of fluid and electrolytes retained by the patients until full recovery. This is usually a very laborious and exacting study, often involving days until the diarrhea stops. This may give rise to changes or mistakes being made while weighing and measuring the input and output. A better way of carrying out a balance study in diarrhea would be to follow a subject through the development of the diarrhea till he needs hospitalization. This would give an opportunity to measure the losses of fluid and electrolytes before a patient would normally enter.

Twenty eight cholera-positive children were studied in 1967-68 for the loss of fluid and electrolytes for a period of time up to 72 hours from the time of admission. However, it was not possible to calculate the amount of fluid and electrolytes lost before the patients were admitted to the hospital. A fair measure of the loss of fluid can be obtained by comparing the admission weight with the convalescent weight. However, the electrolyte composition apparently varies with the state of dehydration and stooling rate. This gives rise to unreliability in the values of lost electrolytes when the preadmission loss is calculated on the basis of admission values of electrolytes without following the patient to recovery. It would seem therefore that cases followed from the onset of disease will provide not only accurate information of the loss of fluid and electrolytes but also an opportunity to study the early pathophysiology and the changes in the concentration of electrolytes as the cholera progresses.

Material and Methods

It is hoped that this study can be combined with the family contact study being conducted by Dr. Barui, et al of Epidemiology Section. Family contacts of index cases will be brought to the hospital and kept for observation. Only contacts who develop diarrhea spontaneously i.e. without using a purgative, will be taken

for this study. It is probable that a few contacts will develop cholera while they are in the hospital, as in the 1967-68 season.

As soon as the contacts are brought into the hospital they will be assigned to a numbered bed and weighed on the special metabolic balance. Each bed will be provided with the respectively numbered bucket and urine container. It will be repeatedly emphasized to the subjects particularly the elderly members of the group that if any of them feels or becomes sick, the attending nurse or doctor must immediately be informed. If a small child becomes sick or wants to defecate he will pass stool into his own bucket, every care being taken not to spill the stool outside the container. For this purpose a few wooden frames will be made so that a child can squat comfortably and pass the stool in the eastern fashion. Co-operative children and elderly subjects can use the lavatory and pass stool in the bucket. It is imperative that the first burst of cholera stool is caught into the bucket. These initial stool samples will be sent for analyses of Na, K, and Cl.

Afterwards the patient will be transferred into a cholera cot, where rectal catheter samples of stool will be collected for analysis of electrolytes and total stool output carefully measured hourly. All vomitus and urine will be weighed and measured and samples sent for electrolyte analysis.

Blood will also be collected hourly for the specific gravity measurements, the maximum allowable limit being 1.034 after which intravenous infusion will be started. Clinical condition of the patient including temperature, pulse, B.P. and respiration will be followed.

As soon as the patient reaches a value of specific gracity of 1.034 and/or looks clinically very dehydrated, he will be weighed again on the balance and an arterial sample of blood obtained for pH and electrolytes with a simultaneous sample of stool. The patient will then be hydrated in the usual manner and returned to the ward.

P R O T O C O L

AGE SPECIFIC IONIC COMPOSITION OF CHOLERA STOOL

by

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Introduction

Studies of the ionic composition in the stool of children with cholera has revealed electrolyte losses which are substantially different from those found in adults (1-5). Fecal losses of sodium, chloride and bicarbonate are lower, and the fecal losses of potassium are higher than in adults.

Attempts to find an appropriate intravenous and oral fluid therapy have been modified by this understanding of these apparent differences. The age at which change in ionic concentration occurs is of significant theoretical, as well as practical, importance in the choice of either intravenous or oral therapy.

Method

The age at which change in ionic content of cholera stool occurs will be determined by analyzing cholera stool in children, adolescents and young adults. Included in this analysis will be several of the products of the metabolism of the Vibrio cholerae which have previously not been studied.

Stool, plasma and urine samples of bacteriologically-proven cholera patients aged 0-16 will be obtained on admission, post-hydration and every six hours for 48 hours. Each will be analyzed for specific gravity, osmolarity and electrolytes. In addition, stool will be analyzed for the NH_3, NO_2 and NO_3 products of vibrio metabolism. Patients will be treated in a routine fashion with intravenous 5:4:1, water in measured amounts and tetracyline. No food will be given during the study. Age, sex, date and time of onset of diarrhea, per cent dehydration, stool rate (c.c./kg./hr.), weight (on admission, post hydration and every six hours), nutritional status and bone age will be assessed.

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PROTOCOL

ADHERENCE OF STOOL IMMUNOGLOBULINS TO VIBRIOS DETECTED BY RABBIT IMMUNIZATION

by

Robert S. Northrup, M.D.

Introduction

The adherence of proteins to bacteria, detected by the subsequent immunization of rabbits with a coated bacteria, has been utilized to demonstrate the participation of various serum proteins in immune reactions with these bacteria. Beernink and Steward (1) showed antibody activity in the IgG and IgM factions of guinea pig serum against E. coli and also demonstrated the appearance of complement to E. coli. We hope in this study to demonstrate the adherence of stool immunoglobulins to Vibrio cholerae.

Methods

Prepare a liquid culture of vibrios (strain 569B) and allow to incubate for approximately 12 hours, thereby producing a maximum number of viable fresh vibrios. Prepare sufficient culture so that approximately 5 cc of packed vibrios can be obtained by centrifugation. Taking half of the liquid culture, kill the bacteria with chloroform and then spin down, washing the vibrios once with saline.

Taking the other half, centrifuge to approximately 2.5 cc of packed vibrios. Add an equal volume of methanol and mix. After 15 minutes spin out the vibrios and wash once with saline.

Take $\frac{1}{2}$ cc. of packed vibrios from each of the two preparations. Mix with 2 cc. of concentrated stool (ten times concentrated). Shake for one hour at room temperature, then centrifuge, discarding the supernate. Washing the resulting packed vibrios, presumably coated with stool immunoglobulins, six times in saline saturated with toluene.

After the final wash, suspend the vibrios in 2 cc. of saline. The procedure may be halted at this point and the saline vibrio mixture frozen until desired.

Mix the 2 cc. of suspended coated vibrios with an equal portion of Freund's adjuvant. Then inject 2 cc. of this antigen-adjuvant mixture into a rabbit, utilizing the foot pads and various other subcutaneous sites. Place the remaining 2 cc. of the mixture in the freezer.

Repeat the injection (2 cc.) after 14 days, this time utilizing only subcutaneous sites.

28 days following the initial injection bleed the rabbit thoroughly. The resulting serum will be tested by Ouchterlony double diffusion analysis against normal human serum, using various dilutions of the normal human serum to prevent a situation of antigen excess interfering with the precipitation of the antigen-antibody complex.

Various stool samples taken from patients at different stages of their disease, will be utilized, as well as jejunal aspirates.

Those sera showing precipitations against normal human serum will be tested by immunoelectrophoresis to determine against which component of serum the antibody activity is directed, and also in Ouchterlony alongside of known specific sera.

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P R O T O C O L

AN IMMUNOCHEMICAL APPROACH TO RAT INTESTINAL WASHINGS

by

Robert S. Northrup, M.D.

Rat intestinal washings (RIW) have been shown in preliminary studies by Dr. K.M.S. Aziz to inhibit the effect of Vibrio cholerae diarrhea factor on rabbit intestinal loops. This information, as well as the heat stability of the inhibitory principle, makes it seem reasonable to postulate that the active inhibitory substance is probably an immunoglobulin representing natural antibody in the rat gut against the cholera toxin. The series of investigations outlined here will attempt to confirm or refute this.

a) Precipitation of the RIW by ammonium sulfate 1.6 and 2.0 molar. Measurement of solid and nitrogen content of the precipitate. Assessment of the inhibitory activity of the precipitate.

b) Lyophilization of the RIW. Measurement of protein (i.e. nitrogen) content of the material per milligram of lyophilized material.

c) Electrophoresis in agar of the lyophilized material, staining the resultant pattern for protein.

d) Preparation of antiserum against rat serum proteins in rabbits, also against RIW and RIW precipitate in rabbits. Analysis of RIW using immunoelectrophoresis with these sera.

e) Double diffusion in agar of RIW against cholera toxin, also of RIW ammonium sulfate precipitate against cholera toxin.

f) Sephadex and DEAE chromatography of RIW with analysis of fractions using immunoelectrophoresis and measurement of toxin inhibitory activity.

g) Starch block electrophoresis of RIW, with analysis of fractions as in (f).

CI/P34
Formerly E/P5

PROTOCOL

PURGING OF FAMILY CONTACTS

R. K. Barui, M.B.B.S.

RESEARCH PLAN

Specific Objectives

To do a comparative study between multiple rectal swabs and purging as the effective technique for the demonstration of Vibrio cholerae in the family contacts of cholera patients.

Method of Procedure

Soon after admission to the P-SCHL hospital and after the diagnosis of cholera is confirmed by the bacteriology laboratory, a study team consisting of one doctor and a lady field assistant will visit the houses of the cholera patients and bring as many people as possible from the family (definition of a family: persons who live and eat together) to the P-SCHL hospital on the same day. An initial rectal swab from each family member will be collected and streaked directly on Monsur's media (Na - taurocholate, K - tellurite and gelatin), gelatin agar and kept in bile peptone enrichment broth for culture for Vibrio cholerae. 50 lambda blood from the fingertips will also be collected for vibriocidal antibody determination by the microtiter mehtod.

All family members will be placed in two groups alternately. To one group, mannitol in water (dose ranging from 15 gm. to 60 gm. depending on age) following an ounce of butter will be given as purgatives. The subsequent four stool samples will be collected in sterile bottles and sent to the bacteriology laboratory for culture for Vibrio cholerae. A daily rectal swab will also be collected from them during their stay in the hospital. From the other group, only a daily rectal swab will be collected during their hospital stay.

All members will be hospitalized for five days.

A second blood sample will be collected from all the family members on the tenth day.

Cholecystokinin may be used instead of a purge.

Background of the Study

Previous studies have demonstrated the value of purging in detecting inapparent infection. Different workers have indicated that family contacts of the patients are uniquely at risk. The present study will help to compare the effectiveness of purging, whole stool examination, and multiple rectal swabs, as a technique to demonstrate the presence of cholera vibrios in the gut.

Facilities Available

During the coming cholera season, we expect sufficient numbers of cholera cases in the P-SCRL hospital whose families can be studied. Bacteriology Section will process the routine culture for Vibrio cholerae and the accommodation for the family contacts can be arranged in the convalescent ward.

SUPPORTING DATA

Previous work done on this Project

During the last cholera season, 46 contacts from 12 families with confirmed cholera patients admitted to the P-SCRL hospital, were purged. Of these 46 contacts, only one person was positive on the initial rectal swab, 3 persons were negative on the initial rectal swab but positive after purging, 5 persons were found negative on both initial rectal swab and on purging, but were positive on subsequent daily rectal swabs.

Results Obtained by Others

Gangarosa et al (1966) reported that 8 (21%) of 38 patients purged 1-2 weeks after their last routine positive stool culture were vibrio positive. Wallace et al (1967) reported that 2 of their 28 cases were found to be vibrio positive after purging and duodenal intubation.

Budget

Cost of Material	₨ 650
Food expenses for family contacts	₨ 1,750
Salary of field team	₨ 3,000
	₨ 5,300

P R O T O C O L

COMBINED JEJUNAL INTUBATION AND PURGING STUDY IN FAMILY CONTACTS OF CHOLERA PATIENTS

by

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Huq, I., D. Bact., Dey, C.R., M.Sc., and
Rahaman, M.M., M.B.B.S., M.Sc., Ph.D.

Many studies now attest to the fact that inapparent infection rates in a cholera-affected area are vastly higher than was previously thought, as many as 100 for each clinical case. Conversely, only a few of these persons actually develop clinically apparent cholera. The factors which may play a role in the acquisition of infection and the subsequent development of a case are theoretically many in number. Upper intestinal antibody may play a major role in protection, particularly in an endemic area such as Dacca. Serum antibody has been shown to correlate with protection, and one must presume that its activity is in the intestine wherein the organism resides.

The presence of other bacteria in the upper intestine, particularly coliforms, may inhibit the development of an infection or case. Most coprophagic animals will not acquire cholera while man, whose upper intestine is normally not exposed to coliform organisms, is susceptible. Social and eating habits in Pakistan are different from those in Western countries and may influence the upper intestinal flora. Alternatively, many organisms have been shown to depend on the presence of a concomitant neighbour organism for maintenance of infection. Thus the presence of coliforms or other flora in the upper intestine may promote the growth of vibrios. The size of the vibrio innoculum could be estimated indirectly by the number of vibrios present in the intestine of family contacts at the time of their arriving at the hospital. Innoculum size must also play a role.

The vibrio has always seemed particularly susceptible to gastric acid, having as it does, a predilection for an alkaline medium. Family members of cholera cases allow a prospective determination of the role of this factor.

Finally, recent verbally-reported bacteriologic and biochemical studies suggest that the V. cholerae diarrheogenic toxin production in the bacterium is inhibited by the presence of moderate concentrations of aromatic amino acids in the culture medium, tryptophan and tyrosine in particular.

Family contacts of cholera patients have a high incidence of infection. Intestinal intubation and sampling from the stomach and the jejunum and ileum will allow the factors mentioned above to be further elucidated. These same patients may also be purged and rectal swabs done, to correlate the upper intestinal findings with the ability of these more routine techniques to demonstrate infection.

Family members who develop diarrhea while under observation in the hospital also provide an opportunity to follow stool composition and electrolyte losses in the initial stages of cholera.

This protocol (CI/P35) presents the methods for handling the overall collaborative aspects of the study. The four protocols (CI/P30, 34, 36 and 37) present the techniques to be used in each particular substudy.

Plan of the Study

Soon after admission to the CRL hospital ward, and after the diagnosis of cholera is confirmed by the bacteriology laboratory, a study team consisting of one doctor and a lady field assistant will visit the houses of the cholera patients, bringing as many people as possible from the family to the CRL hospital on the same day. For purposes of this investigation, a family will be considered to include persons who live and eat together. After the family member reaches the CRL ward, an initial rectal swab will be collected and streaked directly on Monsur's media, gelatin agar and also used to inoculate bile peptone enrichment broth for culture of V. cholerae

Family members over the age of 12 years will then be given an oral tube. This will be a two-lumen tube, one lumen of which will be connected to a small, rubber, mercury-containing bag, the other lumen of which will end in a collection port located approximately 10 cm. above the bag. The tube will be of sufficient length to reach into the ileum (in adults approximately 250 cm.). While the tube is in the stomach, a sample will be obtained for estimation of total titratable gastric acidity and pH.

The tube will then be allowed to pass into the jejunum. After the mercury bag has passed the pylorus it may be inflated with a small amount of air, to encourage its further passage into the intestine. After the tube has passed well into the jejunum, a sample of intestinal fluid of at least 50 c.c. will be obtained. Five c.c. of this sample will be rushed to the bacteriology laboratory for quantitative determination of aerobic and anaerobic flora, including V. cholerae. The other 45 c.c. will

be frozen at -70°C and stored until analysis for coproantibody and aromatic amino acids can be performed. The tube will then be allowed to progress into the ileum and another sample obtained there. This sample will be handled in the same fashion as the jejunal sample. The tube will then be pulled back so that the port will lie approximately at the ligament of Treitz or the upper jejunum and a fatty meal administered. Following the fatty meal, a sample of 5 c.c. at least will be obtained and rushed to the bacteriology laboratory for qualitative vibrio determination.

Mannitol in water will then be administered to half the patients in a dose ranging from 15 to 60 grams depending on the age of the patient. The first four stool samples obtained as a result of this purgative will be collected in sterile bottles and sent to the bacteriology laboratory for qualitative V. cholerae culture. The tube will then be pulled back to the stomach, gastric acidity again determined and a histamine gastric acid stimulation test performed. After these gastric samples have been collected, the tube will be removed.

On admission, a 50 lambda blood sample from the finger tip will be collected for vibriocidal antibody determination by the microtiter method. Family members will be kept in hospital for five days during which daily rectal swabs will be obtained.

The patient will be discharged on the fifth day. A second blood sample will be collected from all family members on the tenth day.

While in the hospital patients will be requested to place all stool in buckets provided for that purpose, and to notify the ward personnel when liquid stool begins. From patients who do pass stool, samples will be obtained from the rectum by rectal catheter and sent to Chemistry Section for electrolyte analysis.

P R O T O C O L

JEJUNAL ASPIRATE STUDY

by

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Studies of the small intestinal juice and mucosa in man have shown that it is normally either sterile or contains low numbers (10^1-10^3) of gram positive organisms such as streptococci, aerobic lactobacilli and fungi (1, 2). During malabsorption (3), diarrhea (4), or other abnormal intestinal states, the small intestine frequently becomes inhibited by bacteria normal to the large intestine, such as E. coli, while no other pathogenic organisms can be found. This study is designed to investigate this point in the frequent nonspecific diarrheas seen on our ward. In conjunction with the combined family contact study, studies of family members of cholera patients will also be undertaken, as noted in that protocol. The methods used are those of Gorbach (1).

Plan

On admission, approximately 5 c.c. of blood will be drawn for titer. The patient will be treated in the usual manner except that no antibiotics will be given. A polyvinyl tube will then be passed into the upper jejunum, well past the ligament of Treitz and its position ascertained by demonstrating a return of alkaline fluid, or by X-ray. The tube may be cleared by injecting 5-10 c.c. of sterile saline, but no fluid should be injected for a period of at least 15 minutes prior to obtaining the sample. The patient may take a light diet as the tube moves into place.

At the time of sampling (2-4 hours after a meal) a volume of fluid equal to one tube dead space (usually 3-5 c.c.) will be withdrawn and discarded. The sample will then be obtained, and immediately taken to the Bacteriology Laboratory where it will be quantitatively cultured both aerobically and anaerobically. A sample of stool will be obtained during this period, by catheter if the patient is passing liquid stool, and also rushed to the Bacteriology Laboratory for quantitative cultures. Solid stool will be diluted in the Bacteriology Laboratory and also cultured quantitatively.

Samples will be obtained in this manner during the first 24 hours in hospital and every 2-3 days thereafter, including at least two samples after the patient has stopped passing liquid stool. Patients who do not show a return of their

intestinal floral pattern to normal will be cultured in the above manner at frequent intervals over a prolonged period, at the discretion of the investigator. Blood samples for titration against the patient's own organisms will be obtained both on admission and discharge (see above - at least 5 c.c. blood) and also at intermediate intervals, if the patient is kept in hospital for a prolonged period.

In the Bacteriology Laboratory quantitative as well as qualitative determinations will be performed on all specimens. Serial dilutions of each fresh specimen will be made and plated on media for the isolation of enteric pathogens and on other special media for assay of fecal and small bowel contants. Both aerobic and anaerobic techniques will be employed. Standard bacteriologic, biochemical and serologic methods will be used in the isolation and identification of each organism. Selected organisms will be preserved for confirmation of identification, or further identification procedures, by other bacteriologic laboratories.

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P R O T O C O L

GASTRIC ACID STUDIES IN CHOLERA CASES

by

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The opinion that gastric acid is the first line of defence against Vibrio Cholerae has long been maintained. It is said that those persons whose gastric acidity is low are susceptible to cholera. Certainly vibrios do not survive well in acid environments, yet whether the patient actually is a low acid secretor chronically, or only temporarily at the time of infection, is unknown. Even in persons secreting adequate acid, the number of vibrios ingested may be too great for all to be killed. For these reasons it seems worthwile to study the gastric acid pattern in the following instances.

Cholera Cases

Cholera cases may be studied after rehydration, or after the diarrheal phase is over, during convalescence. Patients giving a low result may be studied after diarrhea has ceased, in the former case, or after a further period, in the latter case. Lindenbaum has found that the malabsorption of acute cholera diarrhea does not continue into the post cholera period. This may also be so with regard to gastric secretion. Patients treated with tetracycline will not be studied.

Family Contacts

Family contacts of cholera cases will be studied as per the combined Family Contact-Jejunal Aspirate Study. For details please refer to that protocol.

Normals

Family contacts will serve as normals, although normals other than the family contacts may be required.

Methods

The gastric acid studies will be done using two drugs, depending on availability:

1. With Histamine Stimulation
2. With Penta Gastrin (Pentavalent - ICI)

As there are some side effects with histamine it is better to use pentagastrin. Side effects of histamine during the tests are: headache, running of the nose, flushing of body, but with pentagastrin, slight hot feeling is sometimes observed.

Both histamine and pentagastrin can be used either with continuous infusion or with a single dose subcutaneously. As there are variations of opinion with the single dose method, it is better to do the test with continuous infusion method. At least initially, we will plan to use the single dose method, until a suitable pump becomes available.

Doses

Histamine: 40 microgram/kg/hour

Pentagastrin: 5 microgram/kg/hour (Kay et al.)

Instruments Required

1. Continuous aspirator - one (in stock)
2. Continuous infusion pump - one (in stock)
3. Automatic titrater - one (may be done manually).

Procedures

For gastric acidity studies alone, the test will be done as follows: The naso-gastric tube is passed in the morning after overnight fasting. Usually the tube is passed upto 58-60 cm. length. The patient is kept on his back or his left side. If there is any doubt about the position of the tube plane an X-ray of the abdomen will be taken. After the tube is placed in the correct position, the basal collection is started with the continuous aspirator. The basal secretion is collected for 30 minutes, after which the stomach is completely empty. This, multiplied by two, gives the hourly value.

When the basal collection is completed, the pentagastrin or histamine is started with the continuous infusion pump. After the infusion is started the aspirate is collected at 15- minute intervals. In this way 4-6 samples are collected and the volume, p^H, acid in meq/hour and acid meq/liter for each sample are estimated.

Family contacts will be handled as in that protocol. Collections for basal studies will be made just after the tube is passed. Stimulation studies will be performed after the other samples have been collected from the jejunum and ileum.

EPIDEMIOLOGY SECTION REPORT FOR THE 1968 TECHNICAL COMMITTEE MEETING

Introduction

The 1968 fiscal year was characterized by the virtual absence of cholera in Dacca and in Matlab during the usual cholera season which extends from October through January. Instead, sporadic cholera cases began to occur during February and a smaller cholera season was seen in both areas during March through June. Thus, there were not sufficient cases to yield results in some major prospective studies, such as the second year of observation in the 1966-67 vaccine field trial (E/P12), or in the prospective rural and urban community studies set up by Dr. Martin (E/P1 and E/P2). The absence of cholera was not altogether detrimental to the epidemiology program as it provided a "control" year for evaluating the specificity of serological techniques in detecting inapparent infections in community studies (E/P9, E/P10, E/P11).

During the 1968 fiscal year a number of consultants assisted in the epidemiological program. Dr. Willard Verwey and Mr. Howard Williams from the University of Texas Medical Branch, Galveston, came to Dacca and carried out a collaborative study comparing the microtiter and macrotiter systems for assay of vibriocidal antibody. Based on these studies, a decision was made regarding the preparation of reference serum. Drs. Eugene Gangarosa and William Martin from the National Communicable Disease Center, came in January of 1968 to carry out purging studies of convalescent cholera patients. Dr. Robert Armstrong, an EIS officer from NCDC, came in January and February of 1968. Because of lack of cholera cases at this time, he conducted field studies in smallpox in the Dacca and Matlab areas.

This year, Drs. William McCormack and Albert Martin, epidemiologists assigned from CDC, completed their tours of duty in Dacca and returned to the States for medical residencies. Replacing them for two-year tours, are Drs. Kenneth Bart and William Woodward, who joined the Laboratory in June, 1968.

In February, 1968, Dr. Yutake Zinnaka, a WHO consultant, came to Dacca to learn the techniques of the vibriocidal test by the microtiter system which we use. He then went on to the Cholera Research Center in Calcutta and the laboratories in the Philippines to introduce this technique.

PROGRESS REPORT ON

EPIDEMIOLOGY RESEARCH PROTOCOLS

E/P1 Dacca Comparative Urban Cholera Study

E/P2 Naogaon-Nagda-Durgapur Rural Cholera Study

These two prospective community studies, utilizing combined serological and bacteriological techniques, were established by Dr. Martin to more precisely define the epidemiological pattern of cholera. Because of a lack of cholera cases during the 1967-68 cholera season, no results were obtained in this study. Intensive surveillance in these communities has been discontinued, although the base line sera that were collected have been saved for reference purposes if cholera appears in these communities.

E/P3 Antibiotic Treatment Study - Matlab Hospital 1967-68

This study was not carried out during the 1967-68 cholera season because of insufficient cases. It has been initiated during the 1968-69 cholera season.

E/P4 Intradermal Cholera Vaccine Studies

These studies, which have been carried out in the Sylhet tea gardens, have, so far, given conflicting results. The first study suggested that intradermal vaccination was of relatively little value in childhood, although it was as good as 1 ml. or 2 ml. of cholera vaccine given subcutaneously in adults. Presumably this suggested that a large inoculum of vaccine was needed in children as a primary dose, whereas small doses were as effective as large doses for boosting immunity in adults. An attempt to repeat this study showed that intradermal vaccine given either by syringe and needle, or by jet gun using the intradermal head, was less satisfactory than 1 c.c. of cholera vaccine given subcutaneously in producing a vibriocidal antibody response in adults. Further studies in larger population groups are planned.

E/P5 Purging of Family Contacts of Cholera Cases

Family contacts of cholera patients were hospitalized as soon as the index case was identified. An initial rectal swab was obtained. They were then purged with either mannitol, magnesium sulfate, or mannitol following a fatty meal, with the purged specimens being examined bacteriologically. Thereafter the contacts were examined by rectal swabs for the subsequent five days while they remained in the hospital. A total of 200 family contacts was studied, of whom 54, or 27%, were proven to be positive by one of these techniques. Among the 54 positives, the single, initial rectal swab taken on arrival at the hospital, was positive in only 17 (31%). The purged specimen was positive in 29 (54%). This included almost all of those who were positive by initial rectal swabs. The rectal swab taken the day following purging was positive in 35 (65%) of the 54 that ultimately were proven to be positive. Serial rectal swabs for the next four days picked up the remainder that had been missed by the preceding procedures.

This study indicates that no single procedure is ideal for the detection of inapparent infections. A surprising observation was the poor results obtained by the examination of the purged stool and the better results obtained by a rectal swab taken the day following purge. It appears that purging is probably not any more efficient than multiple rectal swabs in detecting inapparent infection, but that none of these techniques are really satisfactory for determining the frequency of inapparent infections.

E/P6 An Assessment of the Risk of Hospital and Laboratory Infections with Vibrio Cholerae

One hundred employees of the CRL were selected for this study. Approximately 30 worked in the Administrative Section, 40 in the laboratories and 30 had direct contact with cholera patients on the ward. Fingertip blood specimens were taken for vibriocidal titers in July, 1967, November, 1967, April, 1968 and June, 1968. These four specimens were titered simultaneously following the last collection. The antibody titers in 99 of these employees were quite constant for the four collection periods, the variation in titers being not more than two-fold (1 tube) between the various collections in 97 of the subjects. The remaining two showed a four-fold variation in titer which was thought to be within the limits of the test system. One individual, an aide-nurse on the ward, had a sixteen-fold rise in titer between the April and June collections. She denied

taking any cholera vaccine during this period. Since this was the time that the maximum number of cholera cases was being seen on the ward, it is felt that this represents serological evidence of inapparent infection in this staff member.

E/P7 Long-Term Serologic Response to Cholera Infection

Long-term serological studies were carried out on 130 cholera patients hospitalized at the Matlab hospital during the 1966-67 cholera season. Bloods were obtained on admission, at 7-10 days and at 1 month, 2 months, 3 months and 6 months following admission. Overall, 88% of the patients showed a four-fold or greater rise in vibriocidal titer 7 to 10 days after admission. These titers fell rapidly with time. The percentage with vibriocidal titers four-fold or greater higher than the admission titer fell to 60% by one month, 35% by two months, 20% by three months and 17% by six months after admission. Because of the rapid fall in titer, particularly in the age group under 5, the results suggest that in order to have a high percentage of the population with sustained titers such as we see in Matlab, not only must the infection rate be extremely high, but also individuals must be repeatedly reinfected. Another striking finding of the long-term serological studies was the fact that children who received only a single injection of cholera vaccine had a better long-term antibody response than children who had been infected with Vibrio cholerae.

E/P8 Serologic Study of Bacteriologically-Proven Cholera Cases At Various Intervals After Illness

This study consisted of obtaining a blood specimen from cholera patients and family contacts who had been previously studied in family studies, after an interval of six months to two years following the acute period. Titrations of these sera revealed that both the hospitalized cholera patients, as well as the family contacts who had confirmed inapparent infection at the time of the index case, continued to have vibriocidal titers significantly higher than were found on the initial blood specimen taken either at the time of admission to the hospital, or at the time of initiation of the family study. Family contacts who did not have inapparent infection at the time of the family study showed no change in titers in these late serum specimens. Of particular interest was the observation that family contacts who had had inapparent infection and had been shown to have a distribution of antibody titers lower than uninfected family contacts at the

time of initiation of the family studies (when the index case was hospitalized), now had a distribution of titers higher than the uninfected contacts, suggesting that the inapparent infection with Vibrio cholerae had effectively enhanced the immunity of the infected individual.

E/P9 Detection of Reinfection with Vibrio Cholerae in the Matlab Vaccine Trial Area

This study involved the collection of paired serum samples during the 1967-68 cholera season on 241 individuals who had had bacteriologically-confirmed cholera during the previous years. The serum specimens were collected in October, 1967 and February, 1968. The timing of collections was to include the usual cholera season. However, since cholera did not appear in the area the study can only be used as a control for methodology. Since 59 of the cases had received cholera vaccine as a part of the vaccine field trial, they will be excluded from the analysis. Of the remaining 182 serum pairs obtained, 117 (64%) showed no change in titer, 25 (13.7%) showed a two-fold rise and 30 (16%) showed a two-fold fall, 5 (3.6%) showed a four-fold rise and similarly 5 (3.6%) showed a four-fold fall. Since there was no cholera over the time period that the serum pairs were collected, the differences are indicative of the variations that can be expected with the methodology utilized.

E/P10 1966 Cholera Vaccine Field Trial - 1967-68 Serologic Surveys

The 1967-68 field trial, as described in protocol E/P12, was designed to test the effect of booster inoculations in the population participating in the 1966-67 vaccine study. Serological surveys were taken in September, 1967 prior to the booster injection, in November, 1967 approximately one month after inoculation and in February, 1968 approximately three to four months after inoculation. The serological survey taken prior to the booster inoculation indicated that there was still a significant difference in the mean titers of the groups who received one or two doses of cholera vaccine one year previously, as compared to the control group, particularly in children over the age of 5 years. The booster inoculations produced a definite improvement in the antibody titers at the end of one month. This was most striking in the children under 5 years. Three months following inoculation, the serological survey suggested that the groups that received the booster injections would have had protection ranging from 60-70%, while the group that received

two doses of cholera vaccine a year previously, but was not boosted, would have only shown 25% protection in children under 5, but about 50% protection in children over the age of 5. Because of insufficient cases occurring during the cholera season, it was not possible to correlate the serological findings with the actual protection obtained in the field trial.

E/P11 A Community Study of Inapparent Cholera Infections -1967-68

This is a continuation of the study initiated in 1966 in Meheran village. In that year, paired serological specimens taken before and after the cholera season, as well as multiple rectal swab cultures of all diarrheal cases, revealed five bacteriologically-confirmed mild diarrheas due to Vibrio cholerae and at least 22 serological conversions, suggestive of inapparent infection with Vibrio cholerae. In the second year of observation, the identical protocol was followed over the same time period extending from November through February. During this time there were no cases of cholera in the Matlab area and no infection detected by rectal swabs in the Meheran village population. The paired serological survey on the population revealed that there was no significant change in the antibody titers from November through February. Thus, this second year of observation with negative bacteriological and serological results, provides excellent control data for a comparison with the positive observations of the first year, thus confirming the usefulness of longitudinal serological studies in communities to detect inapparent infection with Vibrio cholerae.

E/P12 The 1966-67 Cholera Vaccine Field Trial - Second Year of Observation

The plan for the 1967-68 cholera season was to test the duration of protection of the vaccine used in the 1966-67 field trials as well as the effect of a booster dose. In the 1966-67 field trial there were three vaccine groups: the control group, consisting of approximately 10,000 children who received two injections of diphtheria-tetanus toxoids; the 1-dose vaccine group, consisting of approximately 10,000 children who received one injection of cholera vaccine and a second injection of diphtheria-tetanus toxoids; and the 2-injection vaccine group, consisting of approximately 20,000 children who received two doses of cholera vaccine. For the 1967-68 cholera season, booster inoculations were given to the 10,000 children who received one dose of cholera vaccine and to one half of the children who received two doses of cholera vaccine.

The remaining 10,000 children who received two doses of cholera vaccine, as well as the 10,000 children in the control group, received an injection of the control vaccine. A sample of 1,000 children was selected for serological surveys prior to inoculation and one month and three months following inoculation.

The vaccine program was quite successful in that over 97% of the 40,000 children received their booster inoculations. The cholera season was quite late and there were only a total of 32 cases in the vaccinated population, occurring 5-8 months after inoculation. The distribution by vaccine groups was as follows:

Group 000 - 12 cases	Group XXO - 8 cases
Group XOX - 7 cases	Group XXX - 5 cases

(Vaccine O is the control vaccine and Vaccine X is the cholera vaccine.) While there were not enough cases to make the differences statistically significant, the data suggests that there probably was some protection achieved by the booster inoculations, but that during the time period that the cases were occurring, the protection was not substantial.

E/P13 Program and Protocols for the Immunology Laboratory

E/P13 - 1 Quantitation of Total Protein and Immunoglobulins in the Acute Cholera Stood

This study was carried out as a continuation of efforts to determine the immune mechanisms operating within the intestinal lumen. The principal findings were: in 25 cholera patients, severely dehydrated on admission, the mean admission serum protein was 9.9 grams per cent, the levels of immunoglobulins were: IgG, 2,742 mg. per cent, IgA 378 mg. per cent, IgM 231 mg. per cent. All of these values are markedly elevated reflecting the patient's dehydrated condition on admission. The mean value for the serum protein on convalescence was 6.8 grams per cent. In the stool of these 25 patients, the mean stool output in the first 24 hours was 7.4 liters. The mean total protein content was 126 mg. per cent. IgG was 12.6 mg. per cent, IgA 21.9 mg. per cent and IgM 7.1 mg. per cent. This study confirms the preliminary results indicating that the predominant immunoglobulin in the cholera stool is IgA and that there was no specific relationship between the serum and stool immunoglobulin content. An interesting observation in these patients was that there appeared to be no correlation between the rate of stooling and the total protein or the concentration of immunoglobulins found in the diarrheal stool.

In four acute diarrhea patients, not due to Vibrio cholerae, who put out between 3.9 and 7.8 liters in the first 24 hours, the total protein and immunoglobulin content of the stool was similar to that seen in cholera. The total protein was 227 mg. per cent, IgG was 3.25 mg. per cent, IgA was 67.8 mg. per cent and IgM was 20.4 mg. per cent. In two patients with acute diarrhea of a more exudative type, the stool output was much less, being only 100 and 600 ml. The total protein content was higher, being 1092 and 987 mg. per cent and the predominant immunoglobulin was gamma G, being 92.5 mg. per cent. Gamma A was 52 mg. per cent and gamma M was 21.5 mg. per cent. It is probable that in the exudative diarrheas there is leakage of serum proteins into the stool, in contrast to that seen in cholera and similar acute nonspecific diarrheas.

In examining the jejunal aspirates from eight cholera patients during convalescence (after heating to inactivate proteolytic enxymes), the total protein averaged 232 mg. per cent. The immunoglobulins were IgG, 11.6 mg. per cent, IgA, 3.8 mg. per cent and IgM 12.6 mg. per cent. There was considerable difficulty in handling the jejunal secretions because of proteolytic enzymes causing breakdown of the immunoglobulins but it would appear from these studies that IgA was not the predominant immunoglobulin in the normal jejunal secretions, in contrast to the findings in acute cholera stool.

E/P13 - 2 Further Studies of the Characteristics of Vibriocidal Agglutination and Vascular Permeability Factor Neutralizing Antibody

Continuing immunochemical studies to determine the antibody response to cholera reveal that there is both a qualitative as well as a quantitative difference in specific and "natural" vibriocidal antibody. It has long been known that "natural" vibriocidal antibodies can be found in normal human blood, particularly in adults. Serological surveys have indicated that these titers are much lower than are seen in persons who have specific response to either cholera vaccine or cholera infection. Immunochemical studies now reveal that the antibodies are also qualitatively different. The "natural" vibriocidal antibodies in 14 sera from natives of the U.S. and Czechoslovakia have been shown to be exclusively 19S immunoglobulin by density gradient studies, whereas persons who have had specific stimulii with cholera vaccine or clinical cholera, have both 19S and 7S vibriocidal antibodies. Efforts are being made to develop a simple technique for titering only 7S antibodies in order to have a more specific serological test for cholera.

Dr. Eugene Gangarosa of the NCDC is collaborating in this study with particular reference to identifying other organisms that produce nonspecific vibriocidal antibody titers. He has demonstrated that patients infected with Brucella develop high vibriocidal antibody titers. We have obtained some of this sera, and fractionation studies are underway to determine whether this is due to both 19S and 7S immunoglobulins.

E/P13-3 Studies of Opsonizing Antibody in Cholera Serum

Considerable effort has been devoted to establishing assay techniques for opsonizing antibodies in cholera. Initially, studies were carried out in an in vivo model suggested by Dr. Derrick Rowley involving the injection of vibrios previously inoculated with antibody into the peritoneal cavity of mice and determining the vibrio count immediately and at 40 minutes after inoculation. The fall in viable count presumable would represent the organisms that were opsonized, phagocytized and killed by the mouse peritoneal macrophages. Several serum pairs from cholera patients were titered utilizing this technique, and opsonizing antibody titers were found which paralleled the vibriocidal antibody titers; further in fractionating sera, the activity was found in both gamma M and gamma G fractions. One difficulty with this procedure has been the failure to demonstrate satisfactorily by direct microscopic examination of the peritoneal contents, that the vibrios are being ingested by the mouse peritoneal macrophages, rather than just being killed by a direct vibriocidal reaction activated by complement that may be present in the mouse peritoneal cavity.

In order to obviate this problem an in vitro system was developed, utilizing the rabbit peritoneal microphages obtained following glycogen stimulation. These microphages are harvested, washed and a standard suspension placed in a series of tubes and then inoculated with antibody-coated vibrios. Opsonization and phagocytosis can be demonstrated both by a fall in the viable bacteria count as well as by direct microscopic examination of the stained cells. Several serum pairs studied by this technique revealed inconclusive results. Most serum pairs have very low or negligible titers of opsonizing activity. These studies are continuing.

E/P13 -- 4 Cytological Studies of Cholera Stool and Intestinal Secretions with Reference to Phagocytic Cell Population

Preliminary observations of the stool and intestinal secretions of approximately ten patients indicated that the cell population was quite low. The vast majority of the cells seen in the cholera stool represented columnar epithelial cells. Only occasional phagocytic cells were seen, usually located in mucous threads. Because of the rarity of phagocytic cells, it was not possible to demonstrate that they were active in ingesting cholera vibrios.

E/P13 - 5 A Survey of Vascular Permeability Factor Antibody in Various Population Groups

Permeability factor (PF) neutralizing titers were determined on the sera of 31 children ages 1 to 9 years. These included 12 American, 11 East Pakistani and 8 Czechoslovakian residents. PF titers ranging from 3 to 54 antitoxin units/ml. (Craig) were detected in 9 (81%) of the East Pakistani children. The titers were 8 antitoxin units or greater in 7 (64%) of these children. Neutralizing activity was also detectable in 6 (75%) of the Czech sera;however, the titers were low, ranging from 2 to 7 antitoxin units. None of the American sera had detectable neutralizing titers. The significant difference in the PF antibody levels of East Pakistani children as compared to children from non-endemic areas indicates that this test may be useful for epidemiological studies. The finding of 64% of the East Pakistani children (admitted to CRL with nonspecific diarrhea) with titers probably represents previous infection with V. cholerae.

PF titers were also determined on serum pairs collected in the Meheran inapparent infection study during 1966-67 (E/P11). Among 20 serum pairs showing a significant vibriocidal rise, 6 (30%) also showed a rise in PF antibody. Among 20 "control" pairs (no vibriocidal rise), one (5%) showed a PF response. Interestingly, this person was a household contact of one of the bacteriologically-confirmed cases.

E/P13 - 6 See E/P14

E/P13 - 7 Chemical Characterization of the Antigenic Groups of Vibrio Cholerae

It is known from studies on cell wall lipopolysaccharides of various species of salmonella and shigella, that simple sugars such as glucose, methylglucoside, N-acetylglucosamine, etc. play determinate roles in antigenic specificity. The object of the present work has been to prepare a cell wall lipopolysaccharide antigen from Vibrio cholerae and to find out the antigenic determinants in the molecule. Lipopolysaccharide was extracted from cell wall of Vibrio cholerae harvested from broth cultures by the modified Westphal method and was finally purified by ultracentrifugation at 100,000G.

The test to detect the presence of antigen has been a modification of the vibriocidal inhibition test as described by Finkelstein. Specific antisera for this test were prepared in rabbits by immunizing them with the purified Inaba lipopolysaccharide preparation described above, the purified Ogawa antigen supplied by Verwey and a whole cell vaccine containing both Ogawa and Inaba organisms.

After preliminary experiments with the Inaba lipopolysaccharide a procedure was developed for preparation of a lipid-free polysaccharide. Utilizing this procedure on the purified Ogawa lipopolysaccharide provided by Dr. Verwey, it was possible to obtain a partially degraded lipid-free polysaccharide. This polysaccharide contained the Ogawa antigenic determinant as determined by the vibriocidal inhibition test. Because of degradation in the process of preparation, the antigenic determinant has been reduced to a dialyzable fraction. Further studies are underway to characterize this dialyzable antigen.

Because various investigators have reported that the cell wall polysaccharide of V. cholera contains xylose, glucose, galactose, arabinose, glucosamine, fucose, rhammose, galactosamine and glucoronic acid as sugar constituents, all of these but galactosamine and fucose were tested to see if they had any activity in the vibriocidal inhibition test. None of these had inhibitory activity, suggesting that these are not a part of the determinant antigenic group.

E/P14 Studies of Cholera Enterotoxin Antibodies and of Cholera Enterotoxin and Toxoid Present in Field Trial Vaccines

E/P14 - 1 Standardization of a Technique for Titration of Cholera Enterotoxin Neutralizing Antibody

Several lots of crude cholera filtrate containing active enterotoxin prepared by Dr. K.M.S. Aziz were pooled, aliquoted and stored in the deep freeze for these studies. The toxin was titrated in the rabbit ileal loop according to the methods described by Kasai and Burrows and a titration curve was obtained. From this curve a challenge dose which would produce approximately 20 ml. of fluid in a 10 to 15 cm. loop was selected. Further studies on the methodology revealed that the volume of fluid produced in a rabbit intestinal loop with a given challenge dose showed considerable animal to animal variation. In addition, within animals, it became apparent that the more distal loops, i.e. those closer to the ileocecal valve, were more sensitive to the effects of toxin in terms of producing fluid accumulation than the more proximal loops. Within the range of loop-lengths used for routine study, which varied from 9 to 13 cm., it was found that the loop length was not a significant variable influencing the volume of fluid produced by a challenge dose of toxin. Based on these studies, a standard procedure was developed for toxin challenge in order to assay toxin neutralizing antibody. The method is a modification of the technique described by Kasai and Burrows with the antitoxin titer being the reciprocal of the highest serum dilution which produced complete neutralization of the challenge dose of toxin in duplicate tests. The specific studies are described below.

E/P14 - 2 Assay of Enterotoxin Antigen in Field Trial Vaccines

In attempting to define the mechanism whereby the cholera vaccine protects man, it is essential to know whether or not the vaccines stimulate enterotoxin antibody. Groups of rabbits were hyperimmunized intravenously with a series of injections of each of the three field tested vaccines and a fourth group was hyperimmunized with Dr. Aziz's crude toxin. Following the four-week course

of immunization, the animals were bled and the sera titered for enterotoxin neutralizing antibody. This revealed that none of the three field tested vaccines stimulated enterotoxin antibody; however the animals immunized with Dr. Aziz's crude culture filtrate developed high titers of enterotoxin neutralizing antibody. Continuing the study, intestinal loops were prepared in each group of immunized animals and then they were challenged with graded doses of toxin to determine directly the protection they had received by active immunization. Again, this demonstrated that the animals which had received the field tested vaccines showed absolutely no protection to the toxin challenge in the loop. On the other hand, animals immunized with crude enterotoxin preparations showed complete protection when challenged with one standard challenge dose of toxin; however, when this dose was increased four times there was no protection and full-blown positive loops developed. These studies indicate that the vaccines used in the 1963-64, 1964-65 and 1966-67 field trials do not stimulate enterotoxin neutralizing antibodies.

E/P14 - 3 Enterotoxin Neutralizing Antibody Titers in Acute Cholera and Acute Diarrhea Patients

Serum pairs collected on admission and approximately ten days later from 18 cholera patients and 15 acute diarrhea patients negative for Vibrio cholerae were titered for vibriocidal and enterotoxin neutralizing antibodies. The enterotoxin neutralizing titers were tested in tripling dilutions of the serum. Among the 18 cholera patients, 16 showed a three-fold or greater rise in enterotoxin neutralizing antibody and 13 showed a nine-fold or greater rise in titer. Seventeen of the 18 cholera patients also showed a four-fold or greater rise in vibriocidal antibody titer. Among the 15 acute diarrhea patients, a three-fold rise in enterotoxin neutralizing titer was seen in only one individual. None of the acute diarrhea patients had a rise in vibriocidal titer.

An interesting observation was the fact that children characteristically had higher enterotoxin neutralizing antibodies on admission than adults. Thus, among the 10 children ages 2 to 5 years admitted with cholera, 8 had an enterotoxin neutralizing titer of 1:30 or greater. By contrast, among 8 adults, ages 19 to 45 years admitted with cholera, only 2 had an enterotoxin neutralizing titer of 1:30.

The enterotoxin neutralizing titers were determined on 3 cholera patients serially over a period of 21 days. The results indicated that the peak titer was reached on the seventh to tenth day and in two cases showed a significant fall by the 21st day.

E/P14 - 4 Immunochemical Characterization of Enterotoxin Neutralizing Antibody

Sera from convalescent cholera patients were fractionated by Sephadex gel filtration and density gradient centrifugation. By both of these techniques, all of the toxin neutralizing antibody appeared in the fractions containing gamma G globulin. There was no activity in the gamma M-containing fractions. One serum specimen was treated by reduction with 2-mercaptoethanol. This revealed no loss in antibody activity following reduction. These studies are consistent with the enterotoxin neutralizing antibody being a gamma G globulin.

E/P15 Epidemiological Studies of Cholera El Tor in Chittagong-A Comparative Study with Classical Cholera

On June 6, 1968 a patient was admitted to the CRL Ward in Dacca with cholera due to the El Tor biotype of Vibrio cholerae. Epidemiological investigation revealed that the patient had just arrived from Chittagong. Followup at the Chittagong Medical College Infectious Disease Ward revealed that many of the diarrhea admissions were, in fact, infected with Vibrio cholerae Ogawa of the El Tor biotype. Because there were no bacteriological studies and because the cases were occurring sporadically, the physicians treating the patients in Chittagong were not aware of the presence of cholera in the community. Based on these preliminary observations, a detailed protocol was drawn up to study the epidemiological pattern of El Tor Cholera in Chittagong (E/P15). The results of studies performed to date reveal that at the Chittagong Medical College Infectious Disease Ward they had had 653 cases of acute gastroenteritis from March 2 through October 19, 1968. Between June 15, the time of initiating studies, and October 19, 311 cases were admitted, of which 52 (16.4%) were found to be positive for Vibrio cholerae El Tor. Bacteriological surveillance at the Port Trust Hospital and two Railway Hospitals where a smaller number of diarrhea cases were seen, revealed only one isolation of El Tor cholera. Because bacteriological studies have not been performed since El Tor cholera was documented in Chittagong in 1963 and 1964, it is impossible to determine whether the disease has been prevalent in the community since

that time. A review of hospital records revealed a distinct seasonal peak of acute gastroenteritis in March through August, 1967 and in March through August, 1968. The age distribution of the cases in both years is similar, suggesting that El Tor may have been present the previous year; however definitive answers can not be given at this time.

Family studies have been carried out on 35 families. Positive cultures were found in 40 (17.3%) of 231 family contacts. Only two families had a second hospitalized case.

A total of 146 convalescent cases and family contacts were purged with magnesium sulfate, approximately 21 days after the onset of illness in the index case and a rectal swab culture was taken the day following purge. All of these were negative. Twenty-six persons who had confirmed infection had duodenal aspirations performed three months after the last positive rectal swab. All of these were negative.

One housing colony, Latifpur, was studied intensively. There were 82 families, with a total population of 319. There were 11 hospitalized cases with acute gastroenteritis, of which 7 were positive for El Tor cholera. In a community survey, 21 (26%) of the families had one or more infected members. A total of 38 positive contacts was found in the community. The dug well in the center of the community had a positive culture about half way through the investigation. Based on interviews at the time of census, 3 deaths due to diarrhea and 41 symptomatic diarrheas were documented in this community.

Night soil surveillance in Chittagong was begun on August 24, 1968. By October 19, a total of 18 positive cultures for cholera were found in 9,734 samples collected at 12 conservancy centers and 2 housing colonies, giving a recovery rate of 1.8 per 1,000 samples collected. One was classical Inaba and 17 were El Tor Ogawa organisms. Nine were traceable to infected households.

Sporadic cholera cases are continuing to be seen at the Chittagong Infectious Disease Hospital in a pattern that seems distinct from the epidemic occurrence of cholera in Dacca. Since similar hospital surveillance, as well as latrine and night soil sampling are being carried out in Dacca, it is expected that an understanding of the epidemiological distinctions, if any, between El Tor cholera and classical cholera, may be more clearly defined.

COLLABORATIVE STUDIES AND SPECIAL INVESTIGATIONS

1. A Comparative Study of the Vibriocidal Antibody Titration Techniques

This study was done in conjunction with Dr. Willard Verwey, who is using a macrosystem for vibriocidal antibody titrations in the cholera vaccine evaluations which he is carrying out in the United States. These studies revealed an excellent correlation between the two test systems and indicated that the macrosystem was three-fold more sensitive than our microsystem. Based on these comparative studies a decision was made regarding the preparation of a reference serum. Thus, in the spring of 1968, 400 ml. of ten-day convalescent cholera serum were sent to the Division of Biologics Standards of the National Institutes of Health. This serum will be diluted five times with normal human serum for preparation of two liters of reference serum for distribution to laboratories throughout the world.

2. A Search for Chronic Convalescent Carriers of Vibrio Cholerae by Purging

This study was carried out by Dr. Eugene Gangarosa and Dr. Bill Martin, in collaboration with Dr. Majid Mollah in the Clinical Section. Studies in populations affected with El Tor cholera in Calcutta, the Philippines and Iran had indicated that chronic convalescent carriers could be detected in about 3% of the cases. Previous studies by Drs. Mollah and Hirschhorn at this Laboratory on 130 classical cholera cases had failed to demonstrate any chronic convalescent cholera carriers. In consequence, Dr. Gangarosa joined the Laboratory for a month to carry out more intensive purging studies utilizing techniques that had been successful in Iran. In examining over 150 convalescent cholera patients, ranging from two weeks to two years post-illness, in Dacca, Matlab and Chittagong, he was unable to detect a single chronic convalescent carrier. Since all of these studies were performed on patients infected with the classical Vibrio cholerae, the data suggests that the frequency of chronic convalescent carriers may be an epidemiological difference between classical and El Tor cholera.

3. Epidemiological Investigation of a Cholera Epidemic in Multan, West Pakistan

In June, 1968 the CRL was requested to make a retrospective investigation into a cholera epidemic in Multan, West Pakistan. Dr. Woodward conducted this investigation, assisted by members of the Laboratory staff. Over a six-week period in April and May, 1968 about 3,800 cases of diarrhea were admitted to the two hospitals in Multan. Of 130 persons cultured, there were 16 isolations of classical cholera of the Inaba serotype. The majority of cases came from the old portion of the city where living conditions are poor and extremely crowded. The explosive nature of the epidemic suggested a common source of infection, but none could be found. The age and sex incidence of the affected individuals changed during the course of the outbreak, with more young children being hospitalized towards the end of the epidemic. There were 54 deaths in hospitalized cases, or an overall case fatality rate of 1.4%. The mortality was greater at the beginning of the outbreak than at the end. In addition, municipal death registers indicated an excess mortality of about 100 persons during April and May. Local health authorities succeeded in vaccinating a significant portion of the population against cholera. A serological survey taken during the time of the investigation suggested that the population had been highly susceptible and that those who had a history of cholera vaccine had antibody titers suggestive of significant protection from cholera.

DEMOGRAPHIC STUDIES

The regular registration of births, deaths and migrations in the vaccine trial populations is continuing. A detailed demographic report was prepared summarizing the results of the first year of observation in the 110,000 persons under study since May, 1966. In this population, the birth rate was 47/1,000 and the crude death rate 16/1,000, giving a rate of natural increase of 3.1% per year. Collection of data for the second year is complete, and analysis underway.

In the new villages taken for the 1968-69 vaccine field trial, a more detailed census was taken, showing family structure and occupation. Registration of births and deaths is underway in this population so that now the total population under study is over 220,000.

Dr. Shamsa Ahmad, in collaboration with Dr. Melita Gesche of the East Pakistan Research and Evaluation Center, is following up all female deaths in the age group 10 to 50 years in a maternal mortality study. Preliminary results indicate that the maternal mortality rate is about 7/1,000 live births, with the most common single cause of death being eclampsia in primiparas.

PROTOCOL

STUDIES OF CHOLERA ENTEROTOXIN ANTIBODIES AND OF CHOLERA ENTEROTOXIN AND TOXOID PRESENT IN FIELD TRIAL VACCINES

Principal Investigators: A. Ahmed,
A. K. Bhattacharjee,
A. Mahmud in collaboration with
K. M. S. Aziz

Introduction

It is well established that the massive outpouring of fluid seen in cholera can be produced in experimental animals by a cell-free filtrate of Vibrio cholerae. There is accumulating increasing evidence that immunity to this cholera toxin may be significant in protection against the disease. Burrows and Musteikis (1966) have developed a standard technique for titrating cholera toxin activity in the rabbit ileal loop. Utilizing this technique, Kasai and Burrows (1966) have titrated a variety of sera for antitoxin activity in the rabbit ileal loop. They have demonstrated that the outpouring of fluid in the adult rabbit ileal loop could be neutralized by sera prepared from rabbits immunized with whole cell lysates of Vibrio cholerae. In addition they demonstrated that there was a rise in antitoxin titer in cholera patients during the course of the disease. They further demonstrated that there was considerable activity in a pool of normal Bengali sera and very low activity in a pool of normal U.S. sera. Of considerable interest was their observation that sera from rabbits immunized with the cholera vaccine used in the 1963 field trials by the CRL had relatively high antitoxin titers when tested in this rabbit loop system.

Further evidence for the significance of immunity against cholera toxin has been presented by Finkelstein and Atthasampunna (1967). They were able to demonstrate that in adult rabbits actively immunized with a preparation of choleragen there was protection against challenge of intestinal loops with either choleragen or Vibrio cholerae suspensions. More recently, studies from Carpenter's laboratories (personal communication) have indicated that dogs actively immunized with cell-free culture supernatants showed significantly less outpouring of fluid from the chronic intestinal loop preparations challenged with cholera toxin, than did a series of control animals.

With these observations it is imperative that the Immunology Laboratory at CRL establish a standardized technique for titration of cholera toxin and the assay of antitoxin antibodies. The technique described by Kasai and Burrows (1966) has been utilized by Ungar at the Swiss Serum Institute (personal communication) who has found it satisfactory for the standardization of toxin preparations and the titration of antitoxin antibodies in serum. This technique will be set up in the Immunology Laboratory. Toxin will be prepared and standardized in units according to the definition of Burrows and Musteikis (1966) and a convalescent antisera will be standardized against this toxin for reference purposes. The specific objectives of this protocol are :

1) To define the antitoxin antibody response that occurs in the course of cholera, including the time course of the response in relation to the disease, and the specific immunoglobulins responsible for the toxin neutralizing activity.

2) To establish an assay system for the measurement of the toxin and/or toxoid content of cholera vaccines. This includes determining the antigenicity of the vaccines by measuring the antitoxin antibody after various injection schedules into man and animals and assaying the toxin and/or toxoid content of the vaccines by specific absorption studies with standardized antitoxic sera.

3) To separate the vascular permeability factor of Craig from the cholera toxin producing outpouring of fluid in the intestinal loop. This will be done by absorption of toxin preparations with specific antisera.

Methods:

Preparation of Toxin

All toxin preparations will be made from the Vibrio cholera Inaba strain 569b. Two toxin preparations will be made. A whole cell lysate will be prepared according to the method described by Burrows et al. (1965). In this preparation the micro-organisms will be grown for 18 hours on 3% bacto peptone agar at pH 8 and harvested in distilled water. The whole cell lysate will be prepared by treatment of the bacterial suspension at 9 KC per

second in a sonic oscillator for one hour, clarified by centrifugation, and lyophilized. Culture supernatants will be prepared according to the methods established by Craig, Feeley and Aziz (personal communication) and presently being carried out in this Laboratory.

Reference Sera

Serum No. 3814, a ten-day convalescent serum, which has been characterized in terms of total protein, immunoglobulin content, agglutination and vibriocidal antibody and vascular permeability factor neutralizing antibody and has been studied by fractionation on Sephadex chromatography and density gradient ultracentrifugation will be titered and utilized as a reference serum.

Vaccines;

The vaccines to be studied will be the whole cell vaccine used in the 1963 and 1964 field trials, the whole cell vaccine used in the 1966 field trial and the purified Ogawa antigen prepared by Dr. Verwey.

Standardization of Toxin:

The toxin will be standardized in rabbit ileal loops according to the technique of Burrows and Musteikis (1966). Rabbits weighing approximately 2 kg. will be starved for 48 hours. The rabbits will be anesthetized and under aseptic techniques, a laparotomy will be performed. Intestinal loops will be prepared in the distal ileum, beginning just above the vermiform appendix. The ileum will be washed with warm, sterile saline and six 10 cm. loops will be prepared. The first and last loops will be controls. An inoculum in a standard volume of 2 ml. will be injected into each of the other 4 loops and the site of inoculation isolated by a second tie to give a double tie between loops. Following the operative procedure, the animals will be given water ad lib. They will be sacrificed at 18 hours and the reactions read. The reactions will be quantitated by the volume-length ratio. One unit of toxin will be defined as the 50% effective dose.

Titration of Antitoxin:

This will be done according to the technique of Kasai and Burrows (1966). Three units of toxin will be mixed with equal volumes of serial dilutions of inactivated serum, incubated for 1 hour at 37° and then titrated for residual toxicity. The neutralization coefficient will be calculated as defined by Kasai and Burrows (1966) and the dilution of serum required to neutralize two units of toxin will be determined by the graphic-probit method. One antitoxin unit will be defined as that amount of antiserum required to neutralize one unit of toxin.

Fractionation of Toxin-Neutralizing Antibody:

Convalescent cholera sera will be fractionated on Sephadex G200 and by density gradient centrifugation according to the techniques established in the immunology laboratory. These fractions will be titered for toxin neutralizing activity.

Immune Absorption Studies

Data by Kasai and Burrows (1966) indicated that sera from rabbits given the 1963 CRL vaccine produced toxin neutralizing antibody. Data from Feeley's laboratory (personal communication) as well as serological surveys by our Laboratory in the field trial indicate that this vaccine does not produce vascular permeability factor neutralizing antibodies. Immune sera from rabbits immunized with this vaccine will then be utilized to absorb toxin preparations containing both loop toxin and vascular permeability factor. Following absorption, the toxin preparation will be titered for residual activity of skin permeability factor and loop toxin.

Assay of Toxin Antigen in Cholera Vaccine:

The toxin antigenicity in the cholera vaccines will be assayed by immunizing groups of rabbits and humans with a standard course of each of the vaccines. The subjects will be bled at intervals and antisera titered for antitoxin antibodies. The toxoid content of the vaccines will be assayed by absorption of a standardized sera with a defined amount of the vaccine and then titrating the antisera for residual antitoxic activity.

Significance of This Research:

With the intense interest in the cholera toxin responsible for fluid accumulation in the intestines of various experimental animals, it is of paramount importance that this Laboratory has a standard method of assay of toxin and antitoxin. The method described by Kasai and Burrows (1966) has been well standardized and used in Dr. Ungar's laboratory at the Swiss Serum Institute, with excellent results. A direct assay for the cholera toxin producing fluid accummulation in the intestinal loop is necessary since there is increasing evidence that the vascular permeability factor and the diarrhea toxin are two different materials. This is provided by the observation of Kasai and Burrows (1966) that sera from rabbits immunized with the CRL field trial vaccine, contained antitoxin titers. This same sera had been studies by Dr. Feeley (personal communication) and was shown not to contain antibody against vascular permeability factor. A preliminary observation in this Laboratory has indicated that sera from vaccinated Americans never exposed to cholera contains antibody against the toxin responsible for fluid accummulation in the ileal loop of the rabbit, but contains no detectable antibody against vascular permeability factor. Confirmation of this observation will be of major significance as it will establish that these two are distinct toxins and that assay of vascular permeability factor activity cannot be used as an assay or index of loop toxin activity.

The observation by Kasai and Burrows that presently-used cholera vaccines induce antitoxin antibodies, is of considerable significance and required further confirmation. This indicates that the vaccine presently being used in the field trial already contains toxin or toxoid and thus the protection produced by the vaccine may be due to the antitoxin antibodies produced. Thus, preparation of a toxoid vaccine may not be the radical new departure in cholera vaccine that has been considered. It is therefore important to have an assay system for the toxoid content of vaccines that have already been tested, as well as for any new products prior to field testing.

This standard method of assay of antitoxin antibody is vital to the conduct of epidemiological studies to relate this antibody to protection against cholera, as well as determining the correlation of this antibody with vibriocidal titer. This technique will also be of value to standardize toxin preparations for clinical investigation studies being carried out in experimental animals, and for the conduct of collaborative studies with Dr. Ungar's laboratory.

Facilities:

The facilities and equipment presently available at the CRL are adequate to support this project with the exception of the supply of rabbits. To fully support this study will require about 100 rabbits per month. Expansion of the rabbit colony with enlargement of the animal house staff and improved conditions for animals to minimize losses from disease will be necessary.

PROTOCOL

EPIDEMIOLOGICAL STUDIES OF CHOLERA EL TOR IN CHITTAGONG - A COMPARATIVE STUDY WITH CLASSICAL CHOLERA

by

K. J. Bart and M. Khan

Introduction

Cholera due to the El Tor biotype of V. cholerae had been decumented intermittently in Chittagong in 1963 and 1964. On June 6, 1968, after a four-year gap when no cultures were obtained, El Tor cholera was again documented in the Chittagong Municipality. This represented the first El Tor isolated at CRL since 1965.

The present study will utilize a combined bacteriologic and serologic approach to define the epidemiologic pattern of cholera due to this biotype. The specific objectives of this study are to examine the spectrum of illness, pathogenicity, geographic distribution, age and sex characteristics and carrier status; to study the infection rate in families and in the community and the ratio of apparent to inapparent infections; to study the pattern of spread and introduction in affected areas; to compare the effectiveness of paired serology with rectal swabs in detecting El Tor cholera infections; to determine the frequency of long-term carriers; and to compare the preinfection immunity as determined by antibody titer for infected and noninfected individuals. The recognition of El Tor cholera in East Pakistan offers the opportunity to compare the epidemiological characteristics of El Tor cholera with classical cholera in Dacca, using similar methodology in the studies.

Background

Chittagong is the second biggest port of Pakistan and the gateway to East Pakistan. The area at Chittagong Municipality is approximately 40 square miles, with a total estimated

population in 1961 of 364,205, of whom 237,752 were males and 126,453 were females. The estimated population in 1968 is approximately 500,000.

Method

Active case finding is being carried out by hospital surveillance at four hospitals: the Chittagong Medical College Infectious Disease Ward (where most cases of severe gastroenteritis with dehydration in Chittagong are admitted or are transferred to for treatment), the Port Trust Hospital (where the first case of El Tor cholera was documented) and two Railway Hospitals; and in Dacca at the CRL.

Systemic latrine sampling of the municipal conservancy system will be carried out in Chittagong and in Dacca. In Chittagong, this represents approximately 20,000 latrines serving approximately 275,000 people. The Dacca municipal conservancy system serves approximately 150,000 people. Also, there will be individual latrine sampling in two well-delineated housing colonies in Chittagong, i.e. Port Trust North Colony (1,265 latrines representing about 5,000 people) and Pahartoli Wireless Railway Colony (about 1,165 latrines serving approximately 4,800 people), and two well-delineated areas in Dacca, i.e. Rayer Bazar (114 latrines serving approximately 700 people) and Rajnarandhar Lane (44 latrines serving about 1,200 people).

Each day, each of the four hospitals in Chittagong and the CRL ward in Dacca are visited and all cases of acute gastroenteritis admitted during the preceding 24 hours are interviewed and rectal swabs or stool cultures are obtained. Vomitus, if available, is cultures. Serology on each patient is also obtained. Each case of gastroenteritis is swabbed daily for at least three days or for as long as the patient remains in hospital, if less than three days.

Each active case found through hospital surveillance or any person found asymptomatically excreting the El Tor or classical vibrio through latrine sampling, becomes the index case of a family study. The family is considered relatives, friends or visitors who lived, slept or ate within this house or with the family.

The bari (household) of each bacteriologically-confirmed case is visited as soon as the case is identified, if possible within 24 hours.

A family census is taken, listing age, sex, relationship, occupation, vaccination status, and the members are interviewed for information concerning gastrointestinal symptoms, vomiting or diarrhea of any magnitude or recent deaths in the family or bari. A daily individual illness report is completed on each member of the household considered ill. Death reports are completed on any family member who recently died. All families in the bari are also visited enquiring for the presence of illness or death. All neighbour households with illness or death are treated as contacts and a family study is performed. Each member of the family or neighbouring family has a rectal swab and serology drawn on the initial visit (day 1). Rectal swabs are obtained daily for 10 consecutive days and on day 10 a second serology is performed. On day 20 a rectal swab is obtained from each family member and a purge with magnesium sulfate is administered with doses adjusted to age. A post-purge rectal swab and a third serology are obtained on day 21.

Sources of water for various household uses are obtained for culture on days 1, 10 and 21.

At three months each person who has had a positive culture for El Tor has a duodenal aspiration before and after a fatty meal.

PROTOCOL

1966-67 FIELD TRIAL - THIRD YEAR
EVALUATION OF BOOSTER INJECTIONS IN 1966-67 VACCINE TRIAL POPULATION

by

W. H. Mosley and W. E. Woodward

The 1966-67 field trial was an evaluation of a commercial cholera vaccine prepared in the United States to determine the effectiveness of a single dose and two doses in children, ages 0 to 14 years, and to examine the correlation of vaccine effectiveness with the vibriocidal antibody titer. The results of the 1966-67 field trial, in terms of protection, are summarized in Table 1. This indicates that, for the cholera season following injection, a single dose produced 46% protection and two doses produced 64% protection. In 1967-68, the effect of booster doses on part of the population was studied. Again this was combined with serological surveys to evaluate the relationship of antibody titer to protection. Table 1 summarizes the results obtained in the 1967-68 field trial. As Table 1 indicates, there were only a few cases in the 1967-68 cholera season. Thus, it is not possible to draw any definitive conclusions regarding the effectiveness of booster injections or the duration of protection of cholera vaccine. The data does suggest that there was some sustained protection in the group that received two injections in 1966-67 through the 1967-68 cholera season. It also indicates, however, that a booster injection in 1967-68 did not produce substantial protection when compared with the control group.

The 1968-69 field trial will be a continual followup of this population to determine the effect of regular booster injections. Table 1 indicates the vaccine assignments to be given this year. As in previous years, serological surveys will be conducted prior to, and following, the vaccine program and vaccine will be administered by teams going from house to house with jet injectors. The details of the surveillance procedure have been outlined in the original protocol for the 1966-67 field trial (1966 Technical Commitee report).

TABLE 1

SUMMARY OF 1966-67 FIELD TRIAL
(STUDY POPULATION AGES 0-14 YEARS)

Approx. Population	1966-67 Vaccine[a]	1966-67 Cases	1966-67 Protection	1967-68 Vaccine	1967-68 Cases	1967-68 Protection	1968-69 Vaccine
10,000	00	35	-	0	12	-	0
10,000	X0	19	46%	X	7	41%	X
10,000	XX	25	64%	0	8	33%	0
10,000	XX			X	5	58%	X

a Vaccine X = cholera vaccine
Vaccine 0 = tetanus-diphtheria toxoids

PROTOCOL

1968-69 VACCINE FIELD TRIAL
TESTING OF THE PURIFIED INABA ANTIGEN AND OF LYOPHILIZED MONOVALENT INABA AND OGAWA CHOLERA VACCINES

by

W. H. Mosley and W. E. Woodward

The 1968-69 vaccine field trial will test the potency of a purified Inaba antigen, and two lyophilized cholera vaccines, one containing Inaba organisms and the other Ogawa organisms. These three vaccines will be compared to a tetanus-diphtheria toxoid control vaccine. The characteristics of the vaccine and results of preliminary tests are given in appendices 1, 2, 3 and 4.

The study population for the new field trial was selected in villages adjacent to the old field trial areas. Approximately 110 villages, with a total population of 100,000, were selected. A census was taken in these villages during April and May of 1968. Census books were compiled, field staff have been assigned in each of these villages and each family has been issued a family census card. Daily house to house surveillance for acute diarrheal illness, as well as births and deaths, has already been initiated in the majority of these villages. Four additional speedboats have been ordered to provide ambulance coverage for the expanded area and a vehicle will be acquired for those new villages accessible by the Matlab-Chandpur road. In addition, plans have been made to set up an additional cholera treatment center in the most remote part of the new trial area, if it is required, during the coming cholera season.

Vaccines have been assigned only to children ages 3 months to 14 years. There will be four vaccine groups. Each group will contain between 10,000 and 12,000 persons. Three of the groups are for the three new cholera vaccines and the fourth group is for a diphtheria-tetanus toxoid control vaccine.

Prior to the assignment of the vaccine in the census book, the population was stratified in the age groups, 0-4, 5-9 and 10-14. The vaccines were assigned within each of these age groups in strict alternation. The vaccines will be designated by the code letters, F, G, H and I and neither the participants nor the vaccinators will know the designations of the vaccines.

Vaccination will be carried out by field teams, each equipped with four jet guns, one for each of the vaccines. They will proceed from house to house, indentify the children and administer the vaccines which have been assigned and indicate this in the census book with a date stamp. The vaccination program will be under the guidance of a physician stationed in Matlab who will be prepared to treat any adverse reactions which may develop

Only a single injection of vaccine will be given to all participants. The dosage of the purified Inaba antigen will be 150 micrograms administered in a volume of $\frac{1}{2}$ml. The dosage of the lyophilized vaccines will be determined by preliminary testing in a village in the Matlab field trial area. Preliminary studies suggest that 8×10^9 organisms given in a $\frac{1}{2}$ ml. volume are well tolerated, with no more reactions than $\frac{1}{2}$ ml. of commercial cholera vaccine.

Participation in the field trial will be on a voluntary basis. All of the villages selected for the 1968-69 field trial were included at their own request. Many additional villages asked to be included, but could not be taken in this year's study. The program of testing cholera vaccines by the PSCRL in the Matlab area is well known by all the villagers and it has been explained during the time of census and again during the serological surveys. The villagers recognize that, by participating in the field trials, they come under the cholera surveillance program which provides for the rapid recognition and treatment of any suspect cholera cases, with the cure rate in the treatment center being over 99%. This provides the villagers with a measure of protection against cholera that they have never before enjoyed.

Significance Of This Study

The 1968-69 field trial will provide answers to several questions. First, it will demonstrate whether or not a lyophilized vaccine is protective. If it is, this could resolve some of the problems regarding the stability and storage of cholera vaccines. Secondly, the study will determine whether or not vaccines containing only Inaba or only Ogawa organisms can protect individuals infected with the heterologous serotype. If cross protection does exist, and most laboratory tests suggest that it should, then future cholera vaccines would only need to contain one serotype. This could prove to be a major asset in improving the effectiveness of cholera vaccines, since the Inaba organism has characteristically been shown to be more antigenic than the Ogawa organism and incorporating only this serotype could considerably strengthen the potency of the vaccine. The testing of the purified Inaba antigen will indicate whether or not this preparation contains the protective antigen. If this preparation is protective, then such material could be used in preparing adjuvant vaccines.

The serological surveys which will be carried out as part of this trial will be a continuation of the previous studies in the field trial area. These studies with the commercial cholera vaccine have shown that there is a very close correlation between the protective effect of the vaccine and the vibriocidal antibody titer. If this relationship continues to hold up when different types of vaccines are used, then it may be possible in the future to evaluate vaccines by serological tests only.

ATTACHMENT 1

PURCHASE CONTRACT SPECIFICATIONS FOR MONOVALENT CHOLERA FIELD TRIAL VACCINES

1.0 Source of Materials:

Under Purchase Order No. D-837141-6 DBS obtained monovalent (Inaba and Ogawa) cholera vaccines from Merck, Sharp and Dohme who was the lowest bidder.

The material has been tested extensively in the laboratory and in two separate clinical trials (DBS-IND 193 and 206). Response of man was determined serologically and related to laboratory assays. The NIH Cholera Advisory Committee (September 30, 1967) advised that materials prepared in the same manner as used in the two small clinical trials be procured for a large-scale study in East Pakistan. Both protective efficacy and serological response will be determined in the field and again related to laboratory assays. In order to correlate the earlier studies with the large-scale field trial study, it is essential that vaccines be prepared in the same manner as were those obtained by Purchase Order No. D-837141-6. Hence, Merck, Sharp and Dohme, who prepared the first vadcine, would be the sole source for the subject purchase.

2.0 Preparation of Freeze-Dried Monovalent Cholera Field Trial Vaccines:

2.1 Production Strains:

a) Monovalent Ogawa vaccine shall be prepared from *Vibrio cholerae* strain NIH 41.

b) Monovalent Inaba vaccine shall be prepared from *Vibrio cholerae* strain NIH 35A3.

Freeze-dried cultures newly obtained from DBS shall be employed.

2.2 Preparation of Individual Ogawa and Inaba Harvests:

Organisms shall be grown on the surface of Casamino Acids Agar (Casamino Acids, Difco Certified, 3%; NaCl, 0.85%; Agar, 2.0–2.5%; final pH 7.4–7.6) dispensed in appropriate containers (e.g. Blake Bottles, Roux Bottles, Kolle Flasks, Povitsky Bottles). Production bottles shall be inoculated with a minimal volume of inoculum of organisms from a seed culture incubated 4–5 hours at 35°C in 3% casamino acids broth.

After inoculation, production bottles shall be incubated in an inverted position for 18 hours. Growth shall be harvested from each bottle as follows: residual fluid inoculum shall be decanted and discarded and approximately 10 ml. of sterile 0.02 M phosphate buffered saline* (pH 7.2 shall be added. Growth shall be harvested by scraping with a harvesting rake or glass beads in such a manner that the suspended harvest is removed from the bottle within not more than 5 minutes after addition of the harvesting fluid. The pooled harvest from each production bottle shall be passed through a coarse cotton gauze or other suitable inert filter to remove agar particles. The bacterial concentration of the pooled harvest shall be determined in relation to the U.S. Opacity Standard (20 opacity units equal 8 x 10^9 bacteria/ml.) within 2 hours of harvest and before addition of the killing agent. All subsequent calculations of bacterial concentration shall be based on this opacity determination.

2.3 Inactivation with Formalin:

Sufficient neutral formalin (formaldehyde solution, 37%) shall be added to yield a final concentration not exceeding 0.25%. Containers shall be well-mixed and maintained continuously at 4°C. The time interval between addition of formalin and neutralization and drying shall not exceed 3 weeks.

2.4 Neutralization of Formaldehyde:

Immediately before final adjustment of bacterial concentration and prior to drying, 35% sodium bisulfite solution shall be added in an amount sufficient on an equimolar basis to neutralize the formaldehyde calculated from the amount of formalin added. After addition of sodium bisulfite, the pH should be readjusted to 7.2 if necessary.

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* Formula: KH_2PO_4, 0.762 g; $Na_2HPO_4 \cdot 7H_2O$, 3.86 g; NaCl, 8.5 g; Distilled Water, q.s., 1,000 ml.

2.5 Freeze-Drying:

Based on the original opacity determination, each vaccine shall be diluted in 0.02 M phosphate buffered saline (pH 7.2) to a concentration of 100 opacity units and dispensed in 50 ml. vials in 10 ml. amounts to yield 1,000 opacity units per vial. Modification of the opacity unitage and/or volume of the fill will be permitted if necessary for technical reasons. The accuracy of the fill shall be ±2.5%. Freeze-drying shall be carried out in a cabinet-type drying device under conditions adequate to yield a product with not more than 1% moisture. The vials shall be stoppered with butyl rubber stoppers under the original vacuum.

2.6 Number of Vials of Each Vaccine Required:

8,000 vials of each serotype vaccine.

3.0 Diluent:

Diluent for restoration of dried vaccines shall consist of sterile isotonic 0.02 M phosphate buffered saline (pH 7.2) containing 0.25% phenol. Diluent shall be dispensed in 50 ml. bottles in 50 ml. amounts with appropriate overfill to yield a recoverable 50 ml. volume.

3.1 Number of Vials of Diluent:

One vial of diluent shall be supplied for each vial of Ogawa and Inaba monovalent vaccine.

4.0 Tests To Be Performed On Each Vaccine:

a) Integrity of vacuum based on 100% inspection
b) Moisture content
c) Total nitrogen, non-dialyzable nitrogen, and nitrogen precipitable by 1.5 volumes of acetone
d) Residual-free formaldehyde
e) Sterility
f) General safety - when reconstituated with 50 ml. sterile diluent

5.0 Tests To Be Performed on Diluent:

Filled containers of diluent shall be subjected to the following tests:

a) Pyrogenicity

b) Phenol content

c) pH

d) Sterility

6.0 Labelling of Vaccine and Diluent:

Vaccine bottles shall be labelled with a tear-off perforated label designed to meet all requirements of paragraph 130.3 of the Food and Drug Regulations. The permanently attached portion of the label shall bear a code designation to be provided. The tear-off portion shall be labelled "Cholera Vaccine, Serotype Ogawa" (or Serotype Inaba).

Diluent shall be labelled "Diluent for Cholera Vaccine" and shall be in accordance with the above Regulations.

7.0 Materials to Be Submitted Prior to Acceptance:

a) Complete manufacturing protocols and results of all tests performed

b) Five vials of each vaccine and five vials of diluent.

8.0 Criteria for Acceptance:

8.1 Vaccine:

a) Adequate vacuum in all vials

b) Moisture content not exceeding 1.0%

c) Total nitrogen 0.10 to 0.25 mg./ml. when restored with 50 ml. of diluent

d) Residual-free formaldehyde content not more than 0.02%

e) Satisfactory passage of sterility tests

f) Satisfactory passage of safety tests

g) Potency (to be determined by DBS) not less than 0.5 times as potent on a per milliliter basis as corresponding U.S. Ogawa (Lot Og 22) and Inaba (Lot In 12) Reference

Vaccines when restored with 50 ml. of diluent.

8.2 Diluent:

a) Non-pyrogenic

b) Phenol not more than 0.3%

c) pH 7.1-7.4

d) Satisfactory passage of sterility tests.

Addendum: Combinations for Use:

50 ml. vial containing 1,000 opacity units/vial:

When Restored With	Would yield Opacity Units	Bacteria/ml.
50 ml.	20	8×10^9*
25 ml.	40	16×10^9
12.5 ml.	80	32×10^9

*Usual concentration for cholera vaccine

Alternate volumes of diluent to provide accuracy in constituation of vaccine to yield the different concentrations of bacteria.

Volume	No. of Containers
50 ml.	8,000
12.5 ml.	32,000

The purchase order shall specify:

a) Time of delivery

b) Shipment to Dacca, East Pakistan

   1) Quantity

   2) Method of shipment

c) Shipment to NIH

   1) Quantity

   2) Method of shipment

(Determine estimate of need of CRL for 1968 trial)

SAMPLE LABEL

CAUTION: New Drug – Limited by Federal Law to Investigational Use CODE NO. Date Restored:_____	Lot No._____ CHOLERA VACCINE Serotype_____ (Freeze-Dried) When restored with 50 ml. of Diluent contains 8,000 million Vibrios/ml Name of Manufacturer:_____ CAUTION: New Drug – Limited by Federal Law to Investigational Use

Permanently attached to Vial

Perforation

Tear-off Portion

ATTACHMENT 2

RESULTS OF POTENCY AND FREEDOM-FROM-TOXICITY TESTS ON THE MONOVALENT FREEZE-DRIED CHOLERA VACCINE

In the potency tests (Tables 1 and 2), the vaccines were compared with U.S. Reference Cholera Vaccines, Ogawa (Lot OG 22) and Inaba (Lot IN 12). These reference vaccines were prepared for DBS by Merck, Sharp and Dohme using the same methods and were employed by Dr. Hornick in his study conducted under DBS-IND-193 and by Dr. Mosley in his study conducted under DBS-IND-206. Based on these data, it is estimated that the Ogawa vaccine is 1.4 times, and the Inaba vaccine 2.2 times, as potent as the corresponding U.S. reference vaccines. Thus, they appear to be somewhat more potent than those vaccines employed by Drs. Hornick and Mosley in their previous studies. A more thorough evaluation of these data will be performed and reported at a later date, and this will also provide information on cross-protection offered by the vaccines against heterologous serotype challenge.

The vaccines were also subjected to the freedom-from toxicity test required for cholera vaccine (Sec. 5.0 of "Recommendations Relating to the Manufacture of Cholera Vaccine", dated October 26, 1964). To pass this test, 10 mice, injected intraperitoneally with no less than one-half the largest single human dose, must: (1) regain their original weight in 72 hours; and (2) must neither die nor show significant symptoms.

In Table 3, varying doses of the two vaccines were employed. The vaccines passed the test at twice the usual mouse test dose for cholera vaccines (i.e. 10 opacity units/mouse or the equivalent of 0.5 ml. of vaccine containing 8×10^9 vibrios (20 opacity units) per ml.). However, at 4 times the usual test level, the vaccines failed to pass the test. Included in this test for comparison is the CRL vaccine, which when employed at a human dose of 0.4 ml., bordered on reactivity. We believe, however, that the latter vaccine is now somewhat less toxic for mice than it was at the time of the 1963-64 CRL field trials.

The vaccines were also compared in the freedom-from-toxicity test with the corresponding DBS Reference Vaccines as shown in Table 4. We believe that the new freeze-dried vaccines may be perhaps slightly more toxic than the reference vaccines. The latter were very acceptable from a clinical point of view in both Dr. Hornick's and Dr. Mosley's respective studies.

The vaccines were tested for serologic indentity by the slide agglutination technique using absorbed monospecific sera and were found to be true to label.

Attached also is a summary of additional tests performed by the manufacturer as specified by the contract. The results of these tests appear acceptable.

In summary, it is my opinion that these vaccines are satisfactory for use in the projected study. However, in view of their higher potency and somewhat higher toxicity, it is possible that they may be slightly more reactive than the reference vaccines employed by Drs. Mosley and Hornick, and this should be considered if there is any intention of using them at concentrations of greater than 8×10^9 vibrios/ml.

TABLE 1

POTENCY TESTS ON MERCK, SHARP & DOHME FREEZE-DRIED OGAWA VACCINE (LOT 4446G)

Test Date	ED50, ml. x 10^{-4} Lot 4446G	U.S. Ogawa Reference Lot OG 22	Relative Potency
March 14, 1968	0.276 (70–144)*	0.618 (63–160)	2.24
March 21, 1968	0.579 (70–144)	0.825 (78–128)	1.42
April 4, 1968	0.534 (63–160)	0.470 (59–170)	0.880
Geometric Mean	0.441	0.621	1.41

* ( ) = range of 1 S.D. expressed in per cent

TABLE 2

POTENCY TESTS ON MERCK, SHARP & DOHME FREEZE-DRIED INABA VACCINE (LOT 4445G)

Test Date	ED_{50}, ml. $\times 10^{-4}$ Lot 4445G	U.S. Inaba Reference Lot IN 12	Relative Potency
March 15, 1968	0.579 (53-188)*	2.27 (67-150)	3.92
March 28, 1968	0.285 (72-139)	0.534 (63-160)	1.87
April 18, 1968	0.761 (66-151)	0.970 (67-150)	1.27
Geometric Mean	0.468	1.05	2.24

* ( ) = range of 1 S.D. expressed in per cent.

TABLE 3

FREEDOM-FROM-TOXICITY TEST - MERCK, SHARP & DOHME FREEZE-DRIED VACCINES

Vaccine	Dose	Av. Wt. change (g) on day 1	2	3	7	D/10
MSD	40 Op.U.	-1.5	-2.1	-1.4	+2.6	0
Inaba	20 Op.U.	-1.3	-0.4	+0.9	+5.7	0
Lot 4445G	10 Op.U.*	-1.4	0.0	+1.6	+5.7	0
MSD	40 Op.U.	-1.3	-2.2	+0.1	+1.1	4
Ogawa	20 Op.U.	-1.6	-0.9	+0.9	+5.6	0
Lot 4446G	10 Op.U.*	-1.2	+0.2	+2.1	+6.4	0
CRL	0.2 ml.**	-0.8	+0.1	+1.8	+5.8	0
Saline	0.5 ml.	+1.5	+1.9	+3.5	+7.0	0

* One-half usual largest single human dose of 20 Op.U. (8×10^9 vibrios/ml.)

** One-half of single human dose (0.4 ml.) which bordered on toxicity in man at CRL

TABLE 4

FREEDOM-FROM-TOXICITY TEST - MERCK, SHARP & DOHME FREEZE-DRIED VACCINES

Vaccine	Dose	Av. Wt. change (g) on day 1	2	3	7	D/10
MSD Ogawa	20 Op.U.	-2.0	-0.9	+0.3	+4.9	0
Lot 4446G	10 Op.U.*	-1.3	+0.2	+1.6	+5.4	0
DBS Ogawa	20 Op.U.	-1.3	-0.3	+0.9	+5.0	0
Lot CG 22	10 Op.U.*	-1.0	+0.6	+2.1	+6.7	0
MSD Inaba	20 Op.U.	-1.2	-0.6	+0.3	+4.8	0
Lot 4445G	10 Op.U.*	-1.7	+0.6	+1.7	+4.9	0
DBS Inaba	20 Op.U.	-1.6	-0.5	+1.7	+5.5	0
Lot IN 12	10 Op.U.*	-1.2	-0.1	+2.2	+6.0	0
CRL	0.2 ml.**	-1.1	0.0	+2.2	+5.3	0
Saline	0.5 ml.	+0.9	+1.9	+3.3	+6.8	0

* One-half usual largest single human dose of 20 Op.U. (8×10^9 vibrios/ml.)

** One-half of single human dose (0.4 ml.) which bordered on toxicity in man at CRL.

ATTACHMENT 3

INFORMATIONAL MATERIAL
CHOLERA VACCINE (INABA TYPE) NON-CELLULAR CHOLERA ANTIGEN

Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen is an antigenic fraction obtained from cell suspensions of Vibrio cholerae, type El Tor, strain Inaba V86, by physical and chemical fractionation procedures. The antigen is extracted from the cells by brief sonication in the presence of 2.5 M urea and is precipitated by ammonium sulfate. The redissolved antigen is further fractionated by ultracentrifugation and sterilized by treatment with beta-propiolactone. After incubation for the hydrolytic destruction of beta-propiolactone, the antigen, dissolved in 1% lactose, is freeze-dried. This latter step not only stabilized the antigen, but also removes any residue of beta-propiolactone, which is volatile at the temperatures and pressures employed in the freeze-drying step.

This cholera antigen in its present form is designed exclusively as a research tool for the study of the production of immunity against cholera. While the antigen is not pure from either the biochemical or immunological viewpoint, it contains only a limited spectrum of the many antigens in the whole cholera organism. However, the antigens present appear to be those of primary importance in protection against cholera, as judged by the mouse protection test which is used for the potency testing of cholera vaccine in the United States. Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen actively protects mice in this test at ED_{50} dosages below 0.5µg . When injected as a single dose in rabbits or mice, it induces the formation of homologous vibriocidal antibody and, to a lesser degree, heterologous (Ogawa) vibriocidal antibody is produced. Serum produced in rabbits by injections of Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen will passively protect mice against lethal infection with Inaba strains of V. cholerae.

At the present time no recommendations of a dosage for humans can be made. However, since Cholera Vaccine (Inaba Type) non-Cellular Cholera Antigen is an extractive of V. cholerae organisms which are grown as for the preparation of cholera vaccine, the animal safety tests that are applicable to cholera vaccine have been applied to the non-cellular antigen. One hundred and fifty micrograms have been injected into mice and 750 micrograms have been injected into guinea pigs. No deaths or evidences of adverse reaction attributable to the antigen were encountered. The LD_{50} of this antigen by titration intraperitoneally in mice exceeds 900µg. In weight loss toxicity tests performed by the method given in "Recommendations Relating to the Manufacture of Cholera Vaccine (October 26, 1964)", issued by the U.S. Department of Health, Education and Welfare, the non-cellular antigen met requirements for freedom-from-toxicity when injected in 200µg doses.

Cholera vaccines are recommended to meet this standard at one-half the largest single human dose.

Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen is supplied as a sterile, freeze-dried product without preservative in the antigen bottle. A companion vial of diluent for cholera vaccine is also supplied. This diluent is a slightly buffered 8% lactose solution containing 0.5% phenol. It is recommended that this diluent be employed if the antigen is to be used in investigational programs involving human injection. However, there are no presently-known contraindications to the use of phenolized saline as a restoring solution. The amount of antigen in the vial (in milligrams) is stated on the label, and it is suggested tha the antigen be restored to a concentration of 200 ug/ml. and shaken thoroughly before use or before subsequent dilution. In the case of lot no. 82721 F which contains 1.5 mg. per bottle, restoration with 7.5 ml. of diluent will provide the suggested initial concentration. Although there is no evidence of instability or loss of potency on refrigerated storage of antigen solutions over several weeks, it is currently suggested that restored antigen solutions containing preservative be held refrigerated at 2° to 10°C and used for human injection for not longer than seven days after restoration. The freeze-dried antigen should be stored refrigerated below 10°C. Storage below 0°C is not harmful and may be beneficial.

The potency of Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen has been estimated by comparing its mouse protective activity with that of U.S. Reference Inaba Vaccine in mouse protection tests, using an Inaba challenge culture. One hundred and forty micrograms of Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen Lot 82721 F was found to have a mouse protective potency equivalent to 1 ml. of Inaba reference vaccine.

Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen upon restoration is a suspension which will settle upon standing but resuspends readily. Care should be taken to have the antigen evenly suspended before withdrawing material from the bottle.

There are no presently-known specific contraindications for Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen except those which apply to cholera vaccine and other similar immunizing biologicals. It should not be given during an acute illness, during convalescence from surgery or trauma, or in other conditions where general resistance is depressed.

On the basis of reactions observed in animals and analogy with cholera vaccine, the subcutaneous administration of Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen may be followed by erythema, induration and tenderness at the site of injection. Malaise and low grade fever may also occur. Most reactions are initiated within a few hours and subside within 24 to 48 hours. In a small proportion of individuals a delayed reaction of similar character may occur three to eight days after injection.

It is strongly recommended that Cholera Vaccine (Inaba Type) Non-Cellular Cholera Antigen be administered subcutaneously. No contraindications to intramuscular administration are known but, as with other antigens, intravenous administration should be avoided. This antigen has been administered satisfactorily to animals both by syringe and needle and by jet injection equipment.

ATTACHMENT 4

THE REACTOGENICITY OF CHOLERA ANTIGENS

Appended to this report is a copy of the data that have been obtained at the Ramsey Unit of the Texas State Department of Corrections concerning temperatures and local reactions of volunteers who received the following cholera antigens in the indicated doses:

Group 1 - Inaba Pool 3 - 300 mcg in 1.0 ml.

Group 2 - CV (Commercial Vaccine) National Drug Co. Lot 6390 - 0.5 ml.

Group 3 - Experimental monovalent Inaba vaccine, Merck,Sharp & Dohme Lot 44450 - 0.5 ml.

The above antigens were all administered by a jet injection gun, and Groups 1 and 2 were injected on May 18, 1968. Group 3 was injected on June 22 since it was not available for the May 18 experiment.

Groups 1 and 2 represent the principal experimental effort at this time and all members of these groups had been drawn from a single pool of volunteers that received physical examinations and bleedings on May 4. Vibriocidal titers were obtained on these pre-immunization bleedings, and Groups 1 and 2 were subdivided out on the basis of equalizing the geometric mean maximum vibriocidal titer for each group. The properties of age, height, weight and frequency of pre-immunization titers were not significantly different within the two groups. The individuals in these groups were then injected on May 18 within an approximate 30 minute period and temperatures were taken 4, 8, 18 and 24 hours after injection. At the time of the 24 hour temperature reading, observations were made conderning the appearance of the injection site in terms of erythema and induration. The original plan of the experiment had included a normal temperature curve to be obtained on each volunteer on the succeeding weekend; however, an examination of the temperatures recorded indicated that they were so close to "normal" (diurnal variation taken into consideration) that it was considered that control curves would not produce data of significance in the interpretation of reactogenicity.

Group 3, receiving the monovalent Inaba vaccine, was handled in the same way except that physical examinations and bleedings were performed on the same day as the injections, the men being taken from their cell blocks a second time so that injections could be accomplished over a short period of time. Because of the logistics of handling prison inmates, it was agreed with the prison authorities that pre-injection temperatures could not be taken if the injection period was to be kept suitably short.

Table 1 below gives a listing of the mean temperature for each group at each observation interval. There are no significant differences in mean temperature among the three groups at each observation interval, and the largest mean found was 98.53.

TABLE 1

Group Designation	Antigen Injected	Number of Volunteers	Time After Injection (Hours) 4	8	12	18	24
1	In. 3	30	98.50	98.47	98.36	97.53	98.12
2	CV	30	98.06	98.36	98.26	97.30	98.21
3	In.Mono.	31	98.53	98.90	98.45	97.43	98.48

Table 2 lists certain other information that was obtained by observation or calculation. The first column indicates the average maximum temperature (regardless of time of occurrence) for each group. Column 2 indicates the number of individuals having any record of temperatures above 99.0°F. Column 3 indicates the total number of temperature records for each group that exceeded 99.0. Column 4 indicates the number of persons in each group having an observable area of erythema exceeding 2 cm. in diameter, and column 5 gives the mean erythema diameter for the positives in each group. Column 6 gives the number of individuals in each group that were judged to have notable induration at the site of injection 24 hours later. Induration measurements were attempted, but were not very successful. However, all observations were made by a single person.

TABLE 2

Group Designation	Antigen Injected	No.of Volunteers	Av. Max. Temp.	No. Temp. 99.0	Readings 99.0	No.with Eryth.	Av.Eryth. Diam(cm)	No.with Ind.
1	In. 3	30	99.04	11	16	14	6.4	4
2	CV	30	98.85	3	4	13	4.4	2
3	In.Mono.	31	99.05	13	19	14	6.0	9

The evaluation of subjective statements or the behaviour of individuals following injection with vaccines is notoriously difficult, and this is particularly true among prison inmates. From observations of the behaviour of the volunteers, it is my opinion that there were few, if any, instances of notable local pain. No instances of spontaneously-reported, delayed reaction were encountered. It is the opinion of the prison hospital supervisor that if such reactions had occurred they would probably have been reported.

The occurrence of relatively large areas of erytheme (usually not accompanied by induration) should be mentioned. We have seen this previously with both Ogawa lipopolysaccharide and with CRL vaccine. It occurred in this instance with about equal frequency in all groups, and some were quite sizable (up to 10 cm. in greatest dimension). This erythema is not painful and is relatively "cool". It is not known at this time what the mechanism of this reaction may be.

It is concluded from these studies that none of the antigens employed were unduly reactogenic. There is a suggestion that the CV antigen may be somewhat less reactogenic than the other two. This suggestion is based upon a lower average maximum temperature, fewer persons having temperatures above 99°, a smaller average diameter of erythema, and a lower frequency of recorded induration. It is my opinion that all of these antigens can be administered to the type of person represented in these studies without concern over the possibility of an unduly high frequency of immediate undesirable reactions.

PROTOCOL

INAPPARENT INFECTION IN MATLAB CHILDREN

by

W. E. Woodward

Background

Several questions have been raised by indirect evidence presented in the literature. Data from Hong Kong, Calcutta and Meheran have suggested a ratio of inapparent cholera infection to symptomatic cases of as much a 100 to 1. These figures were derived from latrine sampling studies and from investigation of serological conversion in contacts of cases of very mild diarrhea which were vibrio positive. More direct evidence of the numbers of inapparent vs. apparent infections is needed.

There is no consensus in the literature concerning the effect of cholera vaccination on the incidence of inapparent infection.

Serological surveys of vibriocidal antibody titers in the hyperendemic population of Matlab have suggested that more than 50% of individuals have a detectable titer by the age of ten years. This would presuppose an average annual infection rate of at least 5%,,which is approximately ten times greater than the average annual attack rate of symptomatic cases. Other studies conducted by Dr. McCormack have shown that, in the 0-4 year age group, approximately 40% of cholera patients no longer have a significant increase in vibriocidal titer one month after the onset of their disease. In fact cholera vaccination is more effective in maintaining a prolonged titer than is the disease itself. This would indicate that, at least in the younger age groups, the average annual inapparent infection rate must be considerable more than 5%. Studies are therefore needed to determine the incidence of inapparent infection with respect to age, and whether this incidence is influenced by prior vaccination.

Previous vaccination studies have shown a significantly lower case rate in vaccinees than in those receiving placebo, and in addition a significantly lower case rate in those receiving placebo than in those receiving nothing. These differences need to be confirmed.

Study

It is proposed than a population of approximately 1,000 children, 0-10 years, in the Matlab vaccine trial area be examined rigorously over at least one, and probably more than one, entire cholera season. The study population will be randomly selected from the 132 villages under census as of December, 1967 and will include all children of ten years or less in every thirtieth family of the area. Selection of older children for the study is not indicated since this age group is frequently unavailable due to school, work or marriage and subsequent migration.

Many of the children in the study population are already participating in the yearly vaccination program, utilizing vaccine X which contains Inaba and Ogawa organisms. A prevaccination fingertip blood specimen for serology will be obtained from the entire population of children. This will be followed by vaccination of the population according to previous vaccine schedules received. Only those children who have received the full course of vaccine and /or placebo in the past will receive the vaccine or placebo in the present study. Consequently there will be a large population, especially in the very young age groups, which will have received neither vaccine nor placebo. Following the vaccination, a second bleeding of the population will be immediately undertaken in order to detect possible errors in vaccine or placebo assignment.

Following the second bleeding, a rectal culture will be obtained from each child twice a week for the duration of the cholera season, regardless of the child's health. Additional cultures will be obtained from any child suffering from diarrhea. All cultures will be examined for cholera vibrios by the bacteriology staff of the Matlab hospital.

There will consequently be more than 2,000 rectal swabs per week. The swabs will be obtained from the children by the village midwives employed by the CRL. Due to the illiteracy of most of the midwives, tubes of enrichment broth used for transporting the swabs from child to hospital will be previously coded and assigned to individual children. In the event of a positive culture, the remainder of the children in the family will be cultured daily for five days.

After the second bleeding of the population, there will be at least two more bleedings depending upon the character of the epidemic curve. It is planned to bleed on the upswing of the curve and again as the cholera is waning. In the event of a more prolonged cholera season than the usual two to three months, it is planned to bleed the population at approximately one month intervals.

Blood specimens will be analyzed for vibriocidal antibody by the microtechnique of Benenson, after all specimens have been collected at the end of the cholera season. In addition, the sera will be saved for possible determination of skin toxin antibody and/or diarrhea toxin antibody content.

An additional blood will be obtained from the index case and all other children in the family ten days after the positive culture of the index case.

PROTOCOL

DETECTION OF INTESTINAL AND SERUM ANTIBODIES IN CHOLERA BY LATEX FLOCCULATION TEST

by

Ansaruddin Ahmed

Introduction

To date there is no simple and satisfactory assay system for measuring cholera antibody in intestinal contents. The complement-dependent vibriolytic test, which is most frequently used for the estimation of serum antibody, cannot be adopted because of the anticomplementary behaviour of intestinal materials. Agglutination with live Vibrio cholerae also does not work.

For this reason an indirect or passive agglutination test, using chicken RBC as the carrier particles was undertaken last year, and after preliminary progress we decided to use polystyrene Latex as the carrier particle.

The technique of indirect or passive hemagglutination is a very sensitive one and more specific than agglutination of bacterial cells. As little as 0.001 uG of some antibody N (nitrogen) can be detected by this serologic method (Wright and Feinberg, 1952) whereas at least 0.1 uG antibody N is required for the quantitative agglutination test (Heidelberger and Kabat, 1934). Nater et al., 1956 have also found that hemagglutinating titers are usually 5 to 50 times higher than those of baterial agglutination. A precipitating antibody fails to give a positive reaction in high dilutions but if the homologous soluble antigen is made to adsorb on a large particle like tanned RBC, Bentonite particles (Bozicevich et al., 1951) or polystyrene Latex particles (Singer and Plotz, 1957) the sensitivity of the antisera to the flocculation test is increased greatly.

The use of erythorocytes as carriers of antigen has drawbacks. Erythrocytes are liable to significant variations from one batch to another, they possess antigens which may react with antibodies in the sera under investigation, they are fragile and difficult to obtain and store.

Purpose of the Study

1. To design and standardize a simple and specific Latex flocculation (fixation) test that will detect and measure cholera antibodies in intestinal materials like intestinal aspirates and stool.

2. Subsequent studies in the nature of intestinal antibodies by this method if possible, will be carried out.

3. To design a Latex flocculation test that will have higher sensitivity than that of live vibrio agglutination for quantitation of serum antibodies in cholera.

Work Done Elsewhere in our Laboratory

Only a few investigators have done passive hemagglutination in cholera using RBC as the carrier particles. Barua and Sack, 1964 using heat extracted soluble antigen from V. cholerae as the reactant and washed fresh sheep cells as the carrier particles found that this test is much less sensitive than the corresponding bacterial agglutination test. Zinnaka (personal communication), using heat extracted antigen in the presence of approximately 1/20 N NaOH and formolized sheep RBC devised a test that reached up to the sensitivity of bacterial agglutination.

In our Laboratory the following soluble antigens were tried with both tanned and fresh chicken RBC as carriers:

1. Purified lipopolysaccharide Ogawa antigen prepared by Dr. Verwey.

2. Supernatant of a 7 year old, whole cell, high potency B vaccine (which was supposed to contain soluble cell wall antigen due to long keeping).

3. A few batches of supernate from sonicated V. cholerae.

4. A few batches of water phenol extracts of V. cholerae by the Westphal method.

Antigen (2) and one particular batch of antigen (4) proved to be the best reactants and we used fresh chicken RBC because the tanned cells gave nonspecific agglutination probably due to inferior grades of tannic acid.

The test came out to be of a little less sensitivity than that of bacterial agglutination in measuring serum antibody.

Stool antibodies were purified before test by absorption with homologous bacteria, washing the bacteria thoroughly, elution of the supposedly specific antiboyd from the bacteria by 10% barium chloride, dialyzing out the barium salt, and concentrating the antibody by taking out water and electrolytes fromthe dialyzing tests strongly indicated that this type of specifically purified and concentrated antibody from intestinal materials will be detected and probably measured by the indirect hemagglutination test.

Materials and Methods

I Samples to be investigated:

a) Paired and serial samples of sera from cholera and non-cholera diarrhea cases.

b) Small gut aspirates from cholera, and non-cholera diarrhea cases at different stages of disease.

c) Stool samples from similar patients.

II Carrier Particles

Polystyrene Latex particles of uniform size, 0.8 u diameter, obtained from the Dow Chemical Co., Midland, U.S.A., will be suspended in such a way that 0.1 ml. of the stock suspension when mixed with 10 ml. of borate or glycine-buffered saline at pH 8.2 gives 70% light transmission at 650 mu. The sensitization will be done by mixing 0.1 ml. of the suspension and 9.5 ml. of buffered saline and 0.5 ml. of suitably diluted antigen.

Antigen

Antigen will be prepared by the following methods:

1. Sonication of vibrio.

2. Water-phenol extraction of Westphal technique.

3. Extraction by boiling.

4. Extraction by boiling in presence of alkali.

The type of antigen best suited will be selected. The sensitizing dose of antigen will be fixed by simultaneous variation of antigen and antibody.

Diluent

The best-suited buffered saline pH 8.2 will be prepared.

Test Proper

Sensitization of Latex particles will be done by incubation at 37°C waterbath for 1 hour, the particles will be washed once or twice. Serially diluted antibody will be mixed with equal volumes of antigen suspension, incubated for 2 hours at 37°C, centrifuged 3 minutes at 1,500 r.p.m., gently dislodged and flocculation read macroscopically.

After setting the test in tubes the microtiter system will be tried.

Facilities Available

A supply of Latex particles has already been received. No extra personnel are necessary for this study. After initial design and standardization of the test under our direct supervision, the laboratory technician, Mr. N. Huda, will be able to carry out the routine tests.

PROTOCOL

PURGING OF FAMILY CONTACTS

by

R.K. Barui et al.

RESEARCH PLAN

Specific Objectives

To do a comparative study between multiple rectal swabs and purging as the effective technique for the demonstration of V. cholerae in the family contacts of cholera patients.

Method of procedure

Soon after admission to the CRL hospital and after the diagnosis of cholera is confirmed by the bacteriology laboratory, a study team consisting of one doctor and a lady field assistant will visit the houses of the cholera patients and bring as many people as possible from the family (definition of a family: persons who live and eat together) to the CRL hospital on the same day. An initial rectal swab from each family member will be collected and streaked directly on Monsur's media (Na - taurocholate, K - tellurite and gelatin), gelatin agar and kept in bile peptone enrichment broth for culture for V. cholerae. 50 lambda blood from the fingertips will also be collected for vibriocidal antibody determination by the microtiter method.

All family members will be placed in one of two groups alternately. To one group, Mannitol in water (dose ranging from 15 gm. to 60 gm. depending on age) following an ounce of butter will be given as purgatives. The subsequent four stool samples will be collected in sterile bottles and sent to the bacteriology laboratory for culture for V. cholerae. A daily rectal swab will also be collected from them during their stay in the hospital. From the other group, only daily rectal swabs will be collected during their hospital stay.

All members will be hospitalized for 5 days.

A second blood sample will be collected from all the family members on the tenth day.

LIBRARY REPORT FOR FY 1968

The library services have shown an overall increase in obtaining supplementary material for the research investigators.

I. Photocopies: 508 journal articles were requested from NLM, each article containing an average of ten pages. Moreover, 4132 pages were photocopied through PAMSDOC and P-SCRL machines.

II. Exchange: In order to cope with the increasing information demands, the Library is now participating in a scheme being operated by World Health Organization library, known as "International Exchange of Duplicate Medical Literature". More than one hundred medical libraries of the world exchange their surplus journals and books by sending their "want-list" and "offers-list" to the WHO library, which mimeographs and circulates these lists among the participating member libraries. In reply to our want list, we have received a total of 471 back numbers of medical journals and microfilm of nearly 3000 pages of medical journals from different member libraries.

As a further measure of getting more journals of our interest, we inserted an announcement in the "UNESCO Bulletin for Libraries", September 1967, offering "East Pakistan Medical Journal" (Q) and "Journal of Pakistan Medical Association (M), in exchange for other medical journals from all over the world. We contacted medical libraries, medical associations, health directors, doctors, and other institutions in Pakistan to donate copies of Pakistani medical journals and the above-mentioned two journals in particular, so that we may be able to carry out our exchange of journals. We note with pleasure that in less than six months, we have successfully established an exchange agreement with more than ten countries such as Canada, Burma, Czechoslovakia, India, Phillippines, Japan, Denmark, etc. We have started receiving the following important journals under the exchange program:

1. "University Studies" - Karachi University Library
2. "Microbia" - Karachi University Library
3. "Biochemica" - Karachi University Library
4. "The Union of Burma Journal of Life Sciences" Pub. Burma Medical Research Institute, Rangoon
5. Pakistan Journal of Medical Research - Lahore
6. Danish Medical Bulletin - Copenhagen
7. Index Medicus Danicus - Copenhagen
8. Seminar on cholera control: 1968 - New Delhi, India
9. Canadian Medical Association Journal - Toronto
10. Canadian Journal of Biochemistry - Ottawa
11. Canadian Journal of Microbiology - Ottawa
12. Index to Indian Medical Periodicals - New Delhi

13. Folia Microbiologica (in English), Czechoslovakia
14. Physiologia Bohemoslovenica (in English), Czechoslovakia
15. Paediatria Universitatis - Tokyo
16. Acta Universitatis Carolinae Medica (in English) Czechoslovakia
17. Annales des Societes Belges de Medicine Tropicale de Parasitologie et de Mycologie-Antwerp
18. Annual volume of "Recueil des travaux scientifiques" Prince Leopold Institute of Trop-Medicine-Belgium

Similarly, another exchange program is being arranged with U.K. via British National Book list of surplus scientific material.

III. 38 periodical and serial titles have been added to the annual subscriptions (See Table I).

With the increase in information dissemination, the library has started maintaining daily statistics of :

1. Current periodical circulated
2. Original reprints circulated
3. Photocopies provided through (a) P-SCRL, (b) U.S. National Library of Medicine, (c) World Health Organization, etc.
4. Bound journals loaned
5. Books referred to in library
6. Books loaned
7. Books added
8. Bound journals added
9. "Overdue" notices sent out

The annual statistics show nearly twelve thousand books were referred to in the library, and nearly two thousand books were loaned, nearly eight thousand photocopy-pages were provided through P-SCRL and the U.S. National Library of Medicine (See Table II).

Some very important seminars were held in the library during the year. Many outside specialists requested us to supply them with scripts or taped lectures of our seminars. In view of such requests, and in order to preserve the valuable lectures for reproduction, we would like to suggest medical sound library service whereby records and tapes of lectures may be stored for reference or loan. Among the required equipment are clip-on microphones for the speakers, magnetic tapes, a tape recorder and storage cabinets.

The library with its very valuable material such as films, microfilms, back files of journals, reprints, monographs, etc. needs insurance coverage against theft and fire.

There is an urgent need for additional bookcases and increased library space. The demands on the available space of the library have increased to an extent where back files of journals have to be

removed from shelves and piled up aside to accommodate latest bound volumes.

Library Staff

The library activities, as compared with last year's show an increase by 50% in inter-library loans, 200% in photocopy service through P-SCRL and PANSDOC, 30% in circulation of loose journals and reprints to research investigators, and the extra 100% increase of the new venture of exchange of journals.

The two professional librarians and one attendent took the responsibility of the extra work, without affecting the quality of service by giving immediate attention and taking prompt action on every request from the clientele. During Mrs. Molla's leave from April to June, a temporary Library Assistant was appointed. In view of the above-mentioned increased library activities besides other minor routine duties the library will be able to give effective service by systematically preserving and promptly retrieving the rapidly accumulating materials, with the help of a library assistant on permanent basis.

TABLE I

ADDITIONAL PERIODICALS SUBSCRIBED DURING JULY 1967 TO JUNE 1968

1. Advances in Clinical Chemistry
2. " in Immunology
3. " in Internal Medicine
4. " in Pediatrics
5. " in Virus Research
6. Annual Cumulative Index to British Medical Journal
7. Biochemical Preparations
8. Bull. of the Inst. of Statistical Research & Training
9. Circulation
10. Currents in Modern Biology
11. Excerpta Medica: II c Pharmacology & Toxicology
12. Excerpta: 26. Immunology & Serology
13. Goddard's Gazette: Section 705(375) FDa papers
14. Immunology
15. International Review of Experimental Pathology
16. Int. Review of Tropical Medicine
17. Journal of Hygiene, Epidemiology, Microbiology & Immunology
18. Journal of Medical Lab. Technology
19. Journal of Nuclear Medicine
20. The Kidney
21. Lib. of Congress: World List of Future International Meetings
22. Medical Clinics of North America
23. Merck Index
24. Merck Manual
25. Methods of Biochemical Research
26. Methods in Enzymology
27. National Lib. of Medicine: Bibliography of Medical Translations (U.S.A.)
28. National Lib. of Medicine: Bibliography of Medical Reviews (U.S.A.)
29. New Drugs
30. Pakistan Journal of Medical Research
31. Pakistan Journal of Science
32. Pediatric Clinics of North America
33. Physician's Desk Reference
34. Rockefeller University Review
35. Scientific Meetings
36. Special Libraries
37. Standard Methods of Clinical Chemistry
38. Vitamins and Hormones

THE FOLLOWING JOURNALS HAVE BEEN DISCONTINUED

1. Journal of American Water Works Association
2. Journal of Water Pollution Control Federation
3. Library Quarterly

TABLE II.

LIBRARY STATISTICS FOR FY 1967-68

	July-Sept. 1967	Oct.-Dec. 1967	Jan.-Mar. 1968	Apr.-June 1968		Total
1. Current periodicals circulated	1085	1370	1523	1857		5835
2. Original reprint circulated	530	916	1603	1632		4681
3. Photocopy of pages provided:						
(a) from P-SCRL	1346	1001	483	1302		4132
(b) from U.S. National Lib. of Med.	789	1063	445	1394		3691
(c) from World Health Organisation	-	-	-	28		28
4. Bound journals loaned	87	61	87	206		441
5. Books referred to in Library	2328	2840	3218	3518		11904
6. Books loaned	517	439	459	525		1940
7. "Overdue" notices sent out	260	215	133	209		817
8. Books added	157	81	65	206	(IPH 60+CRL 509) =	569
9. Bound journals added	75	67	55	98	(IPH 6 + CRL 295) =	301
10. Maps and Atlases	6 + 7					13

Inter-library loan of xerox copies : Requests made to NLM total 508 articles

Gifts received : Reprints ... 1353
Loose medical journals ... 1569
Microfilmed pages ... 3000

Books - fiction 154
Reports and books 180 (Medical) ... 334

TABLE III-A

LIBRARY ACQUISITIONS

Fiscal Year	CUMULATIVE TOTAL: Books, Bound, Journals and Reports		BOOKS AND REPORTS	
	Accession Number	Annual Additions	Annual Additions	Cumulative Book Total
1962 Jan-June	1-379	379	224	224
1962-63	380-992	613	229	453
1963-64	993-1410	418	202	655
1964-65	1411-1788	378	158	813
1965-66	CRL1789-2445	CRL 657	CRL 320	CRL 1133
	IPH 1-547	IPH 547	IPH 536	IPH 536
1966-67	CRL 2446-3660	CRL1415	CRL 494	CRL 1627
	IPH 548-719	IPH 172	IPH 168	IPH 704
1967-68	CRL3861-4664/A IPH 720-780	CRL 804 IPH 61	CRL 509 IPH 55	CRL 2136 IPH 759

TABLE III-B

LIBRARY ACQUISITIONS

Fiscal Year	BOUND JOURNALS		Periodicals subscribed. Cumulative Total	Inter Library Loan. Journal articles on Xerox from NLM per year	Photocopies provided by PSCRL and PANSDOC Machines per year	Reprints Cumulative Total
	Annual Additions	Cumulative Bound Journals Total				
1962 Jan-June	155	155	-	140	-	200
1962-63	384	539	63	185	-	500
1963-64	216	755	70	203	-	976
1964-65	220	975	80	225	-	1448
1965-66	CRL 337 IPH 11	CRL 1312 IPH 11	146	352	-	CRL2600 IPH 120
1966-67	CRL 921 IPH 4	CRL 2233 IPH 15	158	339	1630pp.	2700 Approx.
1967-68	CRL 295 IPH 6	CRL 2528 IPH 21	196	508	WHO 28pp. 4132pp.	2800 Approx.

PSCRL WARD REPORT

1 July 1967 - 30 June 1968

A total of 1404 patients were admitted to the Ward this year, of which 224 were cases of cholera.

The non-cholera patients, total 1180, were the usual amoebiasis, shigellosis, salmonella and viral infections, plus a large number of children suffering from malnutrition, kwashiorkor-like states and non-pathogenic diarrhoeas.

2458 patients attended the outpatient department during this year.

8927 liters of intravenous fluids were used in the ward.

After the unprecedented heavy season last year, cholera disappeared from Dacca and surrounding areas; no cases of cholera were admitted from 14 June 1967 to 20 January 1968.

Clinical studies on cholera were at a standstill for lack of patients, until PSCRL was asked to help with an outbreak of cholera in a rural area in Chittagong District - the Malumghat Cholera Project was put into operation in November 1967. A separate summary of this project is attached.

When cholera did eventually break out in the Dacca area, in April, a variety of clinical research projects were carried out. These included the pediatric balance study, intravenous acetate study, oral lavage of vibriophage study and the oral glucose therapy study.

It has been a comparatively quiet year and no great pressure has been put on either staff or accommodation for acute and convalescent patients. However, it must be pointed out that bathing and latrine facilities for acute convalescent, attendants, outpatients and others are totally inadequate and some alterations and installations must be made.

Admissions to CRH Ward - 1 July 1967 to 30 June 1968

TABLE 1.

Month	Total Admissions	CHOLERA		NON-CHOLERA CASES	
		Total	Death	Total	Death
July 1967	124	nil	nil	124	4
August	133	nil	nil	133	4
September	108	nil	nil	108	2
October	124	nil	nil	124	2
November	98	nil	nil	98	3
December	80	nil	nil	80	4
January 1968	68	1	nil	67	4
February	60	2	nil	58	3
March	169	27	nil	142	6
April	214	78	nil	136	3
May	148	92	1	56	4
June	78	34	nil	44	2
TOTAL	1404	224	1	1130	41

TABLE II

X-Ray Department

1 July 1967 - 30 June 1968

Type	Total	Patients	Employees & others
Chest X-Rays	1197	728	469
Opaque Medium	64	46	18
Others- abdomen, extremities, etc.	344	163	181
Total	1605	937	668

SUMMARY OF MALUMGHAT CHOLERA PROJECT

On November 17, 1967, the Cholera Research Laboratory received a call for assistance from the Memorial Christian Hospital, Malumghat, because of a large outbreak of cholera moving from Chittagong towards Cox's Bazar.

Because of the absence of cholera in Dacca, the Laboratory responded with an offer to set up a treatment and study centre at the Mission Hospital.

An initial survey team verified the outbreak, surveyed the available facilities and was assured of complete co-operation by both the Hospital staff and local government officials. Following this, a treatment and research team was sent to set up and operate a Cholera Ward at the Mission Hospital. The PSCRL agreed to supply all personnel, supplies, intravenous fluids, medications and transport necessary for the care and study of cholera patients. Also PSCRL agreed to supply the fuel necessary to insure 24-hour operation of the electrical generators necessary to support the research operation.

The PSCRL staff consisted of doctors, nurses, NIH Research Fellow, bacteriology and chemistry technicians, supply officer, drivers and domestic help.

A common dining facility for staff was set up on the mission compound and sleeping accommodations were arranged on the compound, in a nearby government community centre and at the government motels in Cox's Bazar.

A system of regular transport of supplies and equipment from Dacca was set up using PSCRL transport.

Because of the wide geographic scatter of cases, the difficulties of local transport, and ignorance of the fact that cholera therapy was available and free, a system of actively seeking out of cholera patients was set up to ensure adequate numbers of cases to support the clinical research projects. Field teams went out daily in search of cholera cases and spread the news of the available free treatment. Such teams went to villages over a 60-mile area from North to South in search of patients, and to answer reports of suspected cases. Intravenous therapy was begun in the field and continued while the patients were transported back to the Hospital.

Patient recruitment was so successful that during the peak of the epidemic in mid-December ward space in the Hospital became inadequate to handle the patient load, and a hospital tent was erected adjacent to the ward to handle the overflow of patients.

As the epidemic waned in January, the cholera ward was transferred to the tent. The operation continued into mid-February for the study of convalescent cases, although the last positive cholera case was admitted on January 24, 1968.

In total, 314 patients were admitted in the PSCRL cholera ward at Malumghat. 211 proved to be acute cholera infected with V. Cholera, Inaba. There were no deaths among the proven cases, but there was one death from renal failure and ureamia in a suspected case of cholera.

A variety of clinical research projects were carried out at Malumghat. These included the unidirectional flux study, the oral glucose therapy study, the pediatric balance study, the purging study of convalescent carriers, the intravenous acetate study, the c-14 Mannitol permeability study, the oral diamox study, the collection of jejunal biopsies in acute cholera for electron-microscopy and the collection of cholera stool and intestinal samples for immunologic studies.

It demonstrated the capability of the Laboratory and especially the Clinical Ward to move at short notice to a remote rural area and to set up an effective treatment centre and clinical study ward. And it demonstrated again that meaningful clinical research can be done in remote rural areas with a minimum of pre-existing facilities.

The experience at Malumghat should be remembered in the future if the admission rate in Dacca is not sufficient to support the clinical projects planned for a given cholera season.

IV.

PSCRL PUBLICATIONS

and

PRESENTATION AT SCIENTIFIC MEETINGS BY PSCRL STAFF

PSCRL PUBLICATIONS

FY1968

STUDIES PUBLISHED BY PSCRL:

Mosley, W.H., Chowdhury, A.K.M.A., Aziz, K.M.A., Islam, S., Fahimuddin, M.: Demographic Studies in Rural East Pakistan (preliminary analysis of the results of daily registration of births, deaths and migrations in 132 villages in the cholera vaccine field trial area in Comilla District, East Pakistan, May 1966-April 1967). Dacca, East Pakistan, June 1968, 30 pp.

ARTICLES PUBLISHED IN PERIODICALS:

Khan, A.Q., Stockard, J.L.: A trial of ascorbic acid as a prophylaxes agent in families exposed to cholera. J. Pakistan Medical Assn., 17:No.7, pp. 199-202, July 1967.

Phillips, R.A.: Twenty years of cholera research. J. American Medical Assn., 202: pp. 610-614, November 1967.

Mosley, W.H.: Typhoid and cholera vaccines. Letter to the Editor of The Lancet, No. 1, p. 299, February 1968.

Love, A.H.G., Mitchell, T.G., Phillips, R.A.: Water and sodium absorption in the human intestine. J. Physiology, 195: pp. 133-40, March 1968.

Khan, M., Mosley, W.H.: The significance of shigella as a cause of diarrhea in a low economic urban community in Dacca. East Pakistan Medical Journal, 12: No. 2, pp. 45-52, April 1968.

Harvey, R.M., Enson, Y., Lewis, M.L., Greenough, W.B., Ally, K.M., Panno, R.A.: Hemodynamic studies of hypovolemia and acidosis. Circulation, 37: pp. 709-28, May 1968.

Molla, P.: The role of the librarian in medical research. Eastern Librarian, Dacca, East Pakistan, 2: No.4, pp. 25-34, June 1968.

Phillips, R.A.: Asiatic cholera (with emphasis on pathophysiological effects of the disease). Annual Review of Medicine, 19: 1968, pp. 69-80.

Kinzie, J.L., Hare, W.K.: Measurement of intestinal permeability in man by diffusion ratio technique. Federation Proceedings, 27: 1968, p. 385.

Taylor, J.O., Hare, R.S., Phillips, R.A.: Measurement of Na fluxes in human small intestine. Federation Proceedings, 27:1968, p. 286.

Lindenbaum, J., Greenough, W.B., Islam, R.: Antibiotic therapy of cholera. Bull. W.H.O., 36, No. 6, 1967, pp. 871-883.

Lindenbaum, J., Greenough, W.B., Islam, R.: Antibiotic therapy of cholera in children. Bull. W.H.O., 37: No. 4, 1967, pp. 529-38.

Benenson, A.S., Saad, A., Paul, M.: Serological studies in cholera. 1. Vibrio agglutinin response of cholera patients determined by a microtechnique. Bull. W.H.O., 38: No. 2, 1968, pp. 267-276.

Benenson, A.S., Saad, A., Mosley, W.H.: Serological studies in cholera. 2. The vibriocidal antibody response of cholera patients determined by a microtechnique. Bull. W.H.O., 38, No. 2, 1968, pp. 277-285.

Benenson, A.S., Saad, A., Mosley, W.H., Ahmed, A.: Serological studies in cholera. 3. Serum toxin neutralization - rise in titre in response to infection with vibrio cholerae, and the level in the "normal" population of East Pakistan. Bull. W.H.O., 38: No. 2, 1968, pp. 287-295.

Mosley, W.H., Benenson, A.S., Barui, R: A serological survey for cholera antibodies in rural East Pakistan. 1. The distribution of antibody in the control population of a cholera-vaccine field-trial area, and the relation of antibody titre to the pattern of endemic cholera. Bull. W.H.O., 38: No. 3, 1968, pp. 327-334.

Mosley, W.H., Benenson, A.S., Barui, R.: A serological survey for cholera antibodies in rural East Pakistan. 2. A comparison of antibody titres in the immunized and control populations of a cholera-vaccine field-trial area, and the relation of antibody titre to cholera case rate. Bull. W.H.O., 38: No. 3, 1968, pp. 335-346.

Benenson, A.S., Joseph, P.R., Oseasohn, R.O.: Cholera vaccine field trials in East Pakistan. 1. Reaction and antigenicity studies. Bull. W.H.O., 38, No. 3, 1968, pp. 347-357.

Benenson, A.S., Mosley, W.H., Fahimuddin, M., Oseasohn, R.O.: Cholera vaccine field trials in East Pakistan. 2. Effectiveness in the field. Bull. W.H.O., 38, No. 3, 1968, pp. 359-372.

Mosley, W.H., Ahmed, S., Benenson, A.S., Ahmed, A.: The relationship of vibriocidal antibody titre to susceptibility to cholera in family contacts of cholera patients. Bull. W.H.O., 38: No. 5, 1968, pp. 777-785.

McCormack, W.M., Chowdhury, A.M., Jahangir, N., Ahmed, A.B.F., Mosley, W.H.: Tetracycline prophylaxis in the families of cholera patients. Bull. W.H.O., 38: No. 5, 1968, pp. 787-792.

Chowdhury, A.K.M.A., Mosley, W.H.: Statistical approach for estimating the effectiveness of cholera vaccine. Statistics for Development (Institute of Statistical Research and Training, Dacca, East Pakistan,) May 1968, pp. 78-80.

*Aziz, K.M.S., Mohsin, A.K.M., Hare, W.K., Phillips, R.A.: The rat as a cholera model. Nature, 220: pp. 814-815, November 1968.

*Hirschhorn, N., Rosenberg, I.H.: Sodium-potassium stimulated adenosine triphosphatase of the small intestine of man; studies in cholera and other diarrheal diseases. J. Laboratory and Clinical Medicine, 72: pp. 28-, July 1968.

*Hirschhorn, N., Kinzie, J.L., Sachar, D.B., Northrup, R.S., Taylor, J.O., Ahmed, S.Z., Phillips, R.A.: Decrease in net stool output in cholera during intestinal perfusion with glucose-containing solutions. New England J. of Med., 279: pp. 176-181, July 1968.

*Nalin, D.R., Cash, R.A., Islam, R., Molla, M., Phillips, R.A.: Oral maintenance therapy for cholera in adults. The Lancet, II: pp. 370-372, August 1968.

*Hirschhorn, N.: Bile in acute cholera. The Lancet, II: p. 405, August 1968.

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McCormack, W.M., Chowdhury, A.M., Jahangir, N., Mosley, W.H.: Tetracycline prophylaxis in the families of cholera patients. Bull. W.H.O.

Mosley, W.H., McCormack, W.M., Fahimuddin M., Aziz, K.M.A., Rahman, A.S.M., Chowdhury, A.K.M.A., Martin, A.L., Phillips, R.A.: 1966-67 cholera vaccine trial in rural East Pakistan. 1. Study design and results of first year of observations. Bull. W.H.O.

Mosley, W.H., McCormack, W.M., Barui, R.K., Ahmed A., Chowdhury, A.K.M.A.: 1966-67 cholera vaccine field trial in rural East Pakistan. 2. Results of the serological surveys in the study populations: the relationship of case rate to antibody titer and an estimate of the inapparent infection rate with V. cholera. Bull. W.H.O.

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McCormack, W.M., Rahman, A.S.M.M., Chowdhury, A.K.M.A., Mosley, W.H.: 1966-67 cholera vaccine field trial in rural East Pakistan. 3. The lack of effect of prior vaccination or circulating vibriocidal antibody on the severity of clinical cholera. Bull. W.H.O.

Mosley, W.H.: The role of immunity in cholera. A review of epidemiological and serological studies. Texas Reports on Biology and Medicine.

Mosley, W.H., Langmuir, A.D.: The evaluation of cholera vaccine in an endemic area. Bull. of the International Epidemiological Assn.

Mosley, W.H.: The surveillance of cholera. Tropical Medicine in the Promotion of World Health, Final Report of the Eighth International Congresses of Tropical Medicine and Malaria, Teheran, Iran, 1968.

Rosenberg, I.H., Hirschhorn, N.: Bile salts and bile pigments in rice water stool. The Lancet.

Martin, A.R., Vernon, T.M., Mosley, W.H.: Studies of the neutralization of the vascular permeability factor of Vibrio cholerae in man. American J. of Tropical Medicine and Hygiene.

Martin, A.R., Mosley, W.H., Sau, B., Ahmed, S., Huq, I.: Epidemiologic analysis of endemic cholera in urban East Pakistan, 1964-1966. American J. of Epidemiology.

PAPERS READ AT SCIENTIFIC MEETINGS

FY 1968

AUGUST 1967

6TH ANNUAL MEDICAL SYMPOSIUM - JINNAH POSTGRADUATE MEDICAL CENTER, KARACHI, WEST PAKISTAN.

1. "Some Studies on Cholera Patients in Relation to Cholera Toxin" - Aziz, K.M.S., Ph.D.

2. "An Analysis of 41 Consecutive Fatal Diarrhoeal Cases in Children Admitted to the Pakistan-SEATO Cholera Research Laboratory, Dacca" - Rahaman, M.M., Ph.D.

3. "Epidemiologic Pattern of Diarrhea and Cholera in a Semi-urban Community"-Khan, Muslemuddin, M.B.B.S.

DACCA MEDICAL COLLEGE, DACCA, EAST PAKISTAN

4. "Carriers in cholera" - Molla, A. Majid, M.B.B.S.

NOVEMBER 1967

2ND INTERNATIONAL CONFERENCE ON GLOBAL IMPACTS OF APPLIED MICROBIOLOGY, ADDIS ABABA, ETHIOPIA

5. "Economic Aspects of Cholera and Its Control Measures" - Huq, Mr. Md. Imdadul

14TH ANNUAL MEDICAL CONFERENCE OF THE PAKISTAN MEDICAL ASSOCIATION, EAST ZONE, DACCA

6. "Severe Protein-calorie Malnutrition in Children and Vitamin Deficiencies in Hospital Admission" - Rahaman, M.M., Ph.D.

7. "Some Observations on Deaths Attributed to Supernatural Causes" - Chowdhury, Mr. A.L.M. Alauddin

8. "Significance of shigella as a cause of diarrhea in a low economic urban community in Dacca" - Khan, Moslemuddin, M.B.B.S.

JANUARY 1968

PAKISTAN'S FIRST NUTRITION CONFERENCE, DACCA UNIVERSITY, DACCA, EAST PAKISTAN

9. "Pediatric Nutrition" - Rahaman, M.M., Ph.D.

FEBRUARY 1968

3RD AFRO-ASIAN PAEDIATRIC CONGRESS, KARACHI, WEST PAKISTAN

10. "Diarrhoea and severe malnutrition in children - a comparison between fatal and non-fatal case admitted to the Pakistan,SEATO Cholera Research Laboratory, Dacca, East Pakistan" Raheman, M.M., Ph.D.

11. "A prospective Study of Patterns of Morbidity and Growth in Infants in a Low economic Urban Community in East Pakistan" - Ahmed, Shamsa., M.B.B.S.

MARCH 1968

CENTO SEMINAR ON COMBATING PROTEIN-CALORIE MALNUTRITION IN PRE-SCHOOL CHILDREN, NATIONAL HEALTH LABORATORY, ISLAMABAD, WEST PAKISTAN

12. "Protein-calorie malnutrition and diarrhoea in 0-4 year old children in East Pakistan" - Raheman, M.M., Ph.D.

AMERICAN EPIDEMIOLOGIC SOCIETY, VIRGINA, U.S.A.

13. "A Community study of Inapparent Cholera Infections in Rural East Pakistan" - McCormack, W.M., M.D.

APRIL 1968

FEDERATION MEETING OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY, ATLANTIC CITY, U.S.A.

14. "Measurement of intestinal permeability in man by diffusion ratio technique" - Kinzie, J.L., M.D.

15. "Measurement of Na fluxes in human small intestine" - Taylor, James O., M.D.

MAY 1968

SEMINAR ON "STATISTICS FOR DEVELOPMENT" DACCA UNIVERSITY, DACCA

16. "Statistical approach for estimating the effectiveness of cholera vaccine" - Chowdhury, A.I.M.A.

INSTITUTE OF POSTGRADUATE MEDICINE, DACCA

17. "Current Management of Cholera" - Northrup, R.S., M.D.