PAKISTAN-SEATO CHOLERA RESEARCH LABORATORY

TECHNICAL COMMITTEE MEETING

27-29 November, 1967.

Dacca, East Pakistan.

TECHNICAL COMMITTEE MEETINGS

27-29 NOVEMBER, 1967.

M E M B E R S

Chairman

Dr. Colin MacLeod
Vice President for Medical Affairs,
Commonwealth Fund,
New York.

Dr. Ali Nawab Khan
Deputy Director General of Health,
Government of Pakistan,
Islamabad.

Dr. James W. Howie
Director of Public Health
Laboratory Service,
London.

C O N S U L T A N T S

Dr. Andrew Abraham
Research Associate,
Department of Pathology,
Harvard Medical School.

Dr. Graham Bull
Director of Clinical
Research Centre,
Medical Research Council,
London.

Dr. Peter Curran
Associate Professor of
Physiology,
Yale University School of Medicine.

Dr. Robert S. Gordon
Clinical Director, NIAMD,
National Institutes of Health.

Dr. John S. Johnson
Clinical Investigator, NIAID,
National Institutes of Health.

Dr. Alexander Langmuir
Chief, Epidemiology Program,
Communicable Disease Center,
Atlanta.

Dr. Alexander R. Lawton,
Clinical Associate, NIAID,
National Institutes of Health.

Dr. Jean F. Rogier
Chief Public Health Advisor,
US/AID, East Pakistan.

SCIENTIFIC AND PROFESSIONAL STAFF

1.	Dr. Robert A. Phillips	Director.
2.	Dr. Kendrick W. Hare	Deputy Director.
3.	Mr. Patrick G. Talmon	Executive Officer.

Section Chiefs

1.	Dr. James O. Taylor	Chief, Clinical Investigation Section.
2.	Dr. Wiley H. Mosley	Chief, Epidemiology Section.
3.	Mr. Mark P. Tucker	Chief, Maintenance Section.
4.	Dr. K.M.S. Aziz	Chief, Animal Resources Section & Guest Investigator.
5.	Mr. Md. Imdadul Huq	Chief, Bacteriology Section.
6.	Mr. Syed Zafar Ahmed	Chief, Chemistry Section.
7.	Mr. Nuran Nabi	Chief, Administrative Section.
8.	Miss D.G. Torrance	Hospital Supervisor, CRL Ward.

Guest Investigators

1.	Dr. M. M. Rahman	Guest Investigator.

Professional Staff

1.	Dr. W.M. McCormack	Epidemiologist.
2.	Dr. Albert R. Martin	Epidemiologist.
3.	Dr. Robert S. Northrup	Research Physician.
4.	Dr. Joseph L. Kinzie	Research Physician.
5.	Dr.(Mrs.) Ruth S. Hare	Experimental Pharmacologist.
6.	Dr. David R. Nalin	Research Physician
7.	Dr. Richard A. Cash	Research Physician.
8.	Dr. Moslemuddin Khan	Deputy Section Chief, Epidemiology Section.
9.	Mr. A.K.M. Mohsin	Research Associate, Animal Resources Section.

(more)

Profession Staff (Cont'd)

10.	Dr. Ansaruddin Ahmed	Research Associate, Epidemiology Section.
11.	Dr. A.K. Bhattacharjee.	Research Associate, Epidemiology Section.
12.	Mr. K.M.A. Aziz	Research Associate, Epidemiology Section.
13.	Dr. Kh. Abdullah Al-Mahmud	Research Veterinarian, Epidemiology Section.
14.	Dr. Md. Rafiqul Islam	Chief Resident, Clinical Investigation Section.
15.	Dr. A. Majid Mollah	Physician, Clinical Investigation Section.
16.	Dr. Kh. M.M. Toaha	" "
17.	Dr. Md. Zahedul Huq	" "
18.	Dr. Paul S.K. Saha	" "
19.	Dr. Golam Rahman Shaikh	" "
20.	Dr. Waliur Rahman	" "
21.	Dr.(Miss) Lutfa Khandakar	" "
22.	Dr. S. A. Wahid	" "
23.	Dr. R.K. Barui	Physician, Epidemiology Section.
24.	Dr. Moslehuddin Khondakar	" "
25.	Dr.A.S.M. Mizanur Rahman	" "
26.	Dr.(Mrs.) Shamsa Ahmed	Physician, Epidemiology Section.
27.	Dr. Ahmed Rashid	Veterinarian.
28.	Dr.S.Z. Anwarul Quader	"

TECHNICAL COMMITTEE MEETING

27-29 November, 1967.

SUMMARY OF DATA PRESENTED

RESEARCH PROTOCOLS.

APPENDICES.

PAKISTAN - SEATO CHOLERA RESEARCH LABORATORY

ANNUAL REPORT FOR FY67 : DIRECTOR'S COMMENTS

This report is prepared for submission to Dr. Margaret Pittman, Project Officer, NIH Research Agreement No. 196802, and will also be submitted to the Technical Committee for their consideration at their annual meeting, 27-29 November 1967.

The accompanying report was prepared by the Section Chiefs. There is also a report of the Library and Ward facilities; these facilities organization-wise are in the Clinical Investigation Section.

The last project report was submitted in April 1967. Subsequently the Project Officer requested that the Director prepare a project report which would be submitted about 1st October covering the previous fiscal year. This report would then serve as a report to the Technical Committee.

In view of the above, this annual report covers some of the material contained in the last annual report. Also, the Section Chiefs wished to include, in addition to projects completed in FY67, projects continuing from FY67 through this year and new projects instituted since the end of FY67.

The arrival of the Head of the new Maintenance Section and the strengthening of the Administration Section should result in greatly improved research facilities. This has been offset by the death of Professor Kenneth Goodner, and the serious illness of Dr. A. J. H. Tomlinson who had planned to join the laboratory for this coming cholera season.

It is planned in future years that the annual report restrict itself to the previous fiscal year. It is requested that the project officer and members of the Technical Committee and Directing Council review this report and make comments and suggestions on means of improving the report. If feasible, such recommendations will be incorporated in future reports.

At the present time, almost all of the research projects are located in Clinical Investigation Section and Epidemiology Section, with the other Sections providing the necessary laboratory and logistic support. It would be highly desirable if Sections such as Bacteriology and Chemistry could be augmented with appropriate personnel so that research in these areas could be properly expanded.

ADMINISTRATIVE SECTION REPORT FOR FY67

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. Administrative support in this respect is broad in scope and includes consideration of staff, space, utilities, equipment and supplies, and maintenance.

As a result of the expansion of on-going research programs and the instigation of new studies during FY67, the laboratory staff has increased considerably. On July 1, 1966, the laboratory had a total staff of 430 (Headquarters 227, Matlab 203) whereas on July 1, 1967 the number was 576 (Headquarters 290, Matlab 286). The figures for Matlab include boatmen and midwives who are hired on a temporary and seasonal basis. The increase in staff at Matlab can be explained by the decision to increase considerably the vaccine surveillance area. After the arrival of Dr. and Mrs. Hare in September, 1966, the Chemistry Section staff increased considerably as many efficient and effective methods of analyses were introduced by the Hares. Several additional staff were also taken on at headquarters by the Epidemiology Section to cope with the increased data output obtained from the extended surveillance area at Matlab Bazar.

As a result of the above mentioned increase in staff on the research side of the laboratory, a number of new positions were needed on the administrative side to deal with the increased demands from research. The staff of the Administration Section numbered 69 on July 1, 1966 (total staff 430) whereas the figure one year later was 92 (total staff 576).

With the need to provide increased supportive services to the research sections, it became necessary to reorganise the structure of the Administration Section to place it in a better position to serve the needs of the laboratory. Mr. Charles Myers, Management Analysis Officer of NIAID, NIH, was brought out to Dacca in the summer of 1966 for several months to formulate and implement the reorganisation of the Administration Section. As a result, administration was divided into several specialised area, i.e. personnel, finance, transportation, maintenance, general services and supply.

Housing is as acute a problem today as it was one year ago; adequate laboratory and office space is not available to house the large staff. In this connection it is hoped that a favourable decision can soon be reached by USAID/Washington on CRL's request for a new four-storey building. It should be briefly noted that the overflow of cholera patients during the yearly cholera season has to be sheltered in tents and makeshift housing. The laboratory's library is desperately in need of room for expansion.

Utilities is another area where the research work suffers because of lack of supportive facilities such as a proper and constant source of electricity, a hot water supply, and gas for laboratory use. However, we have been fortunate in installing a steam generating plant to provide a dependable source of demineralised water and steam for autoclaves and stills,but not a hot water supply as originally envisaged.

The scope and quality of research equipment has increased and improved steadily over the past year, but there is much room for further improvement. Most of the existing equipment is not of the quality found in a first class research laboratory. An adequate flow of supplies needed for research is another problem. Frequently a research project has been delayed due to the delay in obtaining required supplies. Improvements are being made in reducing this bottleneck but at present this problem remains at times quite frustrating to investigators.

Although not occuring within the period covered by this report, it is worthwhile to mention that with the arrival of our Maintenance Officer from the U.S. in August, 1967, and the consequential organisation of a separate Maintenance Section, it is hoped that many of the maintenance problems of keeping laboratory equipment operational that have plagued the laboratory since its inception will be overcome in time. However, for the purpose of establishing an adequate maintenance shop a sum of approximately $6000 will be required, which, if

it cannot be met from this year's budget, should be budgeted for next year.

To conclude, the nature, scope and quality of research that can be conducted will to a large extent depend on the supporting environment in which the research takes place. Consequently, to the extent that supportive services can be improved, to that same extent will the working environment be propitious to good research.

BREAKDOWN OF P-SCRL PERSONNEL

		Director's Office	Administration	Epidemiology Sec. HQ	Epidemiology Sec. Matlab VTS	Clinical Investigation Section Research	Clinical Investigation Section CRL Ward	Bacteriology Sec.	Chemistry Section.	Animal Resources Sec.	Consultant	TOTAL
JULY 1, 1966	Local	2	69	29	203	27	45	34	10	-	3	422
	Foreign	2	-	2	-	3	1	-	-	-	-	8
JUNE 30, 1967	Local	2	92	37	286	33	45	32	23	*9	3	562
	Foreign	4	-	4	-	3	1	1	1	-	-	14

* The Animal Resources Section was organized on 23 May, 1967, with staff transferred from Bacteriology Section.

ANIMAL RESOURCES SECTION REPORT FOR FY67

Originally the animal house was part of the Bacteriology Section. It became a separate Section in May 1967.

Below is a breakdown of staff in this section as at 30 June 1967:

2 veterinarians

1 technician

1 animal caretaker

5 animal handlers

With breeding and supply, the stock of animals has increased five-fold over FY66. As of 30 June 1967 the animal house contained:

500 rabbits (approx)

250 guinea pigs

1500 mice

500 rats

20 monkeys

25 sheep

3 goats

Last year the animals were fed on pellets imported from the United States. This year we have started preparing animal food in the laboratory according to standard formula and now all animals except the rabbits are fed on this preparation. The laboratory does not have a pellet making machine for preparing rabbit food.

The floors and lower walls of the operation room, two experimental rooms and an animal laboratory have been reconcreted to improve the standard of hygiene.

Following is a list of studies on animals which were carried out by the various sections in the Laboratory during FY67.

Epidemiology Section.

Routine toxin neutralizing tests in rabbits. (Dr. Mosley).

Clinical Investigation Section.

i) Experimental cholera in monkeys. (Dr. Taylor)

ii) Intestinal capillary permeability studies in rabbits. (Dr. Northrup and Dr. Kinzie)

iii) Immunization of rabbits and goats. (Dr. Northrup)

iv) Histochemical studies in rabbits. (Dr. Northrup).

v) Pilot studies of toxin assays by the frog skin potential difference. (Dr. Kinzie).

vi) Intestinal loop on rabbits. (Dr. Aziz)

vii) Skin toxin test on rabbits. (Dr. Aziz)

viii) Toxin innoculation on mice. (Dr. Aziz)

ix) Toxin oral feeding on suckling rabbits. (Dr. Aziz)

x) Monkey intestinal drip. (Dr. Aziz)

xi) Phage on monkeys. (Dr. Hirschhorn)

xii) Toxin neutralization on rabbits. (Dr. Sachar)

Bacteriology Section

i) Antisera production in sheep, rabbits and goats (Mr. Huq)

ii) Blood from chickens, sheep and goats for media and agglutination test. (Mr. Huq)

iii) Passage of rough strains of N.A.G's in mice (Mr. Huq)

iv) Vibrio innoculation on monkeys. (Mr. Iqbalul Hasan)

v) Antisera preparation on rabbits.(Mr. Hasan)

vi) Toxin neutralization on rabbits. (Mr. Hasan)

vii) E. Coli innoculation on suckling rabbits. (Mr. Huq)

viii) Toxin neutralization of E. Coli on rabbits. (Mr.Huq)

These investigators, and Dr. Cash and Dr. Nalin, wish to expand their studies on laboratory animals but the present animal house facilities are inadequate for much more expansion, particularly for operations. Lighting and air conditioning need much improvement. There are no sterilizing facilities at present or ovens for food preparation. More cages will be required if large stocks of animals are required.

The animal house is continuing to supply rabbits, guinea pigs, mice and rats to the Institute of Public Health for their research work.

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BACTERIOLOGY SECTION REPORT
for FY'67

The diagnostic unit of the Bacteriology Section received a total of 53,159 specimens during the year ending 30 September 1967. As in other years, all these specimens were in the form of rectal swabs from patients of the P-SCRL ward in Dacca and people under surveillance by the Epidemiology Section, with the exception of the 5,076 specimens received from pilgrims leaving for Saudi Arabia. This year there has been the longest null period since the laboratory's inception - from July 1967 to October 1967 there has not been a positive isolation.

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

Vibrio Cholera Inaba	-	3481
Vibrio Cholera Ogawa	-	539
Non-cholera vibrios	-	225
Salmonella Group A	-	2
Salmonella Group B	-	17
Salmonella Group C_1	-	5
Salmonella Group C_2	-	3
Salmonella Group D	-	64
Salmonella Group E	-	10
Shigella Group A	-	48
Shigella Group B	-	230
Shigella Group C	-	7
Shigella Group D	-	38

The above chart shows that as in the previous year, the number of Ogawa isolations was very low in comparision to Inaba isolations. Incidentally this year we did not have any isolations of El Tor but there was one isolation of Hikojima.

Three important changes have taken place in the diagnostic unit this year:-

1. With the establishment of the Field Laboratory at Matlab in November 1966 all the field specimens from Matlab VTS as well as Matlab Hospital are processed there under the supervision of experienced Technicians.

2. Our experience with different enrichment fluids for V. Cholerae has clearly shown that subculturing the enrichment fluid after 6-8 hours incubation yields more positive isolation than subculturing after 24 hours. As such we have included in our routine work subculturing both after 6-8 hours and 24 hours.

3. Till last year we were doing routinely isolation of vibrios, salmonellas, shigellas and pathogenic Escherichia coli from patients admitted to the P-SCRL hospital. With the acquisition of more sophisticated medias as well as trained personnel we have extended our routine studies to the isolation of all other pathogenic and non-pathogenic organisms present in the gut of the patients in different stages of their hospitalization. This includes mainly clostridium, staphylococcus, paracolon.

REPORT OF THE RESEARCH UNITS

As in previous years we compared also this year the effectiveness of different culture media used for the isolation of V. Cholerae. All medias

gave the same type of result, with the exception of those where there were difficulties in preparation.

All the vibrios isolated during the period have been characterized according to Feeley's classification. No El Tor strains have been found but isolation of some chicken-cell positive cultures were confirmed.

Our laboratory being the only one of its kind in Pakistan, we receive requests from different Laboratories for supply of diagnostic antisera for the isolation of V. Cholerae. Moreover we use in our routine work every year a sizable amount of antisera. With a view to obtaining a good high titereddiagnostic antisera we tried to immunize firstly rabbits and secondly sheep and goats according to procedures outlined by Professor Goodner. Using Jefferson's strain we were able to obtain serum of the following titer from rabbits and sheep and goats. Incidentally sheep and goats responded in the same way.

Sheep and goats - Final titer after absorption - Inaba - 1/40
Ogawa - 1/120

Rabbits - Final titer after absorption - Inaba - 1/80
Ogawa - 1/320

All these sera have been freeze dried or kept in $-40^{o}C$ freezer with the addition of 0.1% sodium azide.

In vitro studies done in our laboratory have shown that sulphadrugs attack vibrio El Tor at a very low concentration (MIC 0.3 mg/ml) in contrast to true cholera (MIC - 10 mg/ml). Thinking that this may serve as a useful tool in differentiating true cholera and El Tor we followed the procedure mentioned by Han in his Polymixin B test of differentiating true cholera

and El Tor. Tests done with about 150 cultures (50 El Tor & 100 true cholera) have shown that in 90–95% of the cases clear differentiation can be made within 6–8 hours incubation, though over-night incubation gives overlapping results. Considering the shorter incubation time, this test, after some modification, may be routinely used in differentiating true cholera and El Tor.

Search for a drug resistant mutant of V. Cholerae continued. Using low temperature and longer incubation in presence of antibiotic in multiple steps we have been able to get resistant strains of tetracycline up to 20 mcg/ml, chloromycetin up to 10 mcg/ml and streptomycin up to 100 mcg/ml.

Study on the enteropathogenic E. coli in children suffering from gastro-enteritis continued for the second year. Seasonal patterns of the prevalence of pathogenic E. coli have been observed. Changes of the prevalent serotype (from 0127 to 0128) have also been noted. That these entero-pathogenic E. coli are capable of producing diarrhea in infant rabbits has also been confirmed.

Results of a survey on about 500 patients in the ward have shown that 15–20% of the cases contain staphylococcus and 45–50% cases contain clostridium in their stool. Due to non-availability of the typing serum and antitoxin the staphs and clostridia have not been typed.

In order to find out the in vitro effect of culture filtrate of lactobacilli on the growth of V. Cholerae we have started growing lactobacilli and testing these against different strains of V. Cholerae using techniques already used with E. coli strains. The experiment being in the preliminary stage, no comments can be made now.

GENERAL COMMENTS

The main function of the Bacteriology Section at present is routine diagnostic work and supporting the research work undertaken by other sections. This section has suffered heavily during the past year due to non-availability of good autoclaves and media preparation facilities. Following the recommendation of the last Technical Committee, several vital instruments such as autoclaves and microscope have arrived and are awaiting installation. Major renovation work proposed for the section has not yet been done.

The total strength of personnel in the section is just up to requirements. The return of Mr. C. R. Dey, after completion of Dip. Bact., from London, will help us to initiate more detailed diagnostic techniques as well as research work.

CHEMISTRY SECTION REPORT FOR FY67

The Chemistry Section has entered into its second year of existence. During the one year period it went through phased reorganization such as setting up techniques under different research protocols, standardizing procedures, recruiting of technicians, training them for the standard procedures under proposed research protocols and teaching them the importance of quality control and precision in analytical procedures.

This organization and orientation has taken place under the active guidance of Dr. Ruth S. Hare and advice of Dr. Kendrick Hare.

The functions of the Section are multiple in nature but centre around one or two major aspects such as:

a) Providing full laboratory support for all the chemistry involved in clinical investigation protocols.

b) Preparing and supplying the infusion solutions and I. V. fluids required for treatment i.e. the rehydration part of the therapy.

Increase in Staff:

A few Science graduates and undergraduates have been hired to assist with the increasing amount of work under the research protocols. The total strength of staff in this section has gone to 22 against 13. The classification of the staff is

as follows:

a) Senior Research Assistants - 2

b) Research Assistants - 6

c) Research Technicians - 1

d) Laboratory Technicians - 7

e) Glass Washers - 4

f) Laboratory Attendants - 2

and they have been grouped under the following field of activities of this section

a) Routine Chemistry.

b) Research Chemistry.

c) I.V. Fluids.

d) Glass Washing.

Activities:

As reported to the last Technical Committee meeting, there has been a large increase in the activities of the laboratory. Several versatile instruments such as the PE 303 Atomic Absorption Spectrophotometer, PE 202 U.V. recording spectrophotometer, and Corning pH meter have been commissioned and are now in use for research chemistry. The functioning of these instruments has not been ideal as there still are a few arrangements to be made for their proper installation. However, these instrument have not only widened the scope of existing activities but have provided an opportunity for new studies.

Statistics of the activities:

The activities in different spheres are shown in charts and tables.

Chart I shows the methods of analyses and references which we are following in our laboratory.

Appendix 'A' shows some of the procedures for Routine and Research Chemistry as established by Dr. Ruth S. Hare.

Table 1 & 2 show the account of samples done under Routine Chemistry.

Tables 3 to 10 show the research protocols and the chemistry done under those protocols.

Table 11 shows the account of I.V. Fluid.

Proposed expansion of the activities:

Since the research activities are increasing and emphasis is now being given to toxin, antigens and antibodies; it is quite likely that protein chemistry will be investigated, and we can expect this section to be involved. So it is obvious that activities in the field of electrophoresis, chromatography etc. will also be introduced in this section. Instruments like Amino Acid analyzers, Microzone electrophoresis and Immuno-electrophoresis set ups are to be ordered and installed.

Forensic Chemistry:

In the past we have had frequent requests for detection and estimation of poisons in biological fluids and tissues of the patients who come to our Ward in such a condition that poisoning is suspected. The only test we are performing at

present is the arsenic test. In the absence of a well-equipped forensic unit we are handicapped in identifing and estimating the poisonous substances which cause the death of such patients. So it is proposed that arrangements should be made to cover this aspect of analytical chemistry.

We are confident in saying that during this period we have reached a status where other similar laboratories in the country look towards us for guidance in setting up techniques and standard procedures and treat this laboratory as a reference laboratory.

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T A B L E 1

SAMPLE FOR ROUTINE CHEMISTRY

Description of Sample.	Type of Sample.						
	Blood	Stool	Urine	Vomit	Gastric Juices	C.S.F.	Total
Samples from Outside Hospitals (Courtesy analysis)	46	-	-	-	-	-	46
C.R.I. Employees	45	-	-	-	-	-	45
Routine Samples from CRL Ward	130	16	19	2	7	7	181
Samples taken at Admission to CRL Ward	146	-	-	-	-	-	146
Urgent & Emergency sample from CRL Ward	1260	46	16	-	-	3	1325
Total:	1627	62	35	2	7	10	1743

TABLE 2

ROUTINE CHEMISTRY DETERMINATIONS

	Courtesy	Employee	Routine	Admission	Urgent	Total
CO_2	25	4	105	138	1245	1517
Na	35	14	120	132	1132	1433
K	38	14	120	136	1267	1575
Cl	26	13	114	125	1199	1477
Creatinine	15	4	68	47	491	625
Sugar	5	25	40	27	394	491
Urea	3	1	0	0	2	6
Ca^{++}	2	2	7	0	15	26
Mg^{++}	0	0	6	0	15	21
W.B.Sp.Gr.	1	1	11	0	22	35
Sp.Sp.Gr.	3	0	33	9	152	197
Osmolarity	1	0	4	0	12	17
Arsenic	0	0	18	0	0	18
Gastric Juice Acidity	0	0	7	0	0	7
Total Protein	0	0	1	0	2	3

TABLE 3

RESEARCH CHEMISTRY

Protocol : Oral Glucose and Electrolyte therapy in Cholera.

Investigators : Drs. Hirschhorn, Taylor and Sachar.

No. of Patient studied : 8

Determinations	Total Number of Samples Analysed
Sodium	297
Potassium	297
Chloride	297
Carbon dioxide	279
Sugar	236
PEG	20
BSP	17
Osmolarity	43
Creatinine	2
Plasma Sp. Gravity	82
Blood Sp. Gravity	70

T A B L E 4

RESEARCH CHEMISTRY

Protocol : Oral Tetracycline, Glucose and Electrolyte lavage in Acute Cholera.

Investigators : Drs. Northrup, Hirschhorn & Kinzie.

No. of Patients studied: : 10

Determinations	Total No. of samples Analysed
Sodium	789
Potassium	789
Chloride	278
Carbon dioxide	96
Sugar	620
Osmolarity	113
BSP	436
Plasma Sp. Gravity	90
Blood Sp. gravity	88

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TABLE 5

RESEARCH CHEMISTRY

Protocol: Intestinal Electrolyte and Water fluxes and Transmucosal Potential in Acute Cholera.

Investigators: Drs. Taylor and Sachar.

No. of Patients studied: 22

Determinations	No. of samples analysed.
Sodium	658
Potassium	658
Chloride	658
Carbon dioxide	224
Sugar	434
BSP	911
PEG	365
Osmolarity	149
Creatinine	4
Plasma Sp. Gravity	13
Blood Sp. Gravity	13

TABLE 6

RESEARCH CHEMISTRY

Protocol : Bolus Experiment

Investigators : Dr. James O. Taylor

No. of patients studied: 4

Determinations	No. of samples Analysed.
Sodium	655
Potassium	109
PEG	122
BSP	802
Sugar	12

TABLE 7

RESEARCH CHEMISTRY

Protocol: Paediatric Cholera Study.

Investigators: Drs. John Linneman and M. Rahman

No. of Patients studied: 2

Determinations	Total No. of samples analysed.
Sodium	50
Potassium	50
Chloride	50
Carbondioxide	26
Osmolarity	36
Creatinine	26
Sugar	10
Plasma Sp. gravity	10
pH	12

T A B L E 8

RESEARCH CHEMISTRY

Protocol: Comparison of lactate and Acetate I.V. Fluids in the Cholera patients.

Investigators: All CRL Ward Physicians.

Patients studied: 14

Determinations	Total No. of sample analysed.
Sodium	105
Potassium	105
Chloride	105
Carbondioxide	65
Sugar	36
Creatinine	13
Plasma Sp. gravity	70
Blood Sp. gr.	70

T A B L E 9

RESEARCH CHEMISTRY

Protocol: Oral fluid Study.

Investigators: Drs. Haque & Kinzie

No. of patients studied: 2

Determinations	No. of samples analysed.
Sodium	12
Potassium	12
Chloride	12
Carbondioxide	9
Plasma Sp. Gr.	4
Blood Sp. Gr.	5

T A B L E 10

Protocol: Blood Volume Study

Investigators: Drs. Rahman & Kinzie

No. of Patients studied: 1

Determinations	No. of sample analysed.
Sodium	4
Potassium	4
Chloride	4
Carbondioxide	4

TABLE 11

ACCOUNT OF I.V. FLUID (5:4:1)

Month	Production	Consumption in Hospital	Despatch to Matlab	Total Consumption.
December 1966	9,331	6,937	1,224	8,161
January 1967	5,241	3,824	1,469	5,293
February "	1,259	652	0	652
March "	1,481	1,142	588	1,730
April "	2,307	1,700	408	2,108
May "	1,449	1,257	204	1,461
June "	1,052	542	408	950
Total:	22,120	16,054	4,301	20,355

C H A R T 1

CHEMISTRY SECTION

ANALYTICAL PROCEDURES.

Creatinine	:	1. Folin-Wu; J. Biochem. 38, 81(1919) Beckman Model 'B' @ 485 mμ
		2. Ruth S. Hare's specific method. Proc. Society for Experimental Biology & Med. 74, 148 (1950)
Urea	:	Momose, T et al. Determination of urea in Blood and Urine with Diacetyl Monoxime - gluceronolactone Reagent. Clinical Chemistry 11: 113 Beckman Model B-475 mμ
Surgar	:	A photometric Adaptation of the Somogyi Method for the Determination of glucose by Norton Nelson. a) Beckman Model B 670 mμ b) Coleman Junior Spectrophotometer 650 mμ
Specific gravity of whole blood and plasma:		Standard Copper sulfate solution method. Robert A. Phillips, Donald D. VanSlyke, and others. (US Navy Research Unit at the Hospital of the Rockfeller Institute for Medical Research, New York, July 18, 1949.
Sodium and Potasium	:	Baird Atomic flame photometer Model KY-2 (Lithium as internal standard)
Chloride	:	a) Aminco-Cotlov chloride titrator. b) Schales method - by Titration (micro method)
Total CO_2 content	:	Van Slyke Manometric apparatus.
Bromo sulphalein (B.S.P.)	:	Unicam S.P. 600 580 mμ Beckman Model 'B' 585 mμ
Polythylene glycol (P.E.G.)	:	Turbidimetric method. Beckman Model 'B' 415 mμ
Ca and Mg	:	Atomic Absorption Spectrophotometer Perkin Elmer - Model 303.
Na+ K	:	" - " "

Osmolarity	:	Mechrolab Vapur Pressure Osmometer Model 301A.
Total Protein	:	Phenol Reagent, Method. Ref. Daughaday, Lowry & Rosenberg. J. Lab. Clin. Med. 39, 663 (1952)
Absorption Peak Determination	:	P.E. Model 202 Ultra Violet Recording Spectrophotometer.
pH	:	'Corning' Model 12B Research pH Meter.
Arsenic	:	a) Reinch Test b) Gutzeit Method.
Total Acidity in Gastric Juice	:	Ref: Clinical Chemistry, Principle & Techniques Richard J. Henry, Page 909, Chapter 26, (Method of Helmer & Fout).
Total Cholestrol	:	Ref: Zak, B. Am. J. Clin. Path, 27, 583,1957 Bechman Model-B. 620 mμ
Amino Acids	:	Ref: Frame, E.G; Russell, J.A. & Wilhelmi, A.E. J. Biol. Chem. 149, 225, (1943). Beckman Model-B. 475 mμ

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APPENDIX 'A'

SPECIAL CHEMISTRY

Special chemistry will be done only during regular working hours. In the case of blood or stool pH, arrangement may be done in advance in the Laboratory to have it done out of regular hours.

The special chemistries now being done are: pH, osmolarity, Ca, Mg and urea. If any other special chemistry is desired, please communicate with the Clinical Laboratory before doing tests or collecting samples.

Samples for special chemistry (except pH), collected out of hours, may be stored and analysed on the next working day. They should be kept in tightly stoppered tubes; osmolarity samples must be frozen. In the case of plasma or serum Mg, the sample must be centrifuged and the supernatant separated before storate.

1. pH is determined in arterial whole blood, in urine, in rice water stools, or the supernatant of semi-solid stools after centrifugation. It cannot be determined on solid stools, on blood samples which have been exposed to air, or on any specimen collected more than 10 minutes before analysis.

Arterial blood is drawn into a heparinized plastic syringe without exposure to air. One ml of blood is expelled into a heparinized vial (for hematocrit and specific gravity) the syringe is capped and sent immediately to the first floor Laboratory (Dr. Taylor's office). 1.5 ml of blood is sufficient for CO_2 and pH. If other determinations are required, the necessary volume should be drawn but left in the syringe. After the pH is done the remainder can be delivered under oil or into fluoridated vials in the Laboratory.

2. Osmolarity is determined on fresh or frozen samples of plasma, serum, urine, rice water stool or supernatant of semi-solid stool. It can not be determined on solid stool or on any unfrozen specimen stored for more than a few hours. Disposable syringes and tube supplied by the Laboratory should be used. The volume required is 1 ml of whole blood.

(more)

3. Ca and Mg are determined on plasma, serum, urine, rice-water stool or supernatant of semi-solid stool. Neither can be determined on solid stool and Mg cannot be determined on blood samples which have been stored without separation. Scrupulous care must be taken to avoid contamination of specimens. All vessels or tubes used for collecting urine or stool should be washed with distilled water and dried before use. Blood samples should be collected in disposable syringes. Samples should be delivered into tubes provided by the Laboratory and if not under oil should be covered with parafilm or stoppered with a plastic stopper immediately of the collection. 1.5 ml of whole blood are required for both analyses.

4. Urea is determined in plasma, serum or urine. No special collection technique is necessary and the volume required is 1 ml of whole blood.

5. Stool samples, if rice water or semi-solid in character, may be analysed for Na, K, CO_2 and Cl. Requirements are similar to these described for routine blood chemistry, but the volume of semi-solid stool needed is many times that of whole blood.

6. Urine samples may be analysed for Na, K, CO_2, Cl, creatinine and glucose. It is probably pointless to put avoided sample under oil; otherwise the requirements are the same as these for blood.

7. All samples. The volume of whole blood required for all routine and special chemistry is about 10 ml, the volume of urine or rice water stool is about half of that; the volume of semi-solid stool required depends upon the solid content; 5 ml of supernatant after centrifugation is needed. If there is any possibility that the volume is insufficient, indicate the order of importance of the analyses requested.

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BLOOD SAMPLES FOR ROUTINE CLINICAL CHEMISTRY.

1. CO_2 and Cl. Samples must be collected without exposure to air and delivered under oil intotubes supplied by the Laboratory.

2. Na & K. Samples should be collected in disposable plastic syringes and delivered into tubes supplied by the Laboratory. These analyses may be done on samples under oil.

The volume required for the above 4 analyses is 1.5 ml. of whole blood. CO_2 will not be determined unless the sample is under oil. If the sample is hemolysed, only low K values will be reported. If the value for Na is very high, a second blood sample will be requested so that possible contamination may be ruled out. 0.5 ml of whole blood is enough to check Na and K.

3. Creatinine. No special technique of collection is required. The volume required is 1.5 ml of whole blood.

4. Specific gravity. Sample must be in heparinized vial. Volume required is 1.5 ml of whole blood.

5. Glucose. Sample must be in fluoridated vials. Volume required is 0.5 ml of of whole blood.

A total of 5 ml is required for all the above analyses. If that volume is collected and properly distributed it should be possible to make all determinations. However, if there is any doubt about the quantity, please indicate whether the electrolytes or the creatinine is more important.

The blood samples should be sent to the Clinical Chemistry Laboratory promptly after collection. The technician on duty should be called immediately if it is outside of working hours. Prolonged standing will change the CO_2 and K concentrations.

Part I

Present Clinical Research Staff

The Clinical Research Section is now comprised of a staff of 88 persons which includes five NIH Physician Research Associates: Dr. James O. Taylor, Dr. Joseph L. Kinzie, Dr. Robert S. Northrup, Dr. David R. Nalin and Dr. Richard A. Cash and two Guest Investigators:- Dr. M. Mujibur Rahman, M.B.B.S., M.S., Ph.D. who is a physician with doctoral training in nutrition and body composition, and Dr. K. M. S. Aziz, Ph.D. who is a marine biologist from Duke University now specializing in cholera toxin work.

Dr. Md. Rafiqul Islam, the Chief Resident on the CRL Ward, has just returned from a year in the U.K. where he has been having special training in tropical medicine and pediatrics. There are now seven full time ward physicians: Dr. Z. Hoque, Dr. Toha, Dr. Saha, Dr. Golam Rahman, Dr. Waliur Rahman, Dr. L. Khondaker and Dr. Majid Molla. All will be actively involved in research projects in the coming season. Two Senior Residents in Medicine from the University of Maryland will be assisting with the running of the CRL Ward, the first, Dr. Richard Wenzel, from October 1967 thru December 1967; the second, Dr. Music from January 1968 thru March 1968. Their assistance will allow each of the research associates more time for his research projects by lightening the clinical load. It will also improve the quality of the clinical record-keeping and improve the teaching program for the ward physicians.

Dr. Norbert Hirschhorn and Dr. David Sachar returned to the U.S. in May 1967 on completion of their assignments and were replaced in July by Dr. Cash and Dr. Nalin.

Dr. Andrew Abraham, Chief Resident in Pathology at the Peter Bent Brigham Hospital in Boston, will again be on temporary assignment to the Laboratory during the cholera season to assist with the autopsy pathology and to continue electro-micrographic studies in cholera. He will also do a study of viruses in the stool of cholera and other diarrheas.

Part II

Studies Completed in the 1966-67 Cholera Season

The 1966-67 cholera season was the biggest in the history of the Cholera Laboratory. Between September 1, 1966 and January 30, 1967 1,591 proven cases of cholera were admitted to the CRL ward in Dacca. Despite this heavy clinical load, research projects continued uninterrupted. A list of the clinical research projects completed during the season is appended.

Dr. Sachar perfected a technique for measuring transmural electrical potentials across the human intestine. Using this technique he showed that intralumenal glucose and other actively transported sugars cause a dramatic increase in transmural potential difference in the normal intestine. He then went on to show that there is no difference in baseline potential difference or in electrical response to glucose between patients with acute cholera and normals.

Drs. Sachar and Taylor, using multilumen, transintestinal tubes and non-absorbable markers, studied the net movement of sodium and water across 20 cm. segments of ileum and jejunum in acute cholera patients and again after recovery. They showed that there is a net loss of sodium and water

into both the jejunum and ileum in acute cholera, as compared to a net absorption in both areas in convalescence. Intralumenal glucose decreased the net loss of sodium and water in acute cholera and actually caused net absorption in some cases.

Dr. Hirschhorn, with other members of the staff, showed that oral lavage of glucose solutions decreased the net stooling rate in acute cholera. Galactose, another actively transported sugar, caused the same effect, while fructose, a passively transported sugar, did not. He also showed that oral glucose and tetracycline in a lavage could bring an active cholera patient into "positive balance", i.e. a state of no net stool loss but actual absorption of salt and water from the gut, in a matter of hours.

Drs. Kinzie and Northrup, using oral lavage of tetracycline alone, showed a rapid sterilization of the intestine in 2-3 hours and stopping of diarrhea in 35 hours (mean) with a mean total stool output of 6 liters - a significant reduction in both parameters when compared to routine antibiotic therapy in acute cholera.

Immunofluorescent studies of jejunal biopsies by Dr. Northrup and Dr. Crabbé (University of Louvain) demonstrated that the relative proportions of immunoglobulin-producing plasma cells in Bengali cholera patients are the same as normal Belgian and Belgian Congo patients. Also, absolute numbers of plasma cells, taken in fields of maximal density, are approximately the same.

Drs Karchmer and Curlin, CDC Fellows on short term assignment from the EIS, Atlanta, showed that furazolidone and tetracycline in one fifth

the standard oral dose were both as effective as standard high dose tetracycline in reducing the duration and volume of stooling and I.V. requirements in children with acute cholera.

Dr. Andrew Abraham demonstrated that the electro-microscopic appearance of jejunal biopsies in acute cholera shows no characteristic abnormalities.

Dr. Hirschhorn, in a review of all cases of pregnant women with cholera admitted to the CRL ward, showed that (i) the maternal mortality is zero in pregnant cholera patients, (ii) cholera is more severe in the third trimester of pregnancy and (iii) for any season, the incidency of pregnancy is higher in cholera patients than gastroenteritis patients, suggesting that pregnancy may predispose one to clinical cholera.

Dr. A. Majid Molla was unable to demonstrate any cholera vibrio carriers by magnesium sulfate purgation 2 to 6 weeks after the onset of illness. This is in marked contrast to Dr. Gangarosa's finding in the Middle East where 21% of patients were shown to be carriers by this method. These findings may represent a difference between classical cholera (Dr. Majid's study) and El Tor cholera (Dr. Gangarosa's study), a point which would help explain epidemiologic differences between the two strains.

Drs. Sachar and Hoque studied the effects of administering hyperimmune plasma collected from convalescent cholera patients, to acute cholera patients both intravenously and intraintestinally. No significant beneficial result could be detected. The passive rise of antibody titer produced by intravenous administration was small.

Dr. K. M. S. Aziz has developed a method of cholera toxin production which is now yielding high potency toxin. He has also undertaken studies to characterize the properties of cholera toxin.

Part III

Clinical Research Section Studies in Progress During the 1967-68 Cholera Season

Many of the studies planned for the 1967-68 season are extensions of studies done during the 1966-67 season.

Drs. Taylor, Kinzie and R. Hare are continuing the intubation studies of fluxes of sodium and water in cholera with the addition of studies of unidirectional fluxes of these ions. Using a technique where a bolus of a non-absorbable marker plus isotopically-labelled water and sodium (tritium, sodium 22 and/or sodium 24) into the intestinal segments which are delineated by the collecting ports on the intestinal tubes. The rate of disappearance of the isotopes is measured by serial sampling and determining the ratio of isotope to non-absorbable marker as it changes with time. The mean transit time across the segment also allows the calculation of the volume of the segment. Thus it is possible to calculate net movement of sodium and water and unidirectional lumen to plasma flux. The unidirectional plasma to lumen flux is calculated by taking the difference between the net and the lumen to plasma flux. These studies are again being done with and without intralumenal glucose. The results should give clues as to the pathophysiologic mechanisms responsible for cholera and also to the physiologic mechanism involved in the stimulation by glucose of sodium and water absorption.

The advantages of the bolus method, briefly described above, all follow from the extremely small doses of isotope which are needed for each bolus:

many studies can be done without significant radiation exposure to the patient. Since the boluses are small and intermittent, the concentration of isotope in the plasma remains negligible and it is therefore unnecessary to calculate for re-entry of the isotope into the study segment. Also, isotopes such as C-14 labelled urea can be used in boluses since the dose necessary for the bolus is so small. None of these advantages are found with the steady state method.

Dr. Kinzie has developed a method for studying changes in membrane permeability by measuring the relative rates of movement out of the gut of tritiated water and C-14 labelled urea, erythratol and/or mannitol using the bolus technique. These studies should help decide the question of whether membrane permeability is increased in cholera.

Dr. Rafiqul Islam, Chief Resident, will direct a study of oral lavage of glucose, electrolytes and tetracycline as a means of treating cholera, comparing total net stool volumes and total intravenous requirements in patients receiving oral lavage with these parameters in patients receiving only standard intravenous therapy with tetracycline in capsule form. These studies will be done on unselected cholera cases of all degrees of severity and will hopefully show what maximum benefit can be derived from oral therapy at its present stage of development.

Dr. M. Mujibur Rahaman, Guest Investigator, with Dr. Waliur Rahman and Dr. Kendrick Hare will be doing balance studies in pediatric patients with cholera and other diarrheas and will thus hopefully provide more of the basic data necessary to plan a more rational therapy of pediatric cholera and diarrhea in general. Dr. Rahaman also plans to do studies of body composition in children in East Pakistan using a variety of sophisticated techniques.

He plans, too, an evaluation of nutritional status in hospitalized and normal children. Dr. Rahaman's M.S. and Ph.D. work were in the areas of nutrition and body composition, so he is well qualified to carry out these extremely important studies which will gather basic data in these critical areas.

Dr. Robert Northrup has planned an extensive program for the study of a variety of immunologic aspects of cholera. He has just returned from several months of learning immunologic techniques from Dr. Paul Crabbé at Louvain University, Belgium and from Dr. T. Tomasi in Buffalo, New York. He plans extensive studies characterizing the proteins in cholera stool, especially with respect to the immunologic characteristics of stool immunoglobulin fractions, using a wide variety of techniques including column chromatography, immunoelectrophoresis, radioimmunoelectrophoresis and ultracentrifugation. In line with these studies, Dr. Northrup has just demonstrated that cholera stool IgA is secretory in type (i.e. it contains "secretory piece" of Tomasi).

Dr. Northrup is also planning further immunofluorescent studies of small bowel biopsies demonstrating the presence of antibody-producing plasma cells. He plans to look at sites of various antivibrio and antitoxin antibody production by this technique.

Drs. Northrup and Kinzie are planning related autoradiographic studies with labelled vibrio cholera antigens and C-14 labelled Craig toxin.

These studies are complementary to the immunologic studies being done under the direction of Dr. Henry Mosley in the Epidemiology Section

(see Epidemiology Section Report) and in combination these studies should add greatly to our understanding of the immunology of cholera.

Dr. Andrew Abraham of the Peter Bent Brigham Hospital, will be continuing his electro-micrographic studies of small bowel and rectum in cholera patients and normals.

Drs. Nalin and Cash, who joined the CRL staff in July 1967, are in the process of setting up a variety of studies of experimental cholera in monkeys, dogs and sheep. To test the hypothesis that the pancreas may play a role in cholera, either by exocrine outpouring of fluid or by an endocrine mechanism as in the massive diarrhea of certain pancreatic adenomas, they plan to do complete pancreatectomy in the dog and test the ability of cholera toxin to cause diarrhea. To study vascular and blood flow effects on the fluid outpouring in cholera, they plan to do studies with angiotensin (renin) and topical epinephrine. They also plan to study the response of denuded intestinal mucosal surface to cholera toxin to differentiate mucosal and vascular mechanisms. Studies of acetazolamide on the volume and composition of fluid outpouring are planned to confirm and extend the observations of Norris that Diamox reduces the rate of fluid output in experimental cholera. Studies with various adsorbing and chelating agents are planned, to see if these can block the action of cholera toxin. Studies will be done with products of vibrio metabolism, such as nitrites, indoles and ammonia to see if these alone or in combination can cause diarrhea in animal loops.

Drs. Nalin and Cash are also planning studies on human cholera to be done during the cholera season. These include studies of intraintestinal lavage of acetazolamide (Diamox) which has been reported to decrease the rate of

stooling in experimental cholera in animals. They plan to study the effects of Diamox on unidirectional and net fluxes of sodium and water in segments, on the bicarbonate composition and pH of the intestinal contents, and on the rate of stooling with whole gut lavage. They also plan to study the effects of changing the pH of intestinal contents by infusions of bicarbonate (to try to reproduce Diamox effect) and of organic acids (to see if vibrios can be killed, and thus stooling rate reduced, by lowering pH).

The problems of sterilization and storage of bicarbonate solutions have made another source of base desirable in intravenous replacement fluids for cholera. Lactate is expensive, difficult to produce and supports bacterial growth. Acetate is cheap, can be autoclaved and has a long shelf life and therefore would be an ideal source of base in cholera replacement fluids. Dr. Abdul Majid Molla will conduct a study comparing standard bicarbonate replacement solution with a commercially-available acetate solution in the treatment of acute cholera. This study will find out if acetate is appropriate for the rapid correction of severe acidosis in acute cholera.

Dr. Wenzel,,a Senior Resident in Medicine from the University of Maryland, on short term assignment ot the CRL, will do studies on intraperitoneal infusions as a route for rehydration in acute cholera. He also may study the effects of intralumenal calcium and magnesium on sodium and water absorption.

Dr. K. A. Monsur, Director of the Institute of Public Health, in conjunction with Drs. Hoque and Taylor of the P-SCRL,will continue the studies on oral lavage of vibriophage in acute cholera patients, to see if this can result in a killing of all vibrios in the gut and thus shorten the length of

acute cholera. If this is successful, it would be used as a research tool, allowing a killing of cholera vibrios without otherwise changing flora as would occur with antibiotics.

Dr. Hirschhorn is continuing his analysis of bile salts and bile pigments in rice water stools at the Thorndike Memorial Laboratory in Boston. More specimens are being sent to him this year.

Dr. K. M. S. Aziz, Chief of the New Animal Resources Section, is now producing high potency cholera toxin of the Feeley type. His future studies in the characterization of cholera toxin will be outlined by him on his return from the States where he is presently visiting the various laboratories working with cholera toxin.

Part IV
Equipment Required for Future Research

The relationship of severe acidosis and dehydration to renal blood flow and glomerular filtration rate appears to be an extremely important area for future investigation. This is an area where study of patients with acute cholera can contribute much to an understanding of renal physiology, just as Dr. Rejane Harvey's cardiac catheterization studies in acute cholera have contributed a great deal to the understanding of the relationship of acidosis to cardiovascular function.

Drs. Phillips and Hare have proposed studies of renal function in cholera using renal vein catheterization to measure renal clearances in shock and acidosis and in uncomplicated acidosis alone. These studies have been discussed with Dr. Poul Astrup of Copenhagen and he has agreed to collaborate

by sending an investigator from his laboratory familiar with renal vein catheterization studies.

If such a study were to be undertaken here, fluoroscopy equipment, including an image intensifier would be necessary to monitor the placing of renal vein catheters. Such equipment should be budgeted for well in advance of undertaking the project and time allowed for the delays in procurement, shipping and installation. The services of a trained radiologist would be desirable, at least in the early phase of operating this equipment.

Summary

The Clinical Research Section is actively involved in a large number of projects covering a wide range of interests from pathophysiology and therapy of cholera to the immunology of cholera. The unique combination of a large supply of clinical cases of cholera, the excellent laboratory support provided by the Chemistry Section and Bacteriology Section, and the continuing support and guidance of the Director and the Deputy Director and his wife, provide an opportunity for the fruitful study of clinical cholera which cannot be paralleled anywhere else in the world.

Clinical Research Section Studies Completed in the 1966–67 Cholera Season

1. Transmural electrical potentials and their responses to sugars in the intestine of normal subjects and acute cholera patients. Dr. Sachar, Mr. J. R. Saha, Dr. K. Hare.

2. Intubation studies of net movement of sodium and water across jejunum and ileum of patients during acute cholera and after recovery and effects of glucose on these fluxes. Drs. Taylor, Sachar, Mr. J. R. Saha, Drs. K. Hare and R. Hare.

3. Oral glucose solutions for reducing stool output in acute cholera. Drs. Hirschhorn, Kinzie, Sachar, Northrup, Taylor and Phillips.

4. Oral tetracycline lavage in acute cholera. Drs. Northrup, Kinzie and Phillips.

5. Immunofluorescent studies of immunoglobulin producing plasma cells in jejunal biopsies in cholera patients. Drs. Northrup and Crabbe.

6. Furazolidone compared to normal and low dose oral tetracycline therapy in acute cholera. Drs. Karchmer, Curlin and Hirschhorn.

7. Electronmicroscopic studies of jejunal biopsies in cholera and other diseases. Drs. Abraham and Northrup.

8. Clinical review of the effects of cholera on pregnancy. Dr. Hirschhorn, Mr. Alauddin Chowdhury.

9. Search for carriers among convalescent cholera patients by means of cathartic purgation. Dr. Abdul Majid Molla and Dr. Hirschhorn.

10. A technique for measurement of transmural electric potential in intact human intestine. Dr. Sachar and Mr. Saha.

11. The effect of hyperimmune plasma on the clinical course of acute cholera. Drs. Sachar, Hoque.

12. Jejunal biopsy disaccharidasis and lactose malabsorption in cholera and other diarrheal diseases. Dr. Hirschhorn.

13. Some studies on cholera patients in relation to cholera toxin. Dr. K. M. S. Aziz and Mr. A. K. M. Mohsin.

Studies in Progress During the 1967-68 Cholera Season

1. Unidirectional fluxes of sodium and water across the intestinal wall in acute cholera and normals. Drs. Taylor, Kinzie, R. Hare, Phillips.

2. A new method for measuring unidirectional fluxes of isotopically labelled compounds across the intestinal wall, using a bolus injection of isotope and a non-absorbable marker. Drs. R. Hare, Kinzie, Taylor, Phillips.

3. Studies of intestinal permeability characteristics in acute cholera and normals. Drs. Kinzie, Taylor, Hare and Phillips.

4. Oral lavage of a solution containing glucose, electrolytes and tetracycline in the treatment of acute cholera. Dr. Md. Rafiqul Islam with Drs. Majid, Hoque, G. Rahman, W. Rahman, Toaha, Saha, Khondaker and Wenzel.

5. Balance studies in pediatric cholera and other diarrheas in children. Drs. M. Mujibur Rahaman, Waliur Rahman and K. Hare.

6. Studies of body composition in children in East Pakistan. Drs. M. Mujibur Rahaman and K. Hare.

7. Evaluation of nutritional status in hospitalized and healthy children in East Pakistan. Drs. M. Mujibur Rahaman, Golam Rahman and K. Hare.

8. A study of the proteins of cholera stool with special reference to their immunologic properties. Dr. Northrup.

9. The demonstration of Tomasi "Secretory Piece" in the immunoglobulin-A of cholera stool. Drs. Northrup and T. Tomasi.

10. Comparison of techniques for collecting secretions from the normal human intestine. Dr. Northrup.

11. Radioimmunoelectrophoretic analysis of human serum and stool for V. Cholerae binding antibody. Dr. Northrup.

12. Immunofluorescent studies of small bowel biopsies in acute cholera. Dr. Northrup.

13. Autoradiographic studies with labelled V. Cholerae antigens. Dr. Northrup and Dr. Kinzie.

14. Autoradiographic studies with C_{14} labelled Craig toxin. Drs. Kinzie and Northrup.

15. Electron-microscopic studies of small bowel and rectal biopsies in acute cholera and normals. Drs. A. Abraham and Northrup.

16. Comparison of virus isolations in cholera, other bacterial diarrheas and normals. Dr. A. Abraham.

17. Studies in experimental cholera in animals:
    (a) The effect of intraintestinal cholera toxin in pancreatectomized dogs.
    (b) The effect of angiotensin (renin) on the response of dog ileum and jejunum to Feeley-type cholera toxin.
    (c) The effect of topical epinephrine on the response of ileum and jejunum of animals to cholera toxin.
    (d) The response of denuded intestinal mucosal surface to cholera toxin.
    (e) Effects of acetazolamide on cholera toxin induced diarrhea in animal models.

(f) Adsorbing and chelating agents and cholera toxins.

(g) Studies of effects of products of vibrio metabolism: indoles, nitrites and ammonia, in animals' intestinal loops.

(h) Sheep intestinal loops as an animal model for the study of cholera. Drs. Nalin and Cash.

18. Effects of intraintestinal lavage of acetazolamide on fluxes of sodium and water, on intestinal fluid composition and on stooling rate in humans with acute cholera. Drs. Nalin and Cash.

19. Intraintestinal lavage of organic acids in acute cholera: effects on vibrio count and stooling rate. Drs. Nalin, Cash and Phillips.

20. A comparison of a commercially-available intravenous acetate solution with the standard bicarbonate solution in the correction of acidosis in acute cholera. Dr. A. Majid Molla with Drs. Islam, Hoque, Golam Rahman, Waliur Rahman, Toaha, Saha, Khondaker and Wenzel.

21. Intraperitoneal infusions of electrolyte solutions as another route for fluid replacement in acute cholera. Drs. Richard Wenzel and R. Phillips.

22. Oral lavage studies of the effects of intralumenal magnesium and calcium on sodium, potassium and water absorption in acute cholera. Drs. Wenzel and Phillips.

23. Oral lavage of vibriophage in acute cholera: effects on vibrio counts and stooling rate. Drs. K. A. Monsur, Ataur Rahman, Z. Hoque and J. O. Taylor.

24. Bile salts and bile pigments in rice water stool in cholera. Drs. N. Hirschhorn and I. Rosenberg.

25. The purification and properties of cholera toxin. Dr. K. M. S. Aziz, Dr. K. Hare and Dr. R. A. Phillips.

EPIDEMIOLOGY SECTION REPORT FOR FY67

Introduction

The 1966-67 fiscal year was characterized by marked broadening of scope and depth of the program of the Epidemiology Section. This included not only a doubling of the size of the cholera vaccine field trial to include 110,000 persons, but also detailed evaluation of the mechanism of immunity in cholera both by field studies as well as by laboratory characterization of antibodies. Broad community studies were carried out demonstrating the significance of water and boatmen in the transmission of cholera as well as indicating that the infection: case ratio may be as high as 100:1; practical evaluations of high and low dosage tetracycline schedules in clinical cholera were undertaken as well as a study of antibody prophylaxis in the field; continued evaluation of serological and bacteriological techniques in detecting carriers was carried out; patterns of behaviour which may affect disease prevalence were defined, and as a peripheral activity, demographic studies were carried out in the vaccine field trial population providing unique data from a large population group in East Pakistan.

In addition to these expanding research activities, the Epidemiology Section played a major role in conducting training courses for the WHO and for CENTO for physicians from the Middle East area.

New additions to the staff have contributed significantly to the expansion of the program during 1967. These were Miss Aet Jarv, an anthropologist from Australia, who arrived in September 1966, and Dr. Albert Martin, epidemiologist from CDC who arrived in December 1966. Also, Miss Diana Bowen from the U.K. joined the immunology laboratory in March 1967. Significant contributions were also made by a number of short-term investigators. These were Dr. Randy Eichner from CDC who came in July 1966 and carried out a field study evaluating the immunity to vascular permeability factor in convalescent cholera patients. Drs. George Curlin and A. W. Karchmer from CDC arrived in October 1966 and conducted antibiotic trials with furazolidone and low dose tetracycline in cholera patients. In addition, Dr. Karchmer studied an outbreak of cholera in our study population in the Sylhet Tea Gardens, making a significant contribution to the understanding of epidemic cholera in a well-vaccinated population.

Mr. Wallace DeWitt, a bacteriologist from CDC, arrived in November 1966 to set up the field laboratory in the Matlab Bazar vaccine trial area and to evaluate TCBS media and a variety of transport media in the identification of Vibrio cholerae . Drs. Cal Linneman and Tom Vernon from CDC joined us in January 1966. Dr. Linneman assisted in setting up a pediatric clinical study and also carried out a purging study of normal individuals in Matlab with high vibriocidal antibody titers searching for

chronic carriers. Dr. Vernon carried out a field study among family contacts which demonstrated that immunity to vascular permeability factor was not associated with either protection from infection with Vibrio cholerae or modification of the disease. A significant contribution to the immunochemical studies was made by Dr. Paul Crabbe' who joined the immunology laboratory in January 1966 for a few weeks to work in a collaborative project characterizing the immunoglobulins in cholera stools.

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

1. Cholera Vaccine Field Trials.

The 1966 field trial covered a population of 110,000 in the Matlab area. 39,862 children, aged from three months to 14 years, participated in the study evaluating a single dose and a two-dose schedule of standard commercial cholera vaccine. The trial demonstrated that a single dose of cholera vaccine reduced the case rate by 47% during the cholera season; a two-dose schedule reduced the case rate by 66%. The enhanced effect of the two-dose schedule was entirely in children under the age of five years. It was significant that in children under five, one dose produced 32% protection, two doses produced 91% protection in the first three months after

vaccination; however, during the second three months, both the one and two-dose schedules were no longer protective. In older children, aged 5 to 14, the one and two-dose schedule provided the same level of protection averaging about 80% and this protection appeared to extend with some fall-off through the eight month cholera season. Serological surveys in the vaccine trial population confirmed the previously established relationship of vibriocidal antibody titer to protection. The most significant finding in this trial was the fact that it was possible to measure quite accurately the relationship of the cholera case rate to antibody titer. There was a 50% reduction in the cholera case rate with every doubling of the vibriocidal antibody titer. A clinical study in conjunction with this trial revealed that there was no amelioration of the symptoms in vaccinated persons who went on to develop clinical cholera.

2. Mehron Village Study.

This study carried out by Dr. McCormack was designed to estimate the level of inapparent infection in a population utilizing a vibriocidal antibody rise in paired serum specimens as an index of infection. A fishing village with a population of 1600 persons was selected for study and serological specimens were collected from as many individuals as possible in November 1966. Intensive surveillance with culturing of all reported diarrheas over the next three months revealed five cases of mild diarrhea associated with Vibrio cholerae but not requiring hospitalization. The second

serological survey was carried out in February 1967, after the cholera season. There were 30 individuals with significant rises in vibriocidal antibody titer out of 900 serum pairs tested; giving an infection rate of over 3%. These 30 persons were clustered in houses around the five bacteriologically positive diarrheas. Thus, this community, which did not have one clinically apparent case of cholera, had an infection rate of over 3.5% during the cholera season. This rate is more than 10 times higher than the clinical cholera case rate reported for the villages in Matlab. The demonstration of an exceedingly high infection rate in the endemic cholera area and the failure to demonstrate this by intensive bacteriological study of all diarrhea cases has broad implications in our understanding of the epidemiology of cholera.

3. Rayer Bazar Community Study.

The Rayer Bazar Community study was continued through the 1966-67 cholera season. Observations over the past two years have established that cholera was appearing repeatedly in the same area of the community, almost exclusively among persons who used the canal water for drinking and washing purposes. This was in striking contrast to the pattern of acute diarrheal disease and shigellosis which were distributed relatively uniformly over the entire community. This consistent trend of cholera has confirmed the predominant importance of water in the transmission of infection. Because the canal was serving as the main source of infection, a prospective study of the boatmen entering the canal was carried out by setting up a

quarantine station in the canal and culturing all boatmen entering. Over a three month period, 800 cultures were obtained of which four were positive for Vibrio cholerae. Three of the boatmen had clinical disease, discharging infected stools in the canal. In each instance, this was followed by the appearance of secondary cases of members of the community using the canal. Just adjacent to the quarantine station, a boatman entered another canal and developed cholera leading to a major outbreak in the surrounding community. Overall, during the period of study, the five infected boatmen contributed to the contamination of the canal leading to 62 cases in the community.

4. A Relationship of Vibriocidal Antibody Titer to Immunity to Cholera in Family Contacts of Cases.

This study was initiated during the 1965-66 cholera season, but titration of sera and analysis were not completed until 1967. This was a study in family contacts of cholera cases who were bled and then followed by daily rectal swabs for ten days, followed by a second bleeding. A total of 466 contacts in 81 families were followed. The most significant findings were - first, that the secondary infection rate among the contacts was directly correlated with the vibriocidal antibody titer they had on the initial blood specimen, such that the rate fell approximately 50% with each doubling of the antibody titer. A second finding was that, based on the number of persons with a four-fold or greater rise in vibriocidal titer, daily rectal swab collections for ten days

missed approximately 20% of the persons with inapparent infection. This observation has broad implications in terms of current recommendations for identifying carriers in cholera control programs.

As a result of this study, Drs. McCormack and Barui initiated a study comparing the efficacy of multiple rectal swab cultures vs. purging in detecting carriers. Only preliminary data is available to date on 46 family contacts who were studied. One was positive on initial rectal swab culture. This individual and three others were positive following purging. Of the remaining 42 negative on both culture and purge, the striking finding was that in this group subsequently daily rectal swab cultures detected infection in five more. This study is incomplete and will be completed in the 1967-68 season.

5. The Role of Vascular Permeability Factor Neutralizing Antibody in Protection Against Cholera.

Studies of immunity in man to permeability factor (PF) were initiated in August 1966 when Drs. Eichner and Craig came to Dacca and demonstrated that convalescent patients neutralized to toxin when injected into their skin. This study was continued by Dr. Vernon in collaboration with Dr. Craig who followed a series of cholera patients serially and demonstrated that the ability to neutralize PF when injected into the skin paralleled the rise in the serum PF neutralizing antibody titer. This led Drs. Martin and Vernon to follow family contacts of cholera patients to determine if the ability of neutralize PF

was associated with protection from the disease or modification of the symptoms. 142 contacts were studied by initially determining their skin test reaction to permeability factor and following them daily for ten days with rectal swab cultures. The results of this study demonstrated that there was no correlation between the ability to neutralize the cholera permeability factor and the likelihood of being infected with Vibrio cholerae, or the severity of the symptoms in individuals who were infected.

The data available to date on PF neutralizing antibody indicates that the presence of this antibody is probably specifically related to previous infection with Vibrio cholerae and thus may prove to be a valuable epidemiological tool; however, there is no indication that persons with PF neutralizing antibody are any less susceptible to clinical cholera than those without antibody. This is in contrast to the finding that susceptibility to infection by cholera can be definitely related to the vibriocidal antibody titer.

6. Antibiotic Prophylaxis in Family Contacts of Cholera Patients.

The effect of various dosage schedules of tetracycline were evaluated as a prophylactic agent to prevent infection in family contacts of cholera cases. This was done by visiting families on the day of admission of index case, assigning them to one of four treatment groups, and taking daily rectal swab cultures for ten days. The four treatment groups were a placebo

medication four times a day for five days, tetracycline four times a day for five days in a dosage of 250 milligrams for adults and 125 milligrams for children, tetracycline once a day for five days in a dosage of 1 gram for adults and 500 milligrams for children and tetracycline in a single dose on the first day only in a schedule of 1 gram for adults and 500 milligrams for children.

This study established the effectiveness of a five day schedule of tetracycline either in a single daily dose or divided doses in preventing secondary infection among family contacts. A single dose of tetracycline was ineffective as a prophylactic agent.

7. Epidemiological, Bacteriological and Virological Studies of Non-Vibrio Cholera.

This study was conducted by Dr. McCormack in collaboration with the bacteriology section and in cooperation with the virology unit of NAMRU-2. Non-vibrio cholera is an acute diarrheal disease which initially will mimic typical cholera. Clinically, on admission, the patient will present in shock and generally will respond to intravenous fluid administration. The course of diarrhea is characteristically short after the admission to the hospital and the stool does not usually have the typical rice water appearance. Bacteriological and serological studies for cholera are negative.

Epidemiologically, this disease has a distinct seasonal pattern, peaking sharply in March and April. This does not correspond to the diarrhea season in East Pakistan which peaks in July, August

and September or the cholera season which peaks in November and December. The hospitalized patients with this syndrome are characteristically adults with a slight preponderance of males. Field studies have indicated that there is no clustering of these cases within families and that the cases are distributed throughout the city without geographic clustering.

Intensive bacteriologic study using anaerobic and aerobic methods and for specific pathogens as well as for the pattern of intestinal flora has been unrevealing. Specimens sent for virological studies have not been fruitful in identifying a possible pathogen when inoculated into a variety of tissue cultures. The distinctive epidemiological characteristics of this disease including the seasonal pattern and age distribution of cases suggests a single etiology. Lack of secondary cases or of clustering of cases is suggestive of a toxin. Further studies are being carried out to elucidate the cause of this syndrome which is producing significant morbidity in adults in East Pakistan.

8. Serological Response of 6 to 12 Month Old Infants to Cholera Vaccine as a Model for Vaccine Potency Testing.

A study has been carried out in collaboration with Drs. Margaret Pittman and John Feeley. Preliminary studies have demonstrated that in 6 to 12 month old children, a two injection schedule at an interval of one month provides the maximum booster response. Eight different vaccines were tested in this schedule, each in a group of 20 infants. Four were whole cell phenolized vaccines containing both Ogawa and Inaba organisms which showed significant

variations in the mouse potency test. Phenolized vaccines containing only Ogawa and one containing only Inaba organisms, a lyophilized vaccine, and Dr. Verwey's purified Ogawa antigen were tested. One of the whole cell phenolized vaccines was given to the children in graded doses to determine the dose response curve. This study revealed marked differences in the infants' response to the various vaccines. The poorest response was with the purified Ogawa antigen in which only five of 20 infants developed detectable Ogawa titers and none developed Inaba titers.

The best response was with the P-SCRL field trial vaccine used in 1963 in which the mean Ogawa vibriocidal titer was 1:1000 and the mean Inaba titer was 1:1800. Based on the dose response curve to the reference vaccine in these infants, it has been possible to evaluate the relative antigenicity of these vaccines in humans. This model will prove extremely valuable in preliminary tests of future vaccines prior to use in the field trials.

9. Serological Response of Various Population Groups to Cholera Vaccine.

This is a continuing study being carried on by Dr. McCormack. Collaborators in this study have been Dr. Donald Mackay, Medical Director of the Sylhet Tea Gardens, Dr. Geraldine McCormack, Medical Director of the American Consulate Health Unit in Dacca, and Drs. Tom Vernon and Robert Armstrong, PHS Officers at Atlanta, Georgia. These studies have revealed that in the East Pakistan population there is an improved vibriocidal antibody response to a single dose of cholera vaccine with increasing age. Infants

under one year show almost no detectable titer following a single dose of vaccine, whereas by the age of five years, children develop antibody rapidly in a pattern characteristic of a secondary response. A similar age pattern of antibody response has not been found in a study of American children. Whether this relates to nutritional and constitutional factors or exposure to related antigens has not been defined.

Studies of the American population six months after their regular cholera booster reveals that the majority of them have significant vibriocidal titers suggesting that protection produced by vaccine is persisting beyond the six month period in this group. Studies of the effect of a single vs. a two-dose injection schedule has indicated that the only individuals benefiting from the booster dose are those that had an inadequate response to the primary injection. Those persons who developed a good titer following the first dose did not show significant boosting.

10. A Comparison of the Effectiveness of a High Dose of Tetracycline Schedule with Routine Schedule.

In this study, cholera patients admitted to the Matlab Field Hospital were randomly assigned to two programs. One group received 250 milligrams of tetracycline four times a day for five days; the second group received 1 gram of tetracycline on admission, 500 milligrams every two hours for four doses and then 250 milligrams every six hours for five days. The initial high dose was tried because of reports from other laboratories suggesting that this schedule would further shorten the course of diarrhea and intravenous fluid requirements.

A total of 265 patients were tested on these two schedules in a strictly controlled manner, evaluating the clinical course by mean intravenous fluid requirement and mean duration of diarrhea. There was no significant reduction of the course by the high dose regime as compared to the standard tetracyline program.

11. A Prospective Study of Development and Acquisition of Intestinal Pathogens in Infants from Birth through Two Years.

This study has been carried out in Rayer Bazar where infants were followed from birth with weekly visits for the first three months of life, bi-weekly visits for the next three months and monthly visits through the end of the first year. Stool cultures for enteric pathogens were obtained at each of the weekly visits and a stool specimen for parasites was obtained monthly. Observations were made on any clinical illness that the child had, and the child was weighed to determine the growth pattern.

This study was combined with an active medical care program for the child, including immunizations. Out of 113 infants that were initially picked up for study, 83 were followed through the end of the first year. The growth curve revealed that the infants' weight during the first week of life averaged 5.78 pounds, or approximately 2 pounds less than the average American infant. The babies' growth pattern followed that of American infants through the first three months, but then slowed down so that by the age of six months, the Pakistani infants weighed on the average three pounds less than a comparable group of Americans. At nine months, they were five pounds less, and at the end of one year, the average weight was 16.47 pounds or six pounds less

than American infants. During the period of study, a total of 22 infants were found to have pathogenic E. coli including eight in the first month of life. Shigella was detected in nine, including one in the first week of life.

Between the sixth and twelfth month, a large number of infants began to have protozoa and helminths in stool specimens so that by the end of the first year, 34 had protozoa and 18 had helminths. One child had inapparent infection with Vibrio cholerae during the fifth month of life, during the time that cholera was in the locality. These studies of these infants are continuing to define in more detail the pattern of acquisition of intestinal pathogen as they become older.

12. Sociological and Anthropological.

These studies are being carried out by Miss Aet Jarv with particular reference to the patterns of maternal and child care in a collaborative study with the prospective infant study mentioned above. The community reaction to the maternal and child health program being carried out in Rayer Bazar was defined. A detailed description of the observations made in these studies cannot be given in this report; however, it was shown that such factors as the family's attitudes about an unclean period for the mother and child shortly after birth and factors of purdah and belief that certain days of the week had abundant evil spirits influenced the family's participation in the child care program. Weighing a baby was considered bad since it was thought it might attract evil spirits to the healthy child. Customs such as

requiring a week-long ceremony to the Smallpox Goddess prior to permitting a child to be vaccinated influenced the vaccination program.

This study was extended into a more detailed study of families which refused to cooperate in the infant care program. The attitude of dominant members in joint families was shown to be a very significant factor influencing cooperation. An extensive study was made of child rearing in Chatgaon Village adjacent to Dacca. The details of this study will not be reviewed here.

13. Demographic Studies in the Matlab Field Trial Area.

Because of the large population under daily surveillance in the vaccine field trial area, a regular registration of births and deaths was initiated in March 1966 to provide demographic information that is not available in rural East Pakistan. Analysis of the first year's tabulations indicates that the birth rate is 46 per 1,000 and the death rate is 12 per 1,000. Mortality rate is highest in children under one, averaging 125 per 1,000. The crude rate of natural increase in this population is 3.3% per year. Observations of the births reveal a sharp seasonal pattern with almost 50% of the births occurring in the months of September, October and November. Life tables, age specific fatality rates, and gross and net reproduction rates are at present being calculated.

14. Immuno-Chemical Studies of Cholera Antibody.

The immunoglobulins involved in the serum antibody response to cholera have been characterized. By absorption studies it has been shown that antibodies to cholera are found in the Gamma M, Gamma G and Gamma A globulin fractions. In the vibriocidal antibody response, the earliest detectable titer is due to Gamma M globulin which peaks at 7 days followed by a Gamma G response which peaks by the tenth day. A similar pattern is seen for the agglutinating antibody response. Permeability factor neutralizing antibody is exclusively a Gamma G globulin. Over 90% of the vibriocidal activity and 80% of the agglutinating activity is due to Gamma M globulin. Transplacental transfer of vibriocidal antibody has been demonstrated. This is due exclusively to the transfer of Gamma G globulin. Studies of cholera stool in collaboration with Dr. Crabbe' have revealed that the stool contains from 20 to 50 milligrams per cent of Gamma A globulin; 6 to 10 milligrams per cent of Gamma G globulin and trace detectable amounts of Gamma M globulin. Preliminary study suggests that there is agglutinating activity to heat-killed vibrios in the Gamma A globulin fraction. Studies are continuing to characterize the immune response to cholera in intestinal secretions.

EPIDEMIOLOGY PROGRAM FOR FY68

In addition to the continuation of the vaccine field trial, the studies outlined below will have their emphasis on the mild and inapparent infection with Vibrio cholerae utilizing serological techniques as the means of identifying these infections. The study in Mehron Village last year by Dr. McCormack utilizing paired serum specimens as a method of identifying inapparent infections revealed an infection rate of almost 3.5% in the community without a single clinically apparent case of cholera. Serological surveys in the control group in the vaccine field trials suggested that between September 1966 and January 1967 more than 20% have been infected with Vibrio cholerae although the case rate in this group was less than 0.5%. With these findings suggesting an extremely high infection rate in the population, the studies this year have been designed to confirm this observation and to define the role of individuals with inapparent infection in the transmission of cholera. The major specific studies are as follows:

1. 1967 Cholera Vaccine Field Trial.

This will be a continuation of the 1966 field trial designed to answer two major questions: (1) What is the duration of effectiveness of the two-injection schedule that was given in 1966; (2) What is the effect of the booster dose of vaccine in the population immunized in 1966. In the trial there will be four vaccine groups: The control group last year will receive another dose

of ~~diphtheria~~-tetanus toxoid and remain as controls this year. The group receiving a single dose of cholera vaccine last year will receive a booster dose of cholera vaccine this year. The group receiving two doses of cholera vaccine last year (which was twice as large as either the control or the one-dose group) will be divided in two. Half of the two-dose group will receive a booster dose of cholera vaccine. The other half will receive an injection of diphtheria-tetanus toxoid. The vaccine program began on September 20, 1967 and will be completed on October 31. More than 90% of those who received two injections last year are receiving their third injection dose this year. Thus the study population will be approximately 9,000 children in each of the four vaccine groups.

2. Serological Surveys of Matlab Vaccine Field Trial Population.

In September 1967, serum specimens were collected from all children under 14 in every 30th family in the field trial population. Specimens were obtained from 1800 children. These same children will be bled again in November 1967, January 1968 and April 1968. The four specimens from individual children will be titered simultaneously to detect rises in vibriocidal antibody. This study will give us a broad look at the frequency of inapparent infection in the entire field trial population as estimated by the titer rises (and falls) in the serial serum specimens.

3. Inapparent Infections in Children.

A group of 100 children in Matlab have been selected. A serum specimen will be obtained prior to the cholera season, and the children will be cultured daily throughout the cholera season. A second serum specimen will then be obtained and serum pairs will be titered. The frequency of inapparent infection detected by daily culturing will be compared with the frequency found in paired serum specimens.

4. A Study of Re-Infections with Cholera in Convalescent Cholera Patients.

Long-term serological follow-up of 130 convalescent cholera patients last year revealed one child with a significant rise in vibriocidal titer in the Spring cholera season. This study is being extended by a continuing follow-up of that group of convalescents, plus an additional 330 persons who had cholera during the 1963-65 cholera seasons in Matlab. Serial serum specimens will be obtained from these 460 persons at regular intervals through the 1967 cholera season. The frequency of re-infection will be estimated by the number of individuals showing a significant rise in vibriocidal titer.

5. A Study of Factors Influencing the Pattern of Spread of Cholera with Particular Reference to Inapparent Infections.

In this study, three villages in Matlab and three communities in Dacca City with a total of 10,000 persons, have been selected for study. A detailed census and environmental survey has been conducted in each of these six localities. Base line serological

specimens have been obtained from approximately 75% of this population (7500 specimens). Daily house to house surveillance and culturing of all diarrheas will be carried out in each of these communities throughout the cholera season. In addition, in the three Dacca communities, regular routine sampling of water sources will be made. At the end of the cholera season, the second serological specimen will be obtained from the population. From this study, we hope to define the pattern of transmission of both clinical and inapparent infections of cholera and determine the sociological and environmental factors that are significantly related to transmission of the disease. Additionally, in one of the rural villages, migrants will be routinely cultured and given a three day course of tetracycline to determine if this prophylaxis has any significant effect in protecting a village from infection with cholera.

6. A Study of Persons with Inapparent Infection With Vibrio Cholerae.

Because of the high frequency of inapparent infections in family contacts of cholera cases, these contacts will be hospitalized for detailed observation. This will include a comparison of rectal swabs vs. purging in detecting infection in these contacts, and quantitation of vibrio excretion in the stool contents of the infected contacts. Efforts will be made also to obtain samples for culture from various locations in the small bowel to determine the location in the gut of the infecting organisms. Hospitalization will also permit a more careful clinical evaluation of the individuals with inapparent infection to determine if there are mild

symptoms which may escape detection in the field.

7. Antibiotic Studies.

These studies will be carried out in the hospitalized cases in Matlab and will be a continuation of observations made on children in Dacca last year on the effectiveness of a low-dose schedule tetracycline and furazolidone. Cholera patients will be randomly assigned into a control group, a standard tetracycline group, a low-dose tetracycline group (20% of the usual dose), and a furazolidone group. The clinical course in these four groups will be evaluated by standardized techniques and in addition, prior to discharge, patients will be purged to determine the effectiveness of the schedules in eliminating the infection. An additional group will be given chloramphenicol in the usual dosage to study the effect of this drug on the antibody response to cholera.

8. Laboratory Studies of the Immune Response to Cholera.

In addition to providing serological support for the field studies outlined above, the immunology laboratory will be engaged in two major area of study. First will be a standardization of the toxin neutralizing test for use in titrating the fingertip specimens and then an evaluation of this test as an epidemiological tool by comparing toxin neutralizing titers with various populations. Preliminary information suggests that this test could prove as sensitive as the vibriocidal test in detecting inapparent infection and more specific since it would not be influenced by experience with cholera vaccine.

The second area under investigation in the laboratory is a continuing investigation of the mechanisms of immunity in cholera. Preliminary data from Dr. Rowley's laboratory suggests that opsonizing activity can be detected in intestinal secretion. Opsonizing assays are being established in the laboratory. Studies will be carried out to determine the opsonizing activity of convalescent cholera serum and of stool during the course of clinical cholera. If this proves productive, opsonizing activity in the intestinal secretions following vaccination will be studied, and field studies of the relationship of this antibody to immunity will be initiated.

Appendix 1

Epidemiology Section
Personnel 1966-67

The listing below gives the staff of the Epidemiology Section at the beginning and the end of the fiscal year. During this time, the permanent staff increased from 99 to 133 persons, a 34% increase in the staff. The increase in the Dacca staff was due primarily to the transfer of two divisions from Clinical Research Section to the Epidemiology Section during the fiscal year. These were the immunology laboratory which added 7 members to the Epidemiology Section, and Medical Records which added 1 staff member. The only unit in the Epidemiology Section that was expanded was statistics. A fully qualified statistician was hired to head up the unit, and 3 additional statistical assistants were added.

The increase in the Matlab staff is due primarily to the doubling of the field trial area during the fiscal year. This doubling of the study area was accomplished, however, by an increase of the permanent field workers (sanitary inspectors, field assistants and speed boat drivers) from 57 to 75 or only a 32% increase. This was accomplished by a complete reorganization of the field surveillance procedure. The other major additions to the Matlab staff include 6 guards which have become necessary because of several thefts in the field stations, and one cook as we are now feeding patients since they are being kept for studies in the hospital.

A. Foreign Staff

	Position	Date Joined
Dr. W. H. Mosley	Chief, Epidemiology Sec.	June 1965
Dr. W. M. McCormack	Epidemiologist	Jan. 1966
Dr. A. R. Martin	Epidemiologist	Dec. 1966
Miss Aet Jarv	Anthropologist	Sept. 1966
Miss Diana Bowen	Lab. Technologist	March 1967

B. Dacca Staff

Position	July 1966	June 1967
Physician	4	5
Research Associate	2	2
Senior Research Assistant	4	6
Research Assistant	2	2
Research Technician	1	1
Laboratory Technician	3	2
Statistical Assistant	2	5
Medical Records Keeper	-	1
Sanitary Inspector	2	2
Field Assistant	5	4
Aide Nurse	1	1
Interpreter	-	1
Laboratory Attendant	-	1
Security Guard	1	1
Messenger	1	1
Messenger (temporary)	-	1
Total:	28	36
Private Secretary(part-time)	1	1
	29	37

C. Matlab Staff

Position	July 1966	June 1967
Physician	1	1
Wardmaster	1	1
Junior Staff Nurse	1	1
Aide Nurse	2	2
Ward Attendant	3	4
House Cleaner	3	3
Guard	2	8
Cook	-	1
Sanitary Inspector	9	14
Field Assistant	42	51
Speed Boat Driver	6	10
Total:	70	96
Total Dacca and Matlab	99	133

Total

Temporary Staff - Matlab

Lady Field Visitors	77	149
Boatmen	56	41
Total Temporary:	133	190

LIBRARY REPORT FOR FY67

An earlier report included most of the library activities in progress. A brief mention of these includes:

a. The Library supported jointly by the Institute of Public Health and P-SCRL has created great interest and awareness of its increasingly valuable collections among the medical community in Pakistan by regular publication and distribution by post of "Recent additions to the Library". All bound journals are included in the "Union Catalogue of Scientific periodicals in Libraries of Dacca" which informs scientists and other research workers outside the laboratory of our journal resources.

b. Loan facilities are being extended to other Institutions.

c. The library's stocks are increasing vigorously with fresh acquisitions.

d. Among the library services to our research investigators, zerox copies of required articles are being made available through inter-library loan from U.S. National Library of Medicine.

e. Photocopies of locally available journal articles are mostly ordered from Pakistan National Scientific Documentation Center, Dacca, because of the extra heavy load being put on the Laboratory-owned photocopier.

It was noted that many periodicals coming from Britain had breaks in sequences due to numbers being lost in the post. The library of NIH was contacted through Mrs. Doris Parkinson to help with missing back numbers. Similarly the library of the W.H.O. is being contacted for gifts of duplicate medical literature

to fill the gaps in journals. The outcome may result in a soundly based "exchange system for duplicate medical literature". Such a system would augment our journal files in a significant way, especially as the back files of journals are needed more than ever before. This need is mainly increased with incoming guest investigators who require numerous references to articles and periodicals which we do not have, and are not available locally. Much valuable time is lost in waiting for the xerox copies of required articles to arrive from the National Library of Medicine at Bethesda. The heavy demand on inter-library loan services calls for serious consideration of the acquisition of back files of journals. Back files of medical journals in microprint, if available, would be cheaper and space saving.

In order to be uptodate with cholera information, an effort is being made to make card entries for required articles on cholera published from 1963 to date.

A heavy additional strain placed upon the library's available space and personnel increasing due to a number of regular seminars on Medical Topics of interest. Many local physicians and other specialists attend these. The library, at present, is the only available space for such meetings; thus cataloguing, current periodical circulation, binding preparations etc. are curtailed during such meetings.

Library Acquisition during July 1966 to June 1967

1. Acta Pathologica et Microbiologica Scandinavica.
2. Acta Tropica.
3. Biochemical Society Symposia
4. Eastern Librarian
5. ICLA Bulletin. (Int. Committee on Lab. Animals)
6. Information on Lab. Animals for Research.
7. Laboratory Animal Care.
8. Laboratory Animal Digest
9. Medical Annual
10. Pakistan Journal of Family Planning
11. Recent Advances in Clinical Pathology
12. Recent Advances in Gastroenterology

Subscription to the following three journals was discontinued.

1. Water Pollution Abstracts.
2. Medical Officer.
3. Willings Press Guide.

Library Statistics: July 1966 to June 1967.

ACQUISITIONS

	P-SCRL	IPH	TOTAL
Books added	494	195	689
Bound Journals added	921	4	925
Gift books received:			
Bengali	75		
English Fiction	14		
Medical	82		
	P-SCRL	IPH	TOTAL
Total acquisitions	3860	719	4579

Inter-library loan of zerox copies: Requests made to National Library of Medicine total: 339 articles.

Photocopy pages provided to research investigators from P-SCRL photocopy equipment and local order to PANSDOC P-SCRL=220 pp. + PANSDOC+1410 pp. (Exact statistics not available from technician).

Expenditure on library materials: (As collected from the library accession register).

Books:	$ 2010	£228-0-0	Rs. 905.00
Periodical subscriptions:	$ 2000	£390-0-0	Rs. 720.00 approx.
Journals, books, reprints binding:			Rs.4542.90
Photocopy charges to PANSDOC:			Rs. 705.00

Equipment, furniture, etc: Due to lack of space, shelving units cannot be accommodated in the library even though 17 book cases had been requested earlier.

Present Library area:

Present area of the main room of the Library, containing three reading tables and eighteen chairs plus stacks and periodical display cases.

34x30 = 1020 sq ft.

Area of conference room containing a large conference table and twelve chairs plus wall bookcases.

21x12 = 252 sq.ft.

Area of cataloging, storage, book preparation and typing office

21x7 = 147 sq ft.

Total present space = 1419 sq.ft

Future Requirements Estimated:

A. Acquisitions. (in volumes)

Based on FY67 total acquisition = 1614 volumes.

Estimated Cumulative Total Volumes	FY
5000	68
7500	69
10,500	70
14,000	71
18,000	72

B. Space Requirements:

1. Area for housing the collections:

2 book-cases standing face to face require = 48 sq.ft.

30 " " " " = 720 sq.ft.

Existing 40 + 180 book cases estimated (5 years) standing face to face require = 5280 sq.ft.

2. Area for library users:

Table and chair per reader area	=	36 sq.ft.
Table and chair for twenty areas	=	720 "

3. Area for library staff work:

a. Sections for incoming books area	=	45 sq.ft.
b. Sections for books in process area	=	45 "
c. Recent periodicals circulation storage area	=	90 "
d. Storing loose journals for binding area	=	60 "
e. Incoming bound journals area	=	45 "
f. Journals stacks for photocopy reserve area	=	45 "
g. Incoming reprint articles storage area	=	20 "
h. Section for reprints being processed area	=	20 "
i. Staff seating space area	=	180 "
j. Work space area	=	200 "
		750 sq.ft.

4. Grand Total of Estimated Area:

1. For housing collection	=	5280 sq.ft.
2. Reference Section	=	500 "
3. For Library users	=	720 "
4. For staff and work space	=	750 "
5. Extra space for passages, etc.	=	1000 "
6. Cat. cabinets, vert. files, reprint collections	=	500 "
7. Seminars, library exhibitions etc.	=	1000 "
8. Conference room	=	500 "
Total:		10,250 "

C. Equipment, Furniture for FY68.

25 Book cases, 2 filing cabinets, display equipment, photocopy equipment. Estimates for five years would be as follows:

1967-68	1968-69	1969-70	1970-71	1971-72	
24	30	38	44	50	= 186 Total bookcases

(average cost per bookcase = Rs.250 to Rs.300)

Total book cases for shelving likely to be required in next five years similarly vertical file cabinets, catalog cabinets, and other items of library use would be required.

D. Estimated Costs FY68.

For book purchases	$7000	£800	Rs.4000	($40,000)	Next five years.
For Periodicals subscription	5000	£500	Rs.1000	($50,000)	"
Back files of Periodicals:	$20,000				
Photocopying charges			Rs.3000	(Rs.20,000)	"
Furniture, equipment			Rs.10,000	(Rs.70,000)	"

Half way through FY68, the expenses are a guide to future estimates, (from June to October, 1967: $2500 for periodical subscriptions USA, $3075 book orders from USA). The budget needs a planned rise together with the factor of rising costs, e.g. cost of scientific and medical periodical subscription= $30 to $ 40 per year. Cost of a book on scientific or medical subject = $20 Similarly binding costs, equipments, etc. would show a steady rise in cost.

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MAINTENANCE REPORT FOR FY67

One of the several factors that have seriously hampered research has been the lack of the proper organization of maintenance facilities. With the arrival of Mr. Mark Tucker, Maintenance Officer, from the United States in August, 1967, a separate Maintenance Section was established with responsibility for all laboratory equipment and instrument servicing and repair, building maintenance and modifications, plumbing, carpentry, electrical repairs, servicing of outboard engines and the servicing and repair of vehicles not maintained by USAID/Dacca (USAID has standardized on certain trade makes and is seriously limited in providing service to other makes). No new staff is contemplated with this new re-organization.

However, with the proper organization of maintenance there is still a lack of many tools and pieces of equipment that the new Maintenance Officer will need if he is to provide adequate and effective maintenance support to the laboratory. An estimated $6000 is needed for fitting out the Maintenance Section with proper tools and equipment. Although several pieces of "surplus" maintenance equipment were sent to the laboratory from NIH, only two pieces are in operating condition; other items are either incomplete, worn out or were damaged in transit.

Many of the problems of servicing and repairing the fleet of vehicles could be overcome if the laboratory could standardize on one make of automobiles. USAID/Dacca which provides gasoline, oil, servicing and repairs has standardized on Chevrolets and Jeeps, and the laboratory is therefore forced to maintain the other makes which it owns. The laboratory does standardize on Chevrolets and Jeeps for those vehicles donated by the US Government, but cannot on those vehicles donated by other SEATO member countries, such as the United Kingdom and Australia. However, the laboratory is making efforts to standardize on as few different makes as possible.

During FY67 the laboratory received two new Austin sedans from the U.K. and two new Jeep Wagoneers from the U.S. These were a welcomed addition to a fleet of vehicles many of which need replacing. An additional four new replacement vehicles, preferably of the station wagon or Carryall type, should be ordered during FY68.

DACCA WARD REPORT FOR FY67

A total of 3,251 patients were admitted to the ward during this year: 1,734 cases were acute cholera, with 31 deaths. See Tables I and II for breakdown.

Thirty five patients with acute cholera were pregnant, a considerable number being full term. Three living male babies were delivered in the ward, with no complications, and four were stillborn.

The "season" started in September 1966 with severe outbreaks of cholera in a small town 16 miles from Dacca and also in some villages across the river from Dacca. The admission rate steadily increased over the following weeks; the peak figure was 39 admissions in 24 hours, and the highest census, at any one midnight was 147 patients, plus about 100 attendants. One attendant per adult patient, while the patient is in the acute stage, is permitted, and one parent stays with a child patient for the duration of hospitalization. This arrangement relieves the nursing staff to some extent, though increases work in other departments as the attendants are given food.

More than 50 percent of the cholera admissions during this epidemic were below the age of 10 years and approximately 100 children were put on an antibiotic study which necessitated a stay in hospital for 21 days. Without attendants, it would not have been possible to keep these children in hospital for this length of time under study. More convalescent space was needed and a tent and bamboo hut were put up to accommodate these patients.

As the patient load far exceeded anticipation, shortage of chemicals for I.V. fluids, needles, syringes, disposable I.V. sets, etc. occurred and extra supplies had to be bought from the local market and outside Pakistan at considerable cost.

All I.V. fluid for patients was being prepared in the small pharmacy attached to the ward. In November 1966, 3,686 liters were prepared. As this small unit was not equipped to cope with such a huge amount, a larger unit was set up in the Chemistry Section.

It must be mentioned that at no time during this hectic season was the research work stopped. The ward staff, medical, nursing and all others responded to the challenge of coping with epidemic cholera, at the same time maintaining a high standard of care, assisting the research physicians and enabling the research work to be carried on.

After this exceptionally heavy season the number of cholera patients admitted to the ward dropped abruptly from 237 in January 1967 to 15 in February 1967. The number increased very slightly again for the next three months and then in June only two patients with acute cholera were admitted.

A wide variety of clinical entities with diarrhea were treated and investigated also. The number of children suffering from malnutrition and kwashiorkor-like states increased. These children present complicated problems in fluid and electrolyte balance and often have multiple infections on admission. After these problems are handled, these children may require several weeks hospitalization for correction of malnutrition, during which time they require a good diet and good nursing care.

During this epidemic two extra nurses and some domestic staff were employed on a purely temporary basis, but though the patient load has increased over the years, the total nursing staff remains more or less the same - Senior Staff nurses = 6, Junior Staff nurses = 4, Aid nurses = 9.

The X-Ray department has been enlarged, giving improved facilities. Three male nurses have been taught to take simple X-Rays, develop plates, etc. and so relieve the trained X-Ray technician. See table III.

The patient load increased very considerably in 1966-67 and the research program has become more complex and intensified, occasioning the need for more beds and special 'study' rooms.

These two factors have brought to light the urgent need for more bed space, a permanent convalescant ward, 'side rooms' for the nursing of the dangerously ill and dying, and isolation of some cases, separate cubicles for minor procedures such as sygmoidoscopy etc., and even a separate 'delivery' room for the few full term pregnancy cases admitted with cholera. Such expansion would of course include the usual showers, latrines, nurseries, storerooms etc. on an adequate scale.

Without any alterations or installations it is extremely difficult to envisage how the high standard of research and patient care, which the Laboratory's increasing world wide reputation demands, can be maintained.

ADMISSIONS TO CRL WARD - 1st July, 1966 to 30th June, 1967

Table I

	CHOLERA		NON-CHOLERA Diarrhea	
	Admissions	Death	Admissions	Death
Children 10 years and under	901	10	583	18
Males over 10 years	489	1	549	10
Females over 10 years	344	1	385	3
	1734	12	1517	31

Total admissions - 3251

Table II

Month	Total admissions	CHOLERA		NON-CHOLERA (Diarrhea)	
		Total	Death	Total	Death
July 1966	94	14	nil	80	2
August	70	8	nil	62	1
September	131	62	1	69	1
October	294	218	3	76	nil
November	608	444	2	164	2
December	810	627	2	183	1
January 1967	382	237	3	145	3
February	119	15	nil	104	2
March	190	27	nil	163	2
April	258	43	1	215	9
May	189	37	nil	152	5
June	106	2	nil	104	3
Total	3251	1734	12	1517	31

Table III

X-Ray Department - from 1st July 1966 to 30th June, 1967

Type	Total	Patients	Employees
Chest X-Rays	1206	781	425
Opaque Medium	77	51	26
Others - Abdomen, extremities, etc.	729	535	194
Total	2012	1367	645

TECHNICAL COMMITTEE MEETING 1967

CLINICAL INVESTIGATION RESEARCH PROTOCOLS

CI/P1 Unidirectional Fluxes of Sodium and Water Across the Intestinal Wall in Acute Cholera and Normals.

Dr.J.O. Taylor, Dr. J. Kinzie, Dr. Ruth Hare and Dr. R.A. Phillips.

CI/P2 A new method for measuring unidirectional fluxes of isotopically labelled compounds across the intestinal wall, using a bolus injection of isotope and a non-absorbable marker.

Drs. R. Hare, Kinzie, Taylor, Phillips.

CI/P3 Measurement of intestinal permeability in acute and convalescent cholera by diffusion ratio technique.

Dr. J. Kinzie, Dr. J.O. Taylor, Dr. Ruth Hare and Dr. R.A. Phillips.

CI/P4 Oral lavage of a solution containing glucose, electrolytes and tetracycline as a method of treatment in acute cholera.

~~Dr. Md. R. Islam & P-SCRL Ward Physicians.~~

CI/P5 Balance Studies in pediatric cholera patients.

Drs. M.M. Rahman, K. Hare, W. Rahman & R. Hare.

CI/P6 Study of body composition of children in East Pakistan.

M.M. Rahaman, K. Hare and R. Hare.

CI/P7 Study of nutritional status of children in cholera and other diarrheas.

M.M. Rahaman, S.G. Rahman, K.S. Haque & K. Hare.

CI/P8 A study of proteins of the Cholera stool.

Robert S. Northrup, M.D.

CI/P9 Secretory piece in cholera stool.
An Addendum to "Proteins of Cholera Stool" protocol.

CI/P10 Comparison of techniques for collecting secretions from the normal human intestine.

Dr.R. Northrup.

cont'd...2

CI/P11 Radioimmunoelectrophoretic analysis of human serum and stool for V. cholerae binding antibody.

Dr. R.S. Northrup.

CI/P12 Immunohistology studies in cholera - 1967-68.

Dr. R.S. Northrup.

CI/P13 Preparation of Antisera for use in Cholera-related immunologic studies.

Dr. R.S. Northrup.

CI/P14 Withdrawn

CI/P15 Electron microscope study of small bowel and rectal biopsies in acute cholera and normals.

A. Abraham and R.S. Northrup.

CI/P16 Comparison of virus isolations in cholera and other bacterial diarrheas and normals.

A. Abraham

CI/P17 Studies in experimental cholera in animals.

Drs. Nalin & Cash.

CI/P18 Part I: Determination of drug effects on intestinal water and sodium flux, volume and flow rates using Na^{22} and a non-absorbable marker.

Drs. D.R. Nalin & R.A. Cash.

CI/P19 Withdrawn.

CI/P20 Comparison of a commercially-available intravenous acetate solution with standard bicarbonate replacement solutions in the rapid correction of the acidosis of acute cholera and other severe diarrheas.

Dr. A. Majid Molla & P-SCRL CI Section & Ward staff.

CI/P21 Withdrawn.

CI/P22 Withdrawn.

cont'd. 3

CI/P23 Oral lavage of vibriophage in acute cholera: Effects on vibrio counts and stooling rate.

Dr. K.A. Monsur, Dr. Ataur Rahman, Dr.Z. Haque, Dr.R. Northrup & Dr. J.O. Taylor.

CI/P24 Bile salts and bile pigments in rice water stool in cholera.

Drs. N. Hirschhorn & I. Rosenberg.

CI/P25 The purification and properties of cholera toxin.

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

CI/P26 Spinal fluid in asiatic cholera.

Dr. David R. Nalin.

PROTOCOL

UNIDIRECTIONAL FLUXES OF SODIUM AND WATER ACROSS THE INTESTINAL WALL IN ACUTE CHOLERA AND NORMALS

Dr. J. O. Taylor, Dr. J. Kinzie, Dr. Ruth Hare and Dr.R.A.Phillips

Background and Purpose:

Studies of net fluxes of sodium and water in acute cholera done here last year showed that there was a net loss of sodium and water along the whole length of the small bowel in acute cholera. Infusion of glucose into the gut lumen decreased this net loss or actually caused net absorption as long as the glucose was present. In convalescent cholera patients and normals there is net absorption of sodium and water throughout the length of the small bowel and this absorption appears to be variably enhanced by intralumenal glucose.

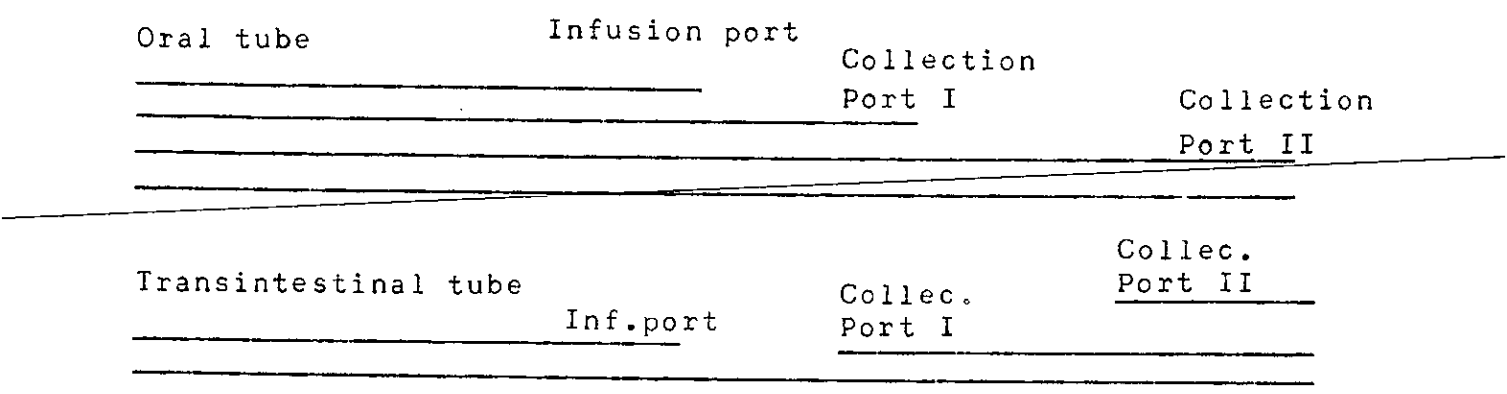
These studies have shown that the whole length of the small bowel is involved in the pathologic process resulting in massive fluid and electrolyte loss in cholera. The colon does not appear to be involved. They have also shown that intralumenal glucose increases net absorption of sodium and water. But they have not shown by what mechanism the bowel suddenly begins to lose large amounts of fluid and electrolytes - or by what mechanism glucose slows or reverses this process. It is hoped that the unidirectional flux studies being done this year will give more clues to the actual mechanism involved in these processes.

Experimental Design:

A technique for measuring unidirectional fluxes across the wall of intestinal segments has been developed here. This method is described more fully in the paper "A new method for measuring unidirectional fluxes of isotopically labelled compounds across the intestinal wall" by Drs. Hare, Kinzie, Taylor and Phillips.

The method is based on the same intestinal intubation steady - state system used last year to measure net fluxes. The patient swallowed a triple lumen tube open at 3 ports:

An infusion port and two collection ports



The ports are positioned by X-Ray monitoring.
Net fluxes are measured by infusing a solution of electrolytes matched in composition to intestinal fluid from that level of the gut in cholera, and a non-absorbable marker (PEG). This solution is infused at a constant rate. Since the marker is not absorbed, once a steady state is achieved the flow rate at each port can be calculated by the formula

(Infusion rate) x (PEG conc in Infusion) =
(Flow rate Port 1) x (PEG conc Port 1) =
(Flow rate Port 2) x (PEG conc Port 2) =

The net flux of water is calculated by simple difference between the flow rate at Port 1 and the flow rate at Port 2.

The ratios of isotope to non-absorbable marker with time given a plot of the rate of disappearance of the isotope from the lumen. The mean transit time across the segment can be determined by the appearance of the non-absorbable marker at the second port. The flow rates at the first and second port are known from the infusion rate and concentration of PEG in the infusion and at each port. From the flow rates and the mean transit time it is possible to calculate the volume of the intestinal segment. The concentration of sodium (or water) is measured directly. From this the unidirectional flux of sodium (or water) can be calculated. The net flux is known from the simultaneous measurement by the steady state method based on change of PEG concentration from infusion to port 1 to port 2. The difference between the net and the unidirectional lumen to plasma flux is the unidirectional plasma to lumen flux.

Procedures:

1. Patients are selected on admission. All patients in shock with a positive dark field are given (after they have been brought into water and electrolyte balance) a single lumen thin polyvinyl tube (lead tube) with a balloon on the end to swallow. The balloon contains 2 cc of mercury. When the balloon passes the pylorus it is inflated with 35 cc of water and allowed to pass through the intestinal tract.
2. A chest X-Ray, stool exam for occult blood, and an EKG are done on the day of admission. Evidence of cardiac or

pulmonary disease or significant GI bleeding excludes a patient from study.

3. When the balloon passes per rectum a study tube is attached to the oral end of the tube and pulled into position by gentle traction on the anal end of the tube. Experience has shown that it is approximately 65 cm from the mouth to the ligament of Treitz and approximately 120 cm from the mouth to the proximal ileum. (approximately 100 cm from the ileocecal valve to the anus). Final tube position is confirmed by X-Ray.

4. One to two liters of 5:4:1 solution are given through the infusion tube and samples collected at port 1 and 2 for PEG blanks. Baseline hematocrit, plasma protein, specific gravity and electrolytes are measured.

5. A solution of electrolytes matching the average composition of intestinal fluid at the level being studied is infused at a constant rate (30 ml/minute). "Average jejunal fluid" (see Table 1) is used for jejunal studies and "average ileal fluid" (see Table 2) is used for ileal studies. Both solutions are made slightly hypotonic by subtracting 20 mEq of NaCl from the true average electrolyte composition of cholera fluid from these levels. This is done so that when 55 mM glucose is added to the solution it does not become markedly hypertonic - which causes a variety of undesired changes including an increase in intestinal volume over the segment.

6. After one to two hours of equilibration with this fluid being infused at a constant rate, simultaneous collections are begun at the first and second collection port by means of peristaltic constant collection pumps. The collection rate at port 1 is 1 ml/minute and the collection rate at port 2 is 5 ml/minute. Thirty minute collections are taken and PEG concentration and electrolytes in the infusion fluid and at each port for the 30 minute pooled collection are determined. Net fluxes of water and sodium are calculated from this "steady state" data.

7. A 1 ml bolus containing 50 mg of BSP and 2.5 μc of Na^{22} and 25 μc of tritiated water is given instantaneously at the first collection port (the port 1 collection pump has been turned off). Constant collection at 5 ml/minute, changing test tubes each minute, is continued at port 2. BSP, Na^{22} counts and tritiated water counts are measured in each of the serial samples collected at port 2 until the bolus is past. This information is used to calculate disappearance rates of Na^{22} and tritiated water and to calculate the mean transit time across the segment.

   1 ml of aliquots from each sample are also pooled for a PEG determination which is used to calculate the port 2 flow rate during the bolus period.

8. Steady state measurements and bolus measurements are alternated. Four steady states and three bolus periods are done per study of a single solution. Thus there are four steady states and three boluses with no glucose. Then glucose is added to the

Facilities Required for the Study:

All of the facilities required for the above study are available at the Pakistan-SEATO Cholera Research Laboratory.

Na^{22}, Na^{24}, and tritiated water can be counted in the Beckman Scintillation counter which has just been installed at the laboratory. Na^{22} and Na^{24} were previously counted on a small Picker Well-type Gamma Counter.

Electrolyte determinations can be accurately measured in the clinical chemistry laboratory. Sodium and potassium are done on a Baird Atomic flame photometer. Chloride is done on a chloride titrimeter. CO_2 is done by the Van Slyke manometric method. Calcium and magnesium are done on the Perkin-Elmer Atomic absorption spectrophotometer.

PEG is done by the turbidometric method of Hyden. BSP is done by a simple colorometric method.

The calculations are done by a qualified statistician under the direction of Dr. Ruth Hare.

TABLE 1

	mEq/L			
	Na	K	Cl	CO_2
Average Jejunal Contents	140	7	136	11
Jejunal Infusion Solution	120	7	116	11

TABLE II

	Na	K	Cl	CO_2
Average Ileal Contents	140	6	126	20
Ileal Infusion Solutions	120	6	106	20

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PROTOCOL

A NEW METHOD FOR MEASURING UNIDIRECTIONAL FLUXES OF ISOTOPICALLY LABELED COMPOUNDS ACROSS THE INTESTINAL WALL.

R. Hare, J. Kinzie, J.O. Taylor, R.A. Phillips.

Background and Purpose:

Visscher and his co-workers (1,2) in 1944 first measured the unidirectional fluxes of sodium and water across the intestinal wall using isotopically labeled material. Visscher's technique has been adopted to study the fluxes of sodium and water in the human intestine using a constant infusion of electrolyte solution with a non-absorbable marker containing the isotopes whose fluxes are to be studied (3,4,5).

Mitchell and his co-workers (6) devised a bolus technique to overcome some of the limitations of this constant infusion of isotope. In this technique, an electrolyte solution without isotope is infused at a constant rate at one end of the gastrointestinal tract. When the patient is purging constantly from the other end (achievement of a "steady state"), an instantaneous bolus of radioisotope is injected at the infusion port. An appearance curve of isotope in the effulent stool is then determined and from this the unidirectional flux of sodium out of the gut can be determined.

We have adapted a bolus technique to the study of unidirectional and net movements of isotopically labeled water, electrolytes and various other solutions across the gut wall of intestinal

cont'd.....

segments. An electrolyte solution containing a non-absorbable marker, but no isotope, is infused at a constant rate through the proximal port of a three lumen tube. Samples are collected at a constant rate from two distal ports, one 15 cm and one 35 cm from the infusion port. The net flux of water can be calculated from the change in concentration of non-absorbable marker (PEG, polyethylene glycol, average molecular weight 4000) according to the method of Cooper, et.al.(7).

To study unidirectional flux of any isotopically labeled compound, one milliliter of a solution of isotope and of a second non-absorbable marker (BSP, bromsulfphthalein) is injected instantaneously through a small tube which exits at the level of the first collection port. Serial one minute samples are then collected at the second collection port until the BSP has disappeared from the second port. A plot of the log of the ratio of isotope to non-absorbable marker against time is linear. A mean transit time across the gut segment can be calculated from the BSP data. A mean flow rate across the gut segment can be calculated from the flow rates at the first and second collection ports. From the mean transit time and the mean flow rate the volume of the intestinal segment can be calculated. The concentration of any substance under study can be directly determined chemically. From this concentration and the volume of the gut segment, the actual amount of the substance being acted upon by the gut mucosa can be calculated. From the

unidirectional transfer rate of the substance (in percentage per unit time) and the actual amount of the substance being acted upon, unidirectional flux can be calculated (in amount per unit time). From the net flux and the unidirectional flux of the substance out of the gut segment, the unidirectional flux into the gut segment can be calculated by simple difference.

The advantages of this "bolus method" in which isotopes are injected only in small instantaneous boluses are:

1) The total amount of isotope given is extremely small, therefore plasma specific gravity remains negligible, minimizing the correction for re-entry of isotope into the gut segment.

2) The total dose of isotope for any given bolus is extremely small, therefore many studies can be done, and a variety of isotopes used simultaneously in each study without reaching unacceptable radiation dosage to the patient being studied.

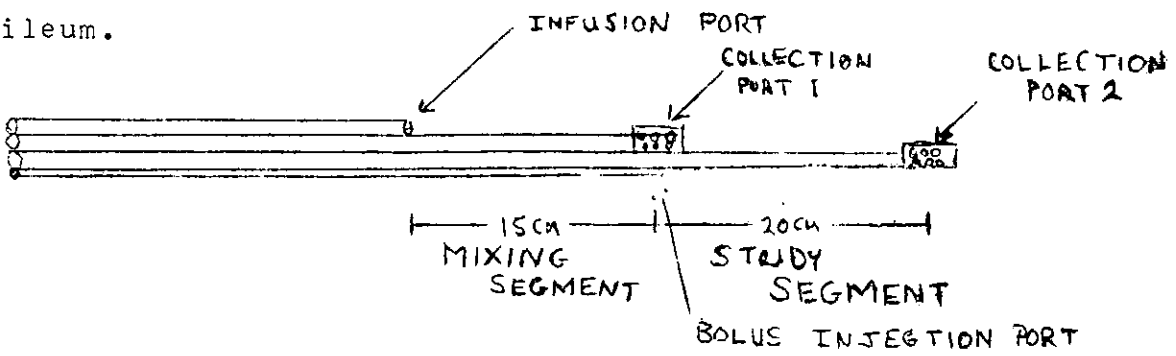
3) A variety of parameters can be monitored simultaneously including: Mean transit time across the gut segment, flow rate across the gut segment, volume of the gut segment, transfer rate of the isotope (which is independent of these other parameters), unidirectional flux out of the segment (which is dependent on all of these other parameters), and net flux. Simultaneous measurement of these parameters allows the relationships between them

to be studied. For example; Is an increase in net flux of a substance out of a gut segment due to a change in the transfer rate (therefore an intestinal wall mechanism) or only due to an increase in pool of substance acted upon at the same rate? Thus, with this method, it is possible to be more precise about the mechanisms of changes in fluxes than with previous methods in human subjects.

4) By repeatedly measuring such parameters as gut segment volume and correlating these with changes in net and unidirectional fluxes, many of the assumptions of the "steady state" method of Cooper, et al (7), which is presently used by so many workers in the field of intestinal absorbtion, can be tested.

Materials and Methods:

A triple lumen study tube to which has been attached a fourth much smaller "bolus injection tube" (see diagram) is swallowed and allowed to pass into position in the jejunum or ileum.



When the tube is in position, an electrolyte solution containing the desired composition and also containing a non-absorbable marker (PEG, Carbowax-4000) is infused through the infusion port at a constant rate using a constant infusion pump set, at the desired rate (30 to 40 ml/minute in our studies).

Samples are collected at a constant rate from the first and second collection port by means of constant collection pumps (Bowman type finger pump with the input end connected to the collection tubes). Port one is set at a collection rate of 1 ml/minute and port 2 at 5 ml/minute. After initial equilibration of 1 hour to achieve a steady state of flow across the gut segment under study, the study is begun by collecting a one half-hour sample from each collection port. The sample collection period at port two is delayed from the collection period at port one by a period equal to the mean transit time from port 1 to port 2 at that given infusion rate. These samples are used for determining PEG concentration at each port as well as the concentration of electrolytes and any other substance under study at each port. From this data the net flux of water can be determined by the method of Fordtrand and Cooper (7) .

After such a "steady state" collection of 30 minutes, port 1 collection pump is switched off, and a 1 ml bolus of radioisotopes and non-absorbable marker is injected "instantaneously" through the small bore tube (dead space 0.5 ml) and this is rinsed rapidly with an additional 2.5 ml of infusion fluid. Beginning at the moment of injection of the bolus, a stop watch is started and serial one minute sampling is begun at port 2. The sample tubes

are changed each minute on the minute. Sampling is continued until no more BSP can be detected in the port 2 fluid. BSP and isotope concentrations are determined in each sample. Aliquots of uniform volume are pipeted from each one minute sample and pooled into a common sample for all of the collection period. PEG is determined on this pooled sample to give port 2 flow rate.

The transfer rate of the isotope across the intestinal wall can be calculated by using the standard equation for exponential decay (or disappearance) of a substance:

$$Q_t = Q_o e^{-\lambda t}$$

Where:

Q_o = the dose in the bolus in cpm of isotope per milligram of BSP

Q_t = the amount of isotope absorbed at time t/in cpm per milligram of BSP.

λ = transfer constant across the intestinal wall.

λ can be calculated as follows:

$$\frac{Q_t}{Q_o} = e^{-\lambda t}$$

$$\ln \frac{Q_t}{Q_o} = -\lambda t$$

$$\lambda = -\frac{\ln \frac{Q_t}{Q_o}}{t}$$

Mean transit time across the gut segment is calculated by the formula:

$$tt = \frac{\Sigma(BSPxt)}{\Sigma(BSP)}$$

where: tt = mean transit time.

Σ(BSP x t) = sum of the products of the concentration of BSP in each sample and time.

and

Σ(BSP) = sum of the concentrationsof BSP in each sample.

Mean flow rate across the gut segment is calculated by the formula:

$$\text{Mean flow rate (ml/min)} = \frac{\text{Port 1 flow rate (ml/min)} + \text{port 2 flow rate}}{2}$$

Gut segment volume can be calculated by the formula:

Gut segment volume (ml) = Mean flow rate (ml/min) x mean transit time (min)

Amount of substance under study in gut segment can be calculated by the formula:

Amount of substance = gut segment volume x mean concentration of substance, where: mean concentration of substance =

$$\frac{\text{Port 1 conc.of substance} + \text{port 2 conc.of substance}}{2}$$

Unidirectional flux out of the gut segment of substance under study can be calculated by the formula:

Unidirectional flux (amt/min)= λ(% min) x amt.of substance in the segment.

The unidirectional flux of the substance into the gut lumen can be calculated from the formula:

Net flux = unidirectional flux out - unidirectional flux in

A positive number means a net flux out.

A negative number means a net flux in.

Where net flux is known from the steady state determination and the unidirectional flux out has been calculated from λ.

Bolus periods and steady state periods are alternated. The P2 flow rate is calculated for the bolus period based on pooled port 2 PEG concentrations. Comparing the port 2 flow rates during bolus and steady state periods confirms whether the segment has the same state of flow in bolus and steady state periods. For most of our studies we do seven 30-minute periods to study a single solution as follows:

Steady state - bolus - steady state - bolus - steady state - bolus - steady state.

The net flux for each bolus is the mean of the net flux for the two steady states bracketing the bolus - these should be almost identical.

The shape of the BSP appearance curve is a check on the validity of the mean transit time, segment volume and thus unidirectional flux measurements. (The transfer rate $\binom{\lambda}{A}$ is independent of this, however). A smooth sharp curve indicates a good study with a valid transit time. An irregular curve indicates some disturbance in the even transit of the bolus across the segment and therefore no valid transit time can be calculated.

Summary:

A new method for measuring unidirectional fluxes of solutes and water across the intestinal wall of gut segments is presented. This method uses intermittent "boluses" of isotope and non-absorbable marker rather than a constant infusion of isotope. The major advantages of this method are: 1) plasma specific activities remain negligible thus minimizing the correction for re-entry of the isotope into the study segment; 2) total radiation dosage is kept very small

allowing repeated studies and use of multiple isotopes simultaneously; 3) multiple parameters are monitored simultaneously, including mean transit time, flow rate, gut segment volume, total pool of substance under study, unidirectional transfer rate and unidirectional and net fluxes. This allows relationships between these parameters to be studied; 4) the validity of many assumptions of the "steady state" method can be checked repeatedly during any study.

PROTOCOL

MEASUREMENT OF INTESTINAL PERMEABILITY IN ACUTE AND CONVALESCENT CHOLERA BY DIFFUSION RATIO TECHNIQUE

Dr. J. Kinzie, Dr. J.O. Taylor,
Dr. Ruth Hare and Dr. R. A. Phillips

Measurement of intestinal permeability to solutes in intact man with the use of perfusion techniques may be approached in two ways.

1) A reflection coefficient i.e. the ability of a solute to act osmotically and draw solvent water into the intestinal tract can be measured[3]. This requires high concentrations of solutes in the gut segment, and moreover is subject to large errors if there is low rate of flow in relation to osmotic pressure gradients, or if the mucosa is in a state of net gut lumen to plasma flux.[5]

2) A method based on "restrictive diffusion"[8,5,6] involves the simultaneous monitoring of diffusion rates with compounds of different molecular radii. A membrane with a small pore size readily distinguishes between large and small molecules permitting the smaller to pass more readily than the large. This difference can be expressed as a ratio of diffusion.

If diffusion of two test substances is measured simultaneously, variables such as pool size, path length and changes in flow rate will cancel in the ratio, which becomes a function of the cross-sectional area of the channels available for diffusive processes. Both solutes are assumed to cross the membrane through the identical set of channels, back diffusion is

assumed to be small due to dilution in an infinite pool, and the molecules must be marked in some way for identification. Tritiated water and a series of carbon-14 labeled compounds of various sizes have proven to be of great value in such studies. THO with a molecular radius of 1.5A, C_{14} labeled urea with a molecular radius of 2.3A, and Erythritol with a molecular radius of 3.3A are of convenient size for the study of pores in biological membranes which have been estimated to have a pore size of 4-9A[2,3,6].

It would be of particular interest to obtain repeated observations of selective permeability in the choleraic intestine during the acute phase and during early and late convalescence, since several workers have postulated that alterations in permeability are important in cholera and there is evidence for altered absorption during the acute recovery phase[9].

Method:

Adult male cholera patients will be intubated following rehydration and achievement of a satisfactory clinical state. A normal chest X-Ray and EKG, without history or physical findings suggesting co-existing disease will be required for all patients undergoing the study. The triple lumen polyvinyl tube to be used has been described elsewhere[1,10]. It possesses an infusion port, with collection ports 15 and 35 cm beyond the infusion port. A thin polyvinyl "bolus" tube also ends 15 cm beyond the infusion port. For jejunal studies, the

infusion port will be positioned 65 cm beyond the teeth, which in East Pakistani adults is just beyond the ligament of Treitz and the position of the tube will be verified immediately after the study by X-Ray. For ileal studies, the infusion port is permitted to pass to 120 cm from the teeth, and tube position is verified before and after the study by X-Ray. After the tube is in place, intestinal contents will be withdrawn and determination of the electrolytes and urea will be performed. A solution approximating the urea concentration but hypotonic with respect to sodium and chloride will be infused at the rate necessary to give a mean flow of approximately 30 cc per minute across the segment being studied.

The composition of infusion solution will be about:

Ileal	Na	120 mEq
	K	6 "
	HCO_3	20 "
	Cl	106 "
Urea		3- 5 mM/L
Jejunal	Na	120 mEq
	K	7 "
	HCO_3	11 "
	Cl	116 "
Urea		3-5 mM/L

After an equilibration period of at least one hour, a series of alternate steady state collections and unidirectional flux studies using the bolus technique will begin. Sampling at the ports is staggered by varying the collection rate during the steady state periods. Four bolus flux studies will be done during the initial infusion, two boluses to contain C^{14} erythritol and two to contain C^{14} urea.

Each bolus will be composed as follows:-

2uc Na^{22}

10uc C^{14}

50uc Tritiated water

50 mgm BSP (Brom sulfalein)

The total dose of irradiation from each bolus, assuming complete absorption, is .07436 Rads.*

Each steady state collection will last 30 minutes and during the bolus periods samples will be collected from port 2 at the rate of 5 cc per minute until observation indicates absence of BSP in the aspirate.

The infusion solution will then be replaced by one identical in electrolyte content but containing 56 mm/liter of glucose as well. After a one hour equilibration period, alternate steady state collections and bolus flux studies will be performed. Re-equilibration with the original solution, followed by steady-state and bolus periods, will complete this initial study.

Stool and urine output will be monitored hourly, and checked against oral input. Intravenous replacement with 5:4:1 will be given as required and plasma electrolytes and

* See appendix, Table 1 for dose calculation and explanations.

specific gravity will be checked every four hours.

All patients studied during the acute phase will be restudied using the same technique 10-12 days following the cessation of diarrhea, and re-studied again two to three months following the illness.

Mean flow rate across the segment during the steady state periods will be determined by the change in concentration of polyethyleneglycol which will be present in the infusion solutions in the concentration of 10 g/L.

Flow rate during the bolus periods will be checked by comparing the PEG concentration of pooled samples with the PEG concentration of the port 2 steady state collections.

~~Radiosodium-22, C^{14}~~ and Tritiated water will be determined by liquid scintillation spectroscopy using a Beckman LS-100 ambient temperature counter and a Dioxane scintillation mixture. Satisfactory separation should be achieved with all three isotopes although quenching due to the presence of bile pigments and BSP may be quite severe in some series. In addition to correction by external standardization, calibration curves for various concentrations of bile pigment and BSP in intestinal aspirate will be determined, and corrections will be used where necessary.

Flux ratios will be obtained by dividing the percent absorption of the test molecule with the percent absorption of tritiated water.

For example:-

$$\text{Flux ratio Erythritol} = \frac{\dfrac{\text{Erythritol cpm expected-cpm observed}}{\text{cpm expected}}}{\dfrac{\text{THO cpm expected-cpm observed}}{\text{cpm expected}}} = \frac{\text{Lambda Ery}}{\text{Lambda THO}}$$

(where cpm expected is the activity which would be present in the sample if no absorption had occurred.) Each bolus will contain a large number of individual samples, and the flux ratio can be validated by comparing it from minute to minute, as well as from bolus to bolus.

Fordtran et al[5] found that only urea/THO fluxes can be accurately measured in the lower intestine of normal humans because of extremely low diffusion rates for larger non-electrolyte non-fat soluble molecules in the ileum.[3]

Although the diffusion of water seems to be restricted across the intestinal epithelium to a greater degree than would be predicted by its molecular size[5], the relative changes in diffusion ratio follow the known differences in normal human jejunum and ileum, in anuran membranes with and without antidiuretic hormone and in stretched vs unstretched artificial membranes.[5] Neither the magnitude nor the direction of water flow alter the urea/THO diffusion ratio in normal patients or in patients with sprue[5]. The method should therefore be valid for study of cholera patients, acutely ill and in convalescence, in spite of the expected changes in net water movement which would be encountered.

Significance of this Research.

If all of the solute species being studied penetrate the intestine via a single set of pores, an increase in the flux ratio

in cholera would indicate an increased radius of these channels. Under such conditions, the relative increase in the ratio Erythritol/THO would be much greater than that of urea/THO. If, however, two different sets of pores are available, one only for water and another for the diffusion of larger molecules, an increase in the flux ratios would be explainable by a relative or absolute increase in the number of larger channels in the membrane.

Evaluation of the sodium, water, urea and erythritol fluxes with and without glucose in acute and convalescent stages of cholera is of interest because recent work at the P-SCRL[9] and elsewhere[7] indicates that the cholera-affected small intestine is capable of responding to this sugar with an increase in net movement of sodium and water. This enhancement of net water and sodium transport has been difficult to demonstrate in convalescent patients[9] and other laboratories have reported negative results with glucose stimulation of net sodium transport in the human ileum.[4] Pore size data may shed some light on this problem.

Facilities required for this Study.

All are presently at P-SCRL.

APPENDIX

Radiation doses to a 40 kg, 160 cm tall man from diagnostic X-ray and Isotope study:

g= geometrical factor, and for such an individual it is 108*

C= mean concentration of isotope in body in microcuries per gm.

T= average life of isotope in body in days (1.443xeffective half life).

E_B=beta particle energy.

K= roentgens per hour per millicurie at one cm due to gamma rays

Formula for total dose of an isotope

Dose (beta plus gamma) = $CT\ (73.8E_B + .0346Ig) \times 1000$ millirad
(for man, rads and roentgens are essentially equal and additive.)

for Na^{22}, K= 13.2, g=108, T=15 days, E_B= .193, 2.02 uc per bolus.

Dose (B + gamma)= 2.0 uc/40,000 x 15 days (73.8x.193 +.036x13.2 x 108)
=44.25 milliroentgens.

For Tritiated water, E_B= .006 Mev, T = 15 days 50 μc per bolus

D_B = 50/40,000 x 15 days (73.8 x .006).

= 19.56 millireontgens per bolus.

For C^{14} labeled urea, EB = .05, 3/4 is absorbed as urea, excreted with T = 1.5 days. 1/4 metabolized by bacteria in the intestine, and absorbed as CO_2 T = 40 days.

D_B= 7.5/40,000 x 1.5 (73.8x.05) + 2.5/40,000x 40(73.8 x .05)

= 10.55 milliroentgens per bolus.

C^{14} erythritol would behave similarly. Total radiation per bolus=74.36 mr/bolus.

This calculation assumes complete absorption of all materials given.

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*Quimby & Feitelbey Radioactive Isotopes in Medicine and Biology, Lea & Febiger, Phil., Pa. 1963, p.117, table 7.

CI/P3

APPENDIX

Dose here per chest X-Ray* with 1 mm Al filter, taken at 36 inches, tube voltage 70-80 KV, filament amperage 75-90 MA.

Chest-exposure time .1 second

96 mr total body radiation.

Abdomen-exposure time .3 second

218 mr.

*From: Radiological Health Handbook Nomogram, p.169 PB121784R, U.S. Govt. Printing Office, 1960.

RESEARCH PROTOCOL

ORAL LAVAGE OF A SOLUTION CONTAINING GLUCOSE, ELECTROLYTES AND TETRACYCLINE AS A METHOD OF TREATMENT IN ACUTE CHOLERA

Chief Investigator: Dr. Md. Rafiqul Islam
Other Investigators: P-SCRL Ward Physicians

Background and Purpose:

Four factors now prevent the widespread application of the highly successful intravenous fluid replacement method of treatment of acute cholera in the rural areas of developing countries where cholera is most prevalent:

(i) the difficulties in preparation and storage of the large amounts of sterile intravenous solutions necessary to treat large epidemics;

(ii) the high cost of such sterile intravenous fluids;

(iii) difficulties in distribution of large amounts of fluid to remote rural health centers, and

(iv) shortages of personnel trained in the administration of intravenous fluid.

Thus, interest in finding a cheap, simple and convenient oral means of treating acute cholera is not new. Phillips and his colleagues first showed that oral glucose and electrolyte solutions decreased the rate of stooling and increased net

absorption in acute cholera. Studies done at this Laboratory last year showed that intralumenal glucose decreased the rate of fluid loss or actually caused net absorption of fluid in acute cholera in 20 cm. segments of both jejunum and ileum. In 11 patients with severe acute cholera, all purging more than 400 ml./hour, oral lavage of 55-100 mm/liter glucose in electrolyte solution has significantly reduced the rate of stooling for as long as it was given. Similar results were found by the Johns Hopkins group in Calcutta. Both groups of investigators have found that 2-3% glucose solutions infused at 1,000 to 1,500 ml./hour gave the optimal results.

Studies done here have shown that infusions of electrolytes alone do not affect net stooling rate (i.e. the total amount of stool passed minus the amount of solution given by mouth).

Oral lavage of solutions containing electrolytes and tetracycline (3 grams given over 7 hours) in patients purging over 400 ml./hour reduced vibrio counts to zero in 2 to 3 hours and caused a reduction of rate of purging by the sixteenth hour of infusion and the diarrhea ended within a mean of 35 hours (as compared with a mean of 49 hours in unselected cholera patients treated with tetracycline in capsule form).

A preliminary study of the effects of combined tetracycline and glucose given orally to 3 patients with acute cholera; two who were purging at a rate greater than 700 ml/hour at the

beginning of the study were brought into positive balance after 8 hours of lavage (18 hours after admission). The third, who was purging at a rate of almost 500 ml/hour prior to study, went rapidly into marked positive balance with glucose and tetracycline.

The proposed solution for oral lavage to be used in this study will contain 5 grams of sodium chloride, 4 grams of sodium bicarbonate, ½ gram of potassium chloride, 25 grams of glucose and 125 mg. of tetracycline. This would give the following composition:

Na	139.8 mEq/l
K	6.75 mEq/l
Cl	86.2 "
CO_2	47.6 "
Glucose	139.0 mM

So far studies of oral lavage of solutions with glucose have been done only in extremely active cholera patients. In this study, it is planned to give oral lavage to patients of all degrees of severity and to compare the total amount of stooling and IV fluid requirements with those of patients receiving routine therapy with intravenous fluids and tetracycline in capsule form. Thus, it is hoped to see what is the maximum benefit that can be achieved with oral therapy at the present time.

Materials and Methods:

1. The first 4 adult patients with clinical cholera arriving each day who are not required for other studies will be selected for study and assigned on an alternating basis to either the routine IV therapy or the oral glucose therapy group. (Patients selected for the routine IV therapy can also be included in the Acetate-Bicarbonate Study). Requirements for inclusion in the study are:

   (i) positive dark-field

   (ii) absence of obvious complications (e.g. coma, fever, etc.);

   (iii) shock of less than 2 hours by history and a history of passing urine within 8 hours prior to admission or a normal creatinine;

   (iv) a normal chest X-ray (chronic lung disease or cardiac disease should exclude a patient from the study). A normal creatinine or chest X-ray is not necessary to begin the study, but by the third hour of study these should be done and found to be normal.

2. All patients, whatever their group, receive routine intravenous rehydration on admission.

3. As soon as a patient has been selected for the oral therapy group, a thin polyvinyl tube with a mercury balloon at the end and an infusion port just proximal to the balloon is swallowed and allowed to pass to 50 cm. from the balloon marker. An X-ray is then taken to confirm the position (the chest X-ray is taken at the same time) and the infusion is begun.

4. The oral infusion fluid will contain BSP to dye it blue and thus distinguish it from IV fluid. All bottles and tubing used for oral infusion must be clearly marked to eliminate the danger of giving oral fluid by vein mistakenly. All oral fluid must be analyzed before administration ~~(although this can be done for 20 liter batches and the~~ administration bottles filled on the ward).

5. Oral infusion will be given by drip at the rate of 1,000 ml/hour (250 drops/minute) for the 1st 8 hours. The infusion solution will be warmed to body temperature before infusion.

6. Careful record of all intake and output will be taken hourly and summarized 4-hourly. Weights will be taken at the beginning of the study and every 4 hours. Blood will be drawn at the beginning of the study and 4-hourly for hematocrit, plasma protein, specific gravity, sugar, Na, K, Cl, CO_2 (and creatinine on the first blood only).

Urine, Na and K will be measured on 4 hour pooled specimens. Rectal catheter specimens will be taken under oil for CO_2, Na, K, Cl and sugar and pooled 4 hour stools will be measured for Na and K.

A doctor and nurse will attend the patients at all times. Vital signs (blood pressure, pulse and respiration) will be measured hourly. Temperature will be taken 4-hourly.

Any evidence of underhydration (weak pulse, decreased blood pressure, poor skin turgor, sunken eyes, poor urine output) will be treated by increasing the rate of IV infusion.

Any evidence of overhydration (puffy eyes, excessive weight gain /greater than 4 kg. positive balance/, fall in plasma specific gravity or excessive urine output) will be treated by slowing the IV and if this is not sufficient, by slowing the rate of oral solution to match net output.

After the initial eight hours of infusion at 1000 ml per hour, the infusion rate will be cut to 500 ml/hour. During the third 8 hour period the oral infusion rate will be cut to (250 ml per hour plus) the average net stool output over the last 4 hours.

With the beginning of the fourth eight hour period, the oral infusion will be stopped if the net stooling over the previous 4 hours has been zero. If not the rate of infusion will be equal to the net output over the last 4 hours. From

this time onward the rate of infusion will be adjusted each 4 hours to the net output per hour of the previous 4 hours. Oral tetracycline capsules 250 mg every 6 hours will be begun when the oral infusion stops.

Careful monitoring of intake and output will be continued until the patient has been off oral infusion for 24 hours without purging. The patient will be followed until the seventh hospital day for bacteriologic relapse.

During the early phase of this study a physician and nurse must attend the patient at all times. Later it may be possible to have the nurse monitor the patient alone, with hourly checks by the physician, although he must always be readily available on the ward.

The physician responsible for the patient will examine the chest frequently and a chest X-ray will be done at the end of the study.

Control patients will have intake and output monitored in the same way as the patients receiving oral infusion.

PROTOCOL

BALANCE STUDIES IN PEDIATRIC CHOLERA PATIENTS

Drs. M.M. Rahman, K. Hare, W. Rahman and R. Hare.

OBJECTIVES:

1. Determine the relationship between the requirement of intravenous fluid and the plasma protein concentration in children.

2. Study the effects of controlled dehydration on the vascular and other fluid compartments of the body in children.

3. Study the effects of infusion 5/4/1 solution on the electrolyte levels (including the levels of Ca^{++} and Mg^{++}) and fluid balances in children.

BACKGROUND

Most of the balance studies in cholera have been limited to the adult age group, although in East Pakistan the disease is more common in children. In most hospitals children have a higher rate of mortality compared to adults. In many centers the amount of fluid required for replacement in children is calculated on the basis of adult formula (Phillips et al 1946) although this has never been adequately tested in the pediatric age group. There are reasons to believe that children do not respond to dehydration in the same way as adults. It has been seen that the average specific gravity and the range of specific gravities of blood or plasma of dehydrated children on admission is appreciably lower than in adults. It is possible that the extracellular compartment of children, being proportionately

larger than that of adults, is able to contribute more to the stool volume. It is also possible that some fluid may be drawn from the intracellular compartment.

It is easy enough to study the blood or plasma volume during the acute phase of the disease, and if this can be combined with the measurement of the vascular as well as extravascular fluid compartments during convalescence, it should be possible to answer some of the questions above.*

The 5/4/1 solution is used at the Laboratory for all cases of cholera and diarrhea irrespective of age. Griffith (Lancet, June 3, 1967) has suggested that this solution and other similar isotonic solutions may be too high in sodium for use in pediatric cases. He has observed a syndrome-complex in Filipino children characterized by fever, tachycardia, vomiting, anuria, convulsion and death. He attributes this to a state of "free" water deficit caused by infusion of solution containing more sodium and chloride than actually lost through stooling. He has also found that children lose more potassium than adults. The 5/4/1 solution used in children in Dacca does not seem to give rise to the syndrome described by Griffith, but we have noticed lethargy and abdominal distension in a substantial number of children, which may or may

* The study of extracellular (as estimated by bromide dilution) and total body water volume needs to be carried out during convalescence because the time required for equilibration of the bromide and alcohol may allow substantial losses of these materials in the stool. If the vascular volume in the acute and convalescent phases is known and if the chloride space (measured during convalescence) is known, the contribution of extracellular fluid to the plasma volume could be calculated theoretically.

not be due to hypokalemia. It is hoped that this study will show if the Dacca children are different from their Filipino counterparts.

With the commissioning of the Perkin-Elmer Atomic Absorption spectrophotometer, study of the Ca^{++} and Mg^{++} balance has been simplified. The 5/4/1 solution does not contain either of these cations (although some commercial solutions do). It would be interesting to see the extent of loss of these elements through stooling and urine, and the effect of infusion Ca^{++} and Mg^{++} - free solution (5/4/1) on their blood levels. Very little extra effort will be required to incorporate this investigation in the balance study.

PROCEDURE:

Subject: Any child more than 6 months of age admitted to the PSCRL ward between 8-30 a.m. to 4 p.m. on week days will be included in the study. The child should give a history suggesting cholera confirmed by dark-field examination.

METHOD:

1. Admission - The child will be weighed and a brief examination of the vital signs including an estimate of the degree of dehydration will be made. An 8 cc sample of arterial blood will be drawn in a heparinized syringe. About 1.5 cc will be placed in a heparinized (green) vial, 1 cc in a fluoride (red) vial and the rest, 5.5 cc, will be left in the syringe for the estimation of blood pH and blood chemistry.

Plasma volume will be measured by injecting T-1824 through one vein and taking a sample of blood from a vein in the other arm after 5 minutes. 2 cc of blood in a heparin-containing tube should be adequate for T-1824, Hct, Hgb and Sp. Gr.

I.V. will be started with 5:4:1 (without glucose) and the amount to be administered will be calculated on the basis of clinical signs of dehydration. A 10 cc sample of stool will be taken through a rectal catheter and placed under oil; then a device will be fitted so that all the stool can be collected in polythene bags. A pediatric urine collector will also be applied.

A detailed history will be recorded with particular emphasis on the exact time of onset of diarrhea and of intake of fluids by mouth or injection. An electro-cardiogram will be made during this examination.

2. After Initial Rehydration - The child will be weighed and a sample of 7 cc of arterial blood obtained for all analyses except for antibodies. A 10 cc sample of stool will be obtained under oil at the same time.

3. Eight, sixteen and twentyfour hours after admission

Initially the studies will be limited to 8 hours and gradually extended to 16 and 24 hours and ultimately up to 3 days .

The clinical condition will be revaluated at the end of 8,16 and 24 hours with records of body weight, urine and stool output, and fluid input. Aliquots of pooled stool (as well as spot samples under oil) and urine will also be sent for examination

every 8 hours. A 7 cc sample of arterial blood will be collected in the usual manner and sent for examination.

At the end of 24 hours, tetracycline 125 mg, 6 hourly for 5 days will be started and diet added as tolerated. The patient will be sent to the ward.

4. Convalescent period.

On the 3rd day after the discontinuation of input/output the patient will be weighed and a blood sample obtained. On the same day the following studies will be carried out. (Details are given in the protocol on Body Composition - CI/P6).

1. Plasma volume - by T-1824 dilution.
2. Chloride space and total body chloride - by the measurement of bromide space and serum chloride.
3. Total body water - by ethanol dilution.

5. Sibling study

Parents will be asked to bring the siblings of the patient to the hospital. A 5 cc sample of blood will be taken from a vein; rectal swabs will be collected for culture and darkfield examination.

LABORATORY DETERMINATION

1. Blood: Admission sample - 1.5 cc for Hct, Hgb, plasma specific gravity (total protein from blood specific gravity) 1 cc for glucose and 1.5 cc for titer. The rest will be used for pH, CO_2, Na^+, K^+, Cl^-, Ca^{++}, Mg^{++}, osmolarity. The rehydration, 8, 16, 24 hours and convalescent specimens will not include titer. (The rehydration

sample will include 1.5 cc for creatinine estimation).

Blood sample (5 cc) from siblings will be submitted for Na^+, K^+, Cl^-, Ca^{++}, Mg^{++}, plasma specific gravity and haematocrit and creatinine.

2. Stool: The four rectal catheter spot samples will be submitted for Co_2, Na^+, K^+, Cl^-, Ca^{++}, Mg^{++}, and osmolarity. The three aliquots of 8-hour pooled stool will be submitted for Na^+, K^+, Cl^-, Ca^{++}, Mg^{++}. (Water content of stool will be measured by drying a sample to constant weight at $104^{\circ}C$ in an oven).

3. Urine: The aliquots from the 8 hour pooled collections will be analysed for Na^+, K^+, Cl^-, Ca^{++}, Mg^{++}, osmolarity and creatinine. Volume and water content of urine will also be measured.

4. I.V. Fluids: 5 cc sample from each bottle will be sent for Na^+, K^+, Cl^- and Co_2 estimation.

PROTOCOL

STUDY OF BODY COMPOSITION OF CHILDREN IN EAST PAKISTAN

M.M. Rahaman, K. Hare and R. Hare.

Diarrhea constitutes the most important cause of morbidity and mortality in the pediatric age group in East Pakistan. Any severe diarrhea causes loss of water and electrolytes from the body leading to disturbance of the fluid spaces which is dramatic in cholera. Higher mortality in children suggests that they do not tolerate dehydration as well as adults. A study of body composition in children involving the fluid and cellular volumes is therefore proposed for the coming season. Anthropometric measurements like height, weight and skinfold thicknesses, yielding data which have some relation with the body composition will also be incorporated in the study. No information regarding body composition in children is available in Pakistan. Because of their relatively poor nutritional status, the various spaces are likely to be different from those in other countries where information is available.

The protocol on body composition has been divided into two parts. The first part is the study of various parameters in apparently "healthy" children to provide base line data for comparison. By necessity this group will have to be chosen from those admitted in the hospital and convalescing from an attack of acute diarrhea but otherwise "healthy".

The second part of the investigation will be part of the "balance" study proposed to be carried out in acute cholera (See Protocol CI/P5).

MATERIALS AND METHODS:

Subjects: Any apparently "healthy" children free from obvious signs of malnutrition and disease will be taken as subjects. They should be convalescing from an attack of acute non-cholera diarrhea and be free from it for at least 4 days before the study is carried out. Any child below 5 years of age will be included. Every attempt will be made to establish the correct age of the child. Total body water, bromide space and plasma volume will be measured.

Total Body Water: This will be measured by ethanol dilution. This alcohol is rapidly absorbed and distributed uniformly throughout the fluid spaces (Harger, Hulpein & Lamp, 1937, Eggleton, 1942). Its rate of metabolism is constant in any given individual and is unaffected by concentration (Kalant, 1962, and others). Gruner (1957) and Gruner and Salmen (1961) reported its use in man for the measurement of total body water.

Bromide Space: Bromide space will be measured by bromide dilution by the method of Finlay and Hare. The volume of distribution of bromide is the same as that of chloride which is chiefly extracellular.

Plasma Volume: The plasma and total blood volume will be estimated by T-1824 (Evan's blue) dilution and hematocrit determination.

All the analytical methods have been developed into micro-techniques so that 0.2 ml of plasma will be sufficient for the estimation of each of the above parameters. In all therefore,

0.6 ml of plasma or a little more than 1 ml of whole blood should be enough for the complete determination of all the fluid spaces.

Technique: After overnight fasting (except for water) the child will be taken to the study room. A sample of control blood (2.5 ml) will be obtained by the following technique:

A measured volume of ethanol as 15 per cent solution mixed with sodium bromide solution and T-1824 will be taken up in a syringe and injected slowly. After exactly 5 minutes a sample of blood will be taken from another vein and used for T-1824 estimation. Two more blood samples will be drawn at the end of the second and third hour and will be used for the estimation of ethanol and bromide. The subject will be given a meal afterwards and discharged from the hospital.

In children with acute cholera, probably only plasma volume (T-1824) can be estimated successfully during the initial phase. As soon as I.V. fluids can be discontinued, all measurements will be made. When the patient is completely recovered, the study will be repeated.

PROTOCOL CI/P7

STUDY OF NUTRITIONAL STATUS OF CHILDREN IN CHOLERA AND OTHER DIARRHEAS

by

M.M. Rahaman, S.G. Rahman, K.S. Haque and K.Hare

The recently published report of the nutrition survey has shown that the population of East Pakistan suffers from a number of severe nutritional deficiences. The most important amongst these are deficiencies of calories, protein, vitamin A, riboflavin and ascorbic acid. Anaemia is also extremely common. East Pakistan is also the endemic focus of cholera. The relationship between malnutrition and susceptibility to cholera in children has not been extensively investigated. It is well known that malnutrition increases the risk of mortality many fold in any kind of diarrhea and children are the worst sufferers from malnutrition in this country. They also constitute the majority of admissions into the PSCRL hospital and mortality is higher in children than in the adults.

The relationship between malnutrition and increased susceptibility to cholera has been the subject of two recent publications from this Laboratory. Rosenberg et al (1966) investigated ascorbic acid, thiamine, vitamin B_{12} and folic acid nutrition in a group of patients suffering from cholera and compared those with patients suffering from other diarrheal diseases. No difference was detected between these two groups. Similarly Khan and Stockard (1967) found no difference in susceptibility to cholera in family contacts treated with ascorbic acid supplements. However, these two studies did not investigate the more important of the nutritional deficiences i.e.,

.....con'd

deficiency of calories, protein, vitamin A, and riboflavin.

Increased mortality in diarrhea associated with severe malnutrition is presently a subject of intense research throughout the world. Due to lack of or unreliability of some vital physical and biochemical data in the malnourished children admitted to this hospital it is almost impossible to assess their nutritional status in retrospective manner. This has made it impossible to compare the rate of mortality in this hospital with that of other centers. Considering these circumstances it was planned to carry out a long term study of the nutritional status of the children admitted to the PSCRL hospital.

MATERIALS AND METHODS

Subjects: All children below 5 years of age admitted to the hospital.

Methods: The admitting doctor will assess the nutritional status and record the findings on the attached form. He will prescribe the required treatment except vitamins or antibiotics fortified with vitamins. The child should be kept NPO (except for water) until blood and the first urine sample is collected. In case of suspected keratomalacia, vitamin A injection must immediately be given as this constitutes a medical emergency.

.....cont'd

On rehydration (the following morning, in the case of those admitted after 4 p.m.) a detailed physical examination for the usual signs of malnutrition will be carried out. A blood sample will be taken and a complete blood count done along with estimation of Hgb. and Hct. Plasma electrophoresis will be carried out along with estimations of total protein Ca^{++} and Mg^{++}. The rest of the sample will be frozen for future analysis of carotene and vitamin A (retinol). A sample of the first urine will be preserved for analysis of riboflavin and creatinine.

CI/P7

ASSESSMENT OF NUTRITIONAL STATUS

CRL #: Age: Sex: Siblings:

Father's profession: Appearance of attendant: ☐ Clean ☐ Fair ☐ Dirty ☐ Very Dirty

DIET
- ☐ Breast milk with mixed feeding
- ☐ Cow's milk (fresh) mixed feeding - quantity
- ☐ Dried milk Brand - quantity
- ☐ Adult diet

PHYSICAL Height ☐ Admission — cm. ☐ Discharge — cm. Weight ☐ Admission — kg. ☐ Discharge — kg.

SKINFOLD Biceps: Triceps: Sub-scapular: Suprailiac:

HAIR
- ☐ Dull
- ☐ Dyspigmentation
- ☐ Thin & sparse
- ☐ Flag sign

FACE
- ☐ Pallor
- ☐ Nasolabial dyssebacea
- ☐ Moon face

LIPS
- ☐ Angular stomatitis
- ☐ Angular scars
- ☐ Cheilosis

EYES
- ☐ Xerosis conjunctivae
- ☐ Keratomalacia
- ☐ Bitot's spots
- ☐ Corneal vascularization
- ☐ Conjunctival or circumcorneal injection

TEETH
- ☐ Mottled enamel
- ☐ Caries

GUMS
- ☐ Spongy or bleeding
- ☐ Pyorrhea

TONGUE
- ☐ Scarlet or raw "beefy"
- ☐ Ulcers
- ☐ Fissures
- ☐ Atrophic papillae

THYROID
- ☐ Enlarged
- ☐ Visible
- ☐ Palpable

SKIN
- ☐ Xerosis
- ☐ Folliculosis and hyperkeratosis
- ☐ Petechiae
- ☐ Ecchymoses
- ☐ Pellagrous dermatoses

SUBCUT TISSUE
- ☐ Oedema
  - ☐ Face
  - ☐ L.Limbs
- ☐ Fat
  - ☐ Normal
  - ☐ Increased
  - ☐ Decreased

(more)

ABBREVIATED FORM FOR THE ASSESSMENT OF NUTRITIONAL STATUS.

Healthy	Apathetic
Wasted	Irritable
Severely wasted	
Wasted with Oedematous legs	Photophobia
Angular stomatitis	Dry conjunctiva
Ulcerated lips	Bitot's spot
Ulcerated tongue	Hazy corner
	Infection
	Blind

PROTOCOL

A STUDY OF PROTEINS OF THE CHOLERA STOOL

Robert S. Northrup, M.D.

Introduction

Although the electrolyte and water contents of the cholera stool have been investigated in great detail, until very recently the proteins of the cholera stool were noted only for their relative lack. Previous studies have indeed shown the protein content of the cholera stool ranged from 0.250 to 2.13 gm per liter. During the previous season, however, in Dacca studies of the protein in the cholera stool were carried out in more detail, with the intention of further analysis of that protein which is present. Through the efforts of Drs. Crabbe' and Mosley, it was found that the cholera stool contained a large amount of immunoglobulin, in particular, much of which was IgA. IgG and IgM were found in much smaller quantity, in reverse proportions to their concentration in serum.

Studies carried out in other laboratories, working with a large variety of physiologic secretions of the normal and abnormal host, have demonstrated a large variety of proteins in secretions. Although the immunoglobulins, with their role in protection and prevention of disease, are of course the major protein components of interest in the cholera stool, it is planned to examine and describe the other proteins of the cholera stool as well, comparing them with proteins from other physiologic secretions. Resistance to or modification of the course of cholera may very well be related to variations in proteins other than the immunoglobulins. Semi-quantitation of these other proteins will help to determine whether or not a difference exists between the protein excreted during cholera and in the normal individual.

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In reference to the immunoglobulins in particular, further characterization of the type of protein secreted into the intestine is of great importance. Much attention is currently being accorded to the presence of the so-called"secretory piece"on IgA secreted into the intestine and saliva. This is of great importance in relation to infection, in that there is some disagreement at the present time as to whether antibody is secreted into the intestine from the serum, or produced locally in the plasma cells of the intestinal mucosa. Burrows showed (1948 - J. Inf. Dis.), in studies of cholera in guinea pigs, that passive immunization with hyperimmune serum could in fact result in the excretion of antibody into the intestine of the guinea pigs. Proof of the presence of "secretory piece" on intestinal IgA, making its sedimentation constant 11S rather than 7S, will suggest that indeed the IgA found in intestinal secretions is more than likely produced locally. Thus we plan to further characterize the IgA of the cholera stool, both through the actual measurement of its sedimentation constant and also through comparing antisera directed specifically against serum IgA, secretory IgA and secretory piece.

Quantitative studies of the secreted immunoglobulins are also of great interest. Studies done here last season by Dr. Crabbe' and Dr. Mosley showed that approximately 200 mg of IgA were secreted into each liter of stool from the average cholera patient. Thus, if the cholera patient were to pass 10 to 15 liters of stool, a not uncommon occurrence in an active patient, he would excrete 2 to 3 gm. of IgA in the stool in a day. Previous studies using labelled IgA in humans suggest that the turnover for this immunoglobulin is between 2 and 3 gm. per day (Tomasi, 1965). Thus it is highly interesting to determine in a number of patients just how much IgA is actually being excreted during the course of the disease and during early convalescence, and also determine precisely the

change, if any, in serum levels of IgA. Our studies in the past have utilized antisera directed against serum IgA, rather than secretory IgA, in the quantitation of protein excretion. Since the diffusion of the heavier 11S secretory IgA would in fact be slower than the lighter 7S, serum IgA, I feel that quantitation of IgA can be made more precise by utilizing immunoplates prepared directly against secretory IgA rather than serum IgA. We thus plan to produce our own antiserum directed specifically against secretory IgA from the cholera stool, in order to solve this difficulty. It is hoped that these studies on IgA quantitation will lead toward more sophisticated studies in the future, using labelled immunoglobulins and, possibly, labelled aminoacids, to study actual turnover of IgA in the cholera patient.

Preliminary studies carried out on cholera stool during August and September 1967, at the laboratories of Dr. J. F. Heremans and Dr. T. Tomasi, in Louvain, Belgium and Buffalo, New York, respectively, cast some doubt on the possibility of quantitatively comparing IgA or other immunoglobulin excretion day by day. Sephadex column chromatography and sucrose density gradient ultracentrifuge studies carried out on lyophilized cholera stool material show the presence of small immunoglobulin fragments in the stool as well as the full-sized molecules. These fragments are immunologically active as antigens for anti-immunoglobulin antisera, but are much smaller in molecular size than the parent proteins. The radial diffusion technique for measuring immunologically specific proteins is well known to be susceptible to changes in particle size. Thus the variation in size of the molecular population in the stool will very likely make strict quantitation by this technique invalid, because no suitable standard could be found for comparison.

Two possibilities exist for circumventing this difficulty. The transit time of the secretions through the bowel, that is to say, the time available for protein digestion by intestinal enzymes in vivo, can be shortened either directly by increasing the volume of stool by means of lavage with fluid or purging with an osmotic cathartic, or indirectly by taking secretions directly from the small intestine. Also, reduction of enzymatic digestion taking place after collection of the sample can be brought about by various techniques, including strict attention to temperature regulation, and the use of enzyme inhibitors. Work of Dr. A. Plaut from Bangkok suggests that short-term heating will reduce enzyme activity. These steps may make stool immunoglobulin quantitation valid. Work is being carried out at present to determine this.

Materials and Methods

Total stool collections will be made from cholera patients at various stages during the disease and early convalescence. The stools will be collected at 4 or 8 hourly intervals in buckets surrounded by ice and salt. Following collection, the stools will be mixed thoroughly and aliquots taken for further analysis. These aliquots will be measured carefully and centrifuged. They will then be concentrated by ultrafiltration either through negative or positive pressure, utilizing a semipermeable membrane. The concentrated product will be dialyzed and subsequently lyophilized. It will be stored in lyophilized state at -70°C. until analysis.

Non-liquid stools will be diluted with an equal volume of saline, mixed thoroughly, and filtered initially through gauze. Subsequently they will be spun in the centrifuge and the supernates removed. This supernatant will then be handled similarly to the supernatant from the stool centrifugations. All

separation operations will be carried out at 4°C. Serum samples will also be collected from the cholera patients daily, and stored frozen until analysis.

Standard methods will be utilized for the analysis of these concentrated stool samples. The analyses will be carried out in Dacca and also in Louvain and Buffalo. These will include the following techniques:

1. Paper electrophoresis with staining for protein by Bromphenol blue; for protein bound carbohydrate with the periodic acid schiff test and for lipopolysaccharide with Alcian blue:
2. High voltage electrophoresis in agar gel;
3. Immunoelectrophoresis against a variety of antisera prepared primarily in rabbits. These antisera will include antiserum directed against all human sera, antiserum specifically reacting against each of the three major human immunoglobulins, IgA, IgM and IgG, antiserum directed against human salivary proteins, human sputum and the secretions from villous tumors of the rectum, all of which will be obtained through the kindness of Dr. J. F. Heremans; and also antiserum directed against cholera stool prepared by injection of stool concentrates at appropriate intervals into rabbits, initially mixed with Freund's adjuvant.
4. Ouchterlony analysis (double diffusion) will be carried out, comparing precipatates produced by sera directed specifically against human serum IgA, human secretory IgA, and human secretory piece.
5. Quantitation of immunoglobulins will be carried out using the immunoplate technique of Fahey. Immunoplates prepared by Hyland Laboratories against serum IgA, IgM and IgG will be used, also immunoplates of our own manufacture using rabbit antiserum directed specifically against stool IgA and against

secretions. A modification of the radial diffusion technique will be employed to measure proteins for which no mono-specific antiserum can be obtained. Suggested by Heremans, this technique will enable comparison of secretory proteins in mixtures containing more than one protein, where two or three precipitin circles are produced in immunoplates. By combining a standard mixture (e.g., the first day stool sample) and an unknown in various proportions, and noting the effect of this on the sizes of the precipitin circles of the resulting combination, it will be possible to identify positively the circles of the unknown by itself. With this identification made, quantitation can be made by measuring the diameter or area of the circles in the usual manner.

In the event that proteolysis cannot be avoided, semiquantitation of immunoglobulins in the secretions will be performed using serial dilutions of the stool material in Ouchterlony against specific anti-immunoglobulin antisera. This technique, being less affectdd by particle size, will be more valid, although less accurate, than the radial diffusion technique in this case.

6. Chromatography with Sephadex and DEAE cellulose columns will be performed; the resulting eluted fractions will be tested for their protein constituents.
7. Analytical density gradient ultra-centrifugation will be carried out on some materials.

In this manner it is hoped that an increase in basic knowledge about cholera intestinal secretions in particular, and intestinal secretions in general, will be gained.

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PROTOCOL

SECRETORY PIECE IN CHOLERA STOOL

An Addendum to "Proteins of Cholera Stool" protocol

Robert S. Northrup, M.D.

Working with Dr. John Bienenstock, of the laboratory of Dr. Thomas Tomasi, in Buffalo, New York, it has been possible to complete one aspect of this study, that regarding the presence of secretory piece on the IgA of the cholera stool.

Stool was obtained from a number of cholera patients during the first or second day after admission to the hospital, and concentrated by negative pressure dialysis at 4 degrees C. The resulting stool pool concentrate was then lyophilized. After transportation of Buffalo, the material was resuspended in saline, and heated at 56 degrees C. for 30 minutes to reduce enzymatic activity. Ouchterlony analysis was then done with various antisera.

Using a rabbit antiserum specific for secretory piece, a precipitin band was produced which was identical with one produced by the antiserum against purified colostral IgA(Figure I). Further, using a rabbit antiserum directed against whole secretory IgA, reactions of identity were produced with both normal human serum and purified colostral IgA, with spurring of the precipitin bands from the stool material and the colostral IgA over the normal human serum.(Figure II). After chromatography of the stool material with Sephadex G-200, the same reactions could be observed with concentrated material taken from the first protein peak eluted from the column.

These reactions demonstrate the presence of secretory piece in the cholera stool, bound to IgA. Thus cholera stool has been shown to contain, like other secretions (tears, colostrum, saliva, etc.), gamma A protein immunologically different from serum IgA, so-called secretory IgA. Ultracentrifugal

analysis of the whole stool material suggests that IgA sedimenting at a rate of 11S is present; this observation can only be considered preliminary, however.

Figure I

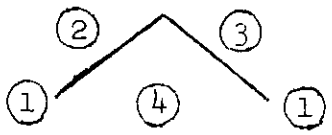
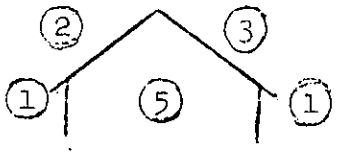


Figure II



1. Normal Human Serum.
2. Cholera Stool Material.
3. Purified Colostral IgA.
4. Rabbit Antiserum Specific for Secretory Piece.
5. Rabbit Antiserum Specific for Secretory IgA (IgA / plus piece).

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PROTOCOL

COMPARISON OF TECHNIQUES FOR COLLECTING SECRETIONS FROM THE NORMAL HUMAN INTESTINE

Robert S. Northrup, M.D.

Introduction:

Studies of intestinal protein secretions from normal and cholera-infected patients currently being carried out at the Pakistan-SEATO Cholera Research Laboratory frequently require the collection of specimens from patients who are secreting non-liquid stool. Various methods have been utilized in the past by other investigators for obtaining intestinal specimens under these conditions. The simplest of these is the collection of the formed stool itself. This is then mixed with saline, either a volume of saline equal to the weight of the stool, or a volume fixed previously, standardised for the time period under study (e.g. 200 cc of saline mixed with the stool from a 24 hour collection period). Studies have suggested that for antibody determinations (Freter, 1962) this method may be misleading, in that the antibody character of the secretions of the upper intestine frequently is lost during passage through the intestine and storage in the large intestine before excretion. A second technique, designed to overcome this objection, is to collect secretions, by means of a suitable collection capsule or tube, directly from the small intestine itself. This technique has been used widely, and is not particularly objectionable to the subject under study. It has the disadvantage, however, that it produces specimens that represent only the secretions of the upper intestine alone, in contrast to the liquid secretions of the cholera patient, our primary object of interest, which represent the total intestine. Thus a third technique has been evolved and

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utilized by other investigators in the past. This involves the artificial production of diarrhea by use of an osmotic laxative such as magnesium sulphate (Epsom salts). The results of this approach, however, in some workers' hands, have suggested that a very high concentration of the osmotic agent or perhaps the magnesium ion itself, alter the proteins or their mechanism of secretion into the intestine, such that the resulting secretory specimen does not represent normal intestinal material, but may contain protein derived from serum (Crabbe' - personal communication). We found, during the previous cholera season, that mannitol delivered in a large volume of isotonic electrolyte solution (25 gm. mannitol in 1 liter 5:4:1) was very effective in producinga liquid diarrhea of equal or slightly greater volume than the administered fluid. It is proposed, thus, to study these three techniques from the point of view both of the character of the protein secretions, and also of their antibody content.

Materials and Methods

Convalescent cholera patients and normal patients will be utilized for the study. The patient will be intubated. He will then be allowed to pass a normal formed stool. This will be collected and preserved at 4°C. while the rest of the procedure is being carried out. Following this, by injecting small quantities of saline through the tubing into the small intestine and subsequently withdrawing into a syringe, a sample of secretion will be obtained from the jejunum and immediately put on ice. The position of the collecting tube will be ascertained precisely by means of X-ray. Following the collection of the intestinal specimen, the patient will be given a liter of 5:4:1 electrolyte

solution diluted in half, containing 25 gm. of mannitol, to drink over a 30 minute period. All stool following this time, except for any definitely formed specimens passed individually, will be collected until the patient spontaneously stops purging. These specimens will be collected on ice.

The formed stool specimen obtained intitially will be weighed and an equal amount of saline will be added to it. It will then be mixed thoroughly, with complete breaking up of any individual particles contained in the mixture. The mixture will then be maintained at $4^{o}C$ for 2 hours, after which it will be filtered through gauze. The filtrate will be centrifuged at 10,000 rpm (11,900 g.) at $4^{o}C$ for 20 minutes and the sediment discarded. The supernate will be concentrated using ultrafiltration through a semipermeable membrane, after which it will be dialyzed against saline at $4^{o}C$ for 24 hours. The dialyzed material will be lyophilized to dryness, in which state it will await further analysis.

The specimens obtained directly from the intestine will be centrifuged, concentrated by ultrafiltration, dialyzed against saline and lyophilized.

The specimens produced by mannitol lavage will be filtered through gauze, centrifuged, concentrated by ultrafiltration and lyophilized.

Using antisera directed against cholera stool, comparative immunoelectrophoresis will be performed on each of the specimens, noting the presence or absence of additional lines in one or the other. By dilutions, Ouchterlony and immunophoretic "titers" will be compared using the anti-stool serum.

Quantitation of the immunoglobulins present in each of these specimens will be performed, using the immunoplate technique and also serial dilutions in Ouchterlony analysis. Immunoplates prepared in Hyland laboratories will be used, containing antiserum directed specifically against each of the 3 human immunoglobulins, IgA, IgM and IgG. Immunoplates made in our Laboratory will also be utilized, incorporating antiserum directed specifically against the secretory IgA of cholera stool. The method of preparation of this antiserum is contained in a separate protocol. Quantitative analysis for albumin will also be performed in this manner.

From a suitable dilution of the lyophilized material from each specimen, the immunoglobulins will be precipitated using 50% saturated ammonuium sulphate. This precipitate will be redissolved in saline, and the resulting solution studied for antibodies. The original solution of non-precipitated lyophilized material will be studied and the results compared for titer (the two solutions will be adjusted to be of approximately equal immunoglobulin content). The antibody in the specimens will be titered using agglutination of heat killed vibrios, or hemaglutination of red cells sensitised with an appropriate vibrio antigen (studies are currently underway to determine which antigen is most suitable for this purpose) or perhaps other techniques. It may also be possible to compare antibody levels in the specimens by Ouchterlony precipitation analysis using various dilutions of the specimen solutions and immunoglobulin solutions for titration. The Farr test may be utilized.

The results of all determinations will be expressed in terms of the total protein content of each of the specimens, as determined both by Lowry analysis and by ultraviolet absorption at 280 µm.

PROTOCOL

RADIOIMMUNOELECTROPHORETIC ANALYSIS OF HUMAN SERUM AND STOOL FOR V.CHOLERAE-BINDING ANTIBODY

Robert S. Northrup, M.D.

In 1962, Morse and Heremans (1), using a technique which they called radioimmunoelectrophoresis studied the serum from a group of diabetics receiving insulin by injection. With this technique they were able to demonstrate the presence of a substance in the serum of these patients which bound insulin, as would an antibody, even though they previously had not been able to demonstrate a precipitating reaction with "immune" serum and insulin. They were also able to demonstrate that this insulin antibody was indeed IgG globulin and was not IgA or IgM. Further, they found that their analysis was semiquantitative, through noting the varying amounts of time necessary to produce lines on the autoradiographic film. This amount of time correlated well with the degree of insulin resistance demonstrated clinically by the patients.

Since then this technique has been used effectively to demonstrate antithyroglobulin antibodies (2), polio virus-binding immunoglobulins in the serum of immunized humans (3) and, very recently, IgA polio antibody in saliva, duodenal fluid and urine (4), The advantages of the technique are as suggested above: precise localization of a particular antibody to one of the three types of immunoglobulin, IgA, IgM or IgG; the ability to demonstrate an antibody which by other techniques cannot be demonstrated; and the ability to semiquantitate the analysis.

Previous work in this laboratory by Mosley and Ahmed has suggested that coproantibody to V.cholerae may be in large part IgA immunoglobulin

I expect by this study to confirm their findings using this very sensitive technique, and also to quantitate in a rough way the coproantibody response during the typical course of cholera. I also expect that this technique will show us which antigen is most strongly bound by stool IgA. This antigen will then most likely be suitable for our immunohistologic work.

Materials and Methods.

Specimens: Patients with bacteriologically proven cholera will be studied. Patients with diarrhea but without demonstrable Vibrio cholerae in the stool will be used as controls. Some patients will be treated with tetracycline, to remove vibrios from the stool; tetracycline does not affect antibody response to clinical cholera but vibrios and vibrio products in the stool may absorb coproantibody. The stool will be collected on ice during the course of diarrhea and in early convalescence. Liquid stools will be filtered through gauze to strain out large impurities and then centrifuged at 10,000 rpm for 15 minutes at 4°C. The resulting supernate will be divided in half. The first half will be concentrated by ultrafiltration using negative pressure dialysis, and dialyzed against distilled water in the cold for 24 hours. To the other half, saturated ammonium sulphate will be added, bringing the concentration in the stool to 50% saturation and precipitating the immunoglobulins. This mixture will be allowed to stand overnight in the cold and will then be centrifuged. The precipitate will be dissolved in saline, then reprecipitated with ammonium sulfate. It will then be redissolved with saline and dialyzed against saline for 36 hours in the cold. These two solutions of the material will be used

for the analytical procedure.

Softer formed stools will be diluted with an equal weight of saline, thoroughly broken up and mixed, and allowed to stand for one hour in the cold. The mixture will then be strained through gauge, and centrifuged at 10,000 rpm for 15 minutes at 4°C. The resulting supernatant will be divided in half and handled as the liquid specimens described above.

Serum will be collected from the patients concomitantly with the stool collection, and analysed as such.

Antigens: A variety of vibrio antigens have been prepared and will be tried in the system. The techniques for preparation include sonication alone, sonication with urea, phenol extraction, extraction with diethylene glycol, extraction with heat and acid, and extraction with ether. Precipitation tests against immune human convalescent serum and hyperimmune anti-vibrio goat serum suggest that the lipopolysacchride phenol extract and the urea sonicate might prove the most effective. One stool has produced precipitation with the sonicate. Protein content will be measured in each by the Lowry technique.

Labelling of Antigen: The antigen to be used will be labelled using a technique similar to that described by Bale et al[5]. Briefly, Iodine-125 will be used as the labelling agent. The antigen to be used will be dialyzed against phosphate buffered saline pH 8.1. A stock solution of iodine chloride is prepared, 2.0 molar in sodium chloride, 0.02 molar in potassium chloride and 1.0 molar in

HCl (see 5). It is planned that approximately 4 molecules of iodine will be conjugated with each molecule of antigen. The I-125 solution will be buffered with the buffered saline, pH 8. A calculated amount of iodine chloride solution and the I-125 solution are then added to the buffered antigen solution with rapid and vigorous mixing of the three solutions. One minute later a 6.25% solution of bovine serum albumin is added to the solution of iodinated antigen to protect against radiation effects and the destruction of protein in dilute solutions. The iodinated antigen is then dialyzed against buffered saline to remove unbound I-125.

Radioimmunoelectrophoresis procedure: For the analytic procedure it is necessary to have on hand suitable antisera reacting specifically with each of the three human immunoglobulins. Antiserum reacting specifically with IgA, IgM and IgG will be obtained from Hyland Laboratories and, after suitable testing to ensure purity and specificity, will be employed for this step. Using standard techniques, the material to be tested (concentrated stool, immunoglobulin precipitate from stool or serum) will be electrophoresed in agar for 20 to 30 minutes at a current of 100 ma in veronal buffer. To the trough will then be added the various antisera, anti-IgA, anti-IgM, anti-IgM or anti-whole human serum. These will be allowed to diffuse overnight in the cold, after which non-precipitated protein will be removed by washing with saline for over 24 hours. The labelled antigen under study will then be applied to the trough for another 24 hrs.

period, following which the slides will be washed to remove any unbound antigen.

The autoradiograph will then be made from the slide. This will be done in two ways. In order to obtain a general idea of the amount of label present on a slide, X-Ray film will be applied to the slide and after appropriate intervals (2 days, 4 days, 6 days, 2 weeks etc.), will be developed to ascertain if a line is present. In similar fashion, stripping film designed specifically for autoradiogrphy will be applied to a duplicate of the section to which X-Ray film has been applied. After development of the X-Ray film has shown that exposure has been sufficient, the slide with stripping film will be developed and the autoradiographic pattern observed. Records will be kept by photographing the slides after each step.

Using the above-described techniques, it is expected that we will be able to demonstrate the presence of binding activity for the vibrio antigen in the precipitated immunoglobulin lines of the immunoelectrophoresis, in particular, we expect, in the precipitated IgA.

Reference.

1. Morse, J. H. and Heremans, J.F.: J.Lab. Clin.Med.,59:891,1962.
2. Goodman, H.C., Enum, E.D. and Robbins, J.: J.Immunol.92:843,1964.
3. Ainbender, E. el al: Proc.Soc.Exp.Biol. and Med.119:1166,1965.
4. Berger, Ainbender, et al: Nature 214, 420, 1967.
5. Bale et al: Proc. Exp.Biol. & Med. 122: 407, 1966.

PROTOCOL

IMMUNOHISTOLOGY STUDIES IN CHOLERA - 1967-68.

Robert S. Northrup, M.D.

During the 1966-67 cholera season in Dacca, preliminary studies of the jejunum and ileum from the immunohistologic point of view were carried out. These were of two general sorts. The first, carried out in collaboration with Dr. Paul Crabbe, was a study of the immunoglobulin-producing plasma cell population of the jejunal and ileal mucosa. This study, which is currently being completed, showed that the native Bengali mucosa is similar to mucosa of populations elsewhere in the world; i.e. the number and relative proportion of plasma cells producing each of the three types of immunoglobulin, IgA, IgG, IgM, are approximately the same as in other areas of the world, with a predominance of IgA-producing cells. The second was an investigation into the antibody-producing character of the mucosal plasma cells; it is with regard to this area of investigation that this protocol has been developed.

During the 1966-67 season, preliminary studies were carried out using an indirect fluorescent labelling technique to label the antibody-producing plasma cells. It is planned to continue these investigations, using a wider variety of antigens and antisera with the same technique, and also to utilize other techniques, in particular, direct labelling of the antigen, to find the answer.

Methods

Adult cholera and non-cholera patients will be utilized for the study. These patients will be treated with replacement fluids; some will be treated with tetracycline on the possibility that vibrio

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antigenic products diffusing into the mucosa from the intestine may block further indirect labelling. Tetracycline would, of course, by killing off the vibrio population of the intestine, remove these antigens. A biopsy will be obtained from the small intestine, snap frozen on the cryostat chuck, and sectioned. The slide on which the section has been placed will be fixed for three minutes in methanol, after which the unlabelled antigen to be used will be applied. The antigen solution will be incubated with the section for thirty minutes, after which the slide and section will be rinsed in phosphate buffer. Following this, a labelled antiserum to the antigen will be applied to the section, and similarly incubated for thirty minutes. After incubation non-absorbed serum will be washed off in buffer, the section mounted, and the slide examined with the fluorescent microscope. In this manner it is planned to examine sections from the mucosa of patients at various times during the course of the disease and early convalescence. Sections will be obtained and examined similarly from non-cholera patients with diarrhea and also from normal patients. It is planned to test a wide variety of antigens and antisera for their haemaglutinating and precipitating properties prior to the season and in this manner obtain a variety of antigens and antisera, some of which, it is expected, will be effective in labelling the antibody-producing cells.

It is also planned to employ a direct labelling technique. Here the vibrio antigen will be labelled itself, either with fluorescein or Iodine-125. After the biopsy has been obtained and sectioned,

the labelled antigen will be applied to it and, after a suitable time period, the slide will be rinsed as previously described in phosphate buffer. In the case of fluorescent-labelled antigens, the section will then be examined under the fluorescent microscope. In the case of antigens labelled with I-125, a piece of stripping film will be placed over the section and the film plus biopsy and labelled antigen incubated for various time periods at $4^{o}C$. Following this the section and film will be developed and examined under the light microscope for the appearance of the typical silver grains on the film, over the antibody-producing cells which have taken up the labelled antigen.

It is hoped that positive results will be obtained, both with the indirect fluorescent labelled procedure and also with the direct autoradiographic technique. If the latter technique does produce good positive results, it is planned to utilize this for a double labelling procedure in the following manner. The fluorescent section will first be labelled with the I-125 labelled antigen. It will then be incubated with an antiserum specifically directed against one of the immunoglobulins, in particular anti-IgA, which has been labelled previously with fluorescein. In this manner it will be possible to demonstrate that a particular cell which has produced an antivibrio antibody is also one which produces IgA, IgM or IgG.

Positive findings in the mucosa of the intestine will be correlated with studies of antibody activity in serum and stool specimens obtained concomitantly with the mucosal biopsies. Total numbers of cells producing antibody thus will be correlated with

the amount of antibody which is being produced, and will also, it is planned, be correlated with total numbers of cells producing the individual immunoglobulins.

It is expected that large numbers of antigen and antisera may very well have to be studied in order to find that particular combination which will produce the desired results. The antigen and antiserum-producing techniques which are planned are described in a separate protocol.

Fluorescent antibody studies of bacteria are also planned. Here vibrios will be incubated with stool protein, then washed, after which labelled antiimmunoglobulins will be applied. Alternatively, vibrios in the cholera stool itself will be studied in the same manner for the presence of immunoglobulin coatings. Suitable controls, a very important item here, will be used. In this manner the absorption of antibody to the vibrio will be observed. Labelled anti-complement antisera, and incubation of the organisms with purified stool proteins, may help to determine whether complement has any place in coproantibody reactions.

PROTOCOL

PREPARATION OF ANTISERA FOR USE IN CHOLERA-RELATED IMMUNOLOGIC STUDIES

Robert S. Northrup, M.D.

Introduction

In performing various immunologic studies related to cholera infections during the 1967/68 cholera season, we will require in our Laboratory a variety of antisera. Among these antisera are antiserum directed against whole human serum, antiserum directed against the three serum immunoglobulins, IgA, IgM and IgG, and antiserum directed against IgA secreted in the cholera stool. This protocol is designed to set forth those techniques which will be used in the production of these antisera.

Materials and Methods:

Each antiserum will be prepared in at least two rabbits with the resultant product compared for antibody potency. This comparison will be done in two ways; the first using immunoelectrophoresis against the antigen, comparing the number and amount of precipitin lines; the second through incubating 0.5 ml. aliquots of the antiserum with varying quantities of the antigen in 0.5 ml. for one hour at 37°C. and then 24 to 48 hours at 4°C. The resultant precipitate will be washed three times with 1.5 ml. cold saline and finally dissolved in 2 ml. $\frac{N}{10}$ NaOH. The protein in this solution will be estimated by measuring the u-v absorption at 280 mμ. and by Lowry analysis. (See reference No.1).

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The technique for producing anti-whole human serum will be as follows: normal human serum will be diluted 25 times in saline. One day 1 22-25 c.c. of pre-immunization blood will be withdrawn from each animal to be immunized. Following this, 0.5 cc of the dilute normal human serum will be emulsified with 0.5 cc of Freund's adjuvant (Defco). This material will be injected in divided does into the hind legs of each animal intramuscularly. On day 8, the same dose of dilute serum and Freund's adjuvant will be used (1.0 ml.total); this will be injected intramuscularly into the hind legs as before and also intradermally in two sites on the back of the rabbit. A booster of the same dose will be administered on day 35 into the hind legs always with complete adjuvant and the antiserum will be harvested on day 42. Following this, the comparative studies between sera from different animals will be performed as described above.

Antiserum directed against stool is already in the process of being prepared by a method similar to the above. If more antiserum becomes needed it will be prepared in the following manner. A pool will be made of aliquots taken from various concentrated stool specimens. This will be millipore filtered and dialyzed against distilled water for 2 days at $4^{o}C$. Following this the protein content of the stool pool will be measured using the Lowry technique and the concentration of the stool will be adjusted to approximately 2 to 6 mg. of protein per ml. One half ml of this concentration-adjusted material will be emulsified with one half ml of Freund's adjuvant as described above. Following the obtaining of 22 to 25 ml.

of control blood, the emulsified antigen will be injected into the hind legs in a similar manner and schedule as described for the anti-whole human serum. Following the harvesting on day 42 of the resultant antiserum, an aliquot will be absorbed with normal human serum in order to precipitate antibodies directed against serum factors, leaving only antibodies directed specifically against stool antigens. This will be done in the following manner: to the rabbit antiserum, normal human serum lyophilized will be added. The two sera will be mixed thoroughly and allowed to stand for a few hours in the cold. The resultant precipitate will be removed by centrifugation following which normal human serum will again be added to the supernate. If a precipitate forms, this again will be centrifuged and the absorption procedure repeated until no further precipitate forms. Ouchterlony test will determine whether absorption has been complete. By this technique absorption will have been continued until no precipitating antibodies against human serum factors will remain in the rabbit antiserum.

Antisera directed specifically against IgG, IgM and both serum and stool IgA will be prepared in a somewhat different manner. (See reference 2.) A precipitin line consisting of the desired antigen combined with antibody will be obtained by immuno-electrophoresis. The antigen-containing material will first be electrophoresed. Then serum which contains a mixture of antibodies, including a precipitant appropriate to the desired antigen, will be placed in the longitudinal trough. Two days will be allowed for diffusion and development of lines of

precipitate. Following this the agar zone containing the appropriate Ag-Ab precipitate arc will be excised, care being taken to avoid contamination with other unwanted precipitin arcs. The tiny piece of agar will be washed in 20 ml.of physiological saline solution for 48 hours at room temperature to remove diffusable substances. Then the agar with its contained precipitate will be broken up in 0.2 ml. of physiological saline and emulsified in Freund's complete adjuvant in the ratio of 1:4. Control serum will be obtained from the rabbits. Then the antigen-adjuvant mixture will be injected into toepads and various intradermal sites of a rabbit. The same procedure will be repeated 10 days later. Two weeks after the second injection a serum sample will be taken which will be tested as described above for antibody level and specification. If sufficiently high titer is not present, the injection can be repeated.

Antiserum directed against stool IgA will also be prepared using stool IgA purified in another fashion. Here, using Sephadex and DEAE cellulose column chromatography,with suitable testing of the eluted fractions, the IgA in the stool will be obtained in a relatively pure preparation. This preparation will then be adjusted to approximately 2 mg. protein per c.c., emulsified with adjuvant as described above in the sections related to preparing anti-whole human serum and, after obtaining control serum from the rabbits to be used, injected intramuscularly, 1 c.c. of the antigen-adjuvant mixture being injected on day 1 and day 10 as described above. This will be absorbed after harvest with cord serum and purified γ-M obtained from Sephadex.

The purity of the anti-immunoglobulin antisera will be tested using immunoelectrophoresis and double diffusion in agar. Only those antisera producing a single line of antigen antibody precipitation will be utilized for investigative work. Less specific antisera will be improved by appropriate absorption.

References

1. Binaghi, R.A.: Journal of Immunology 97: 159, 1966.

2. Goudie, Horne and Wilkinson: Lancet, 1224, Dec. 1966.

PROTOCOL

ELECTRON MICROSCOPE STUDY OF SMALL BOWEL AND RECTAL BIOPSIES IN ACUTE CHOLERA AND NORMALS

A. Abraham and R. S. Northrup

The 32 small intestinal and 2 rectal biopsies obtained during the 1966-67 cholera season from 11 patients in acute and early convalescent cholera have been examined at the light microscopic level, using 0.5 micron, toluidine blue stained sections. Material from control and Vibrio cholerae inoculated rabbit ileal loops taken at 6 hours was also studied in the same manner. A block of the human material and control and positive blocks from the rabbit small intestine have undergone preliminary examination with the electron-microscope.

In both the rabbit and human material, the increased resolution made possible by the thin sectioning of the biopsies has brought into evidence an interesting finding not emphasised or noted previously by other workers. Many villi exhibit a striking separation of epithelial and subepithelial cells, extending from an intact capillary endothelium up to the desmosomes and tight junctions of the epithelial cells. This finding was noted by Dammin et al.(1) in the mucosa of experimentally infected guinea pigs. Diamond (2) and also Wheeler (3) demonstrated in gall bladder epithelium that this change was associated with rapid water movement across the mucosa, in their case water absorption. This finding in the

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human biopsies is of interest in view of the large movements of sodium and water demonstrated by Taylor et al. (4) in the cholera affected intestine. Careful search of the tissue blocks as yet unexamined with the electron-microscope is planned in order to further document this observation.

Other than this, the biopsies showed essentially the same appearance at the light microscopic level as biopsies studied by Gangarosa et al.(5) and Kent and Lindenbaum (6). They illustrate again that biopsies taken in early convalescence are not adequate for proper controls. As the muscosal pattern after one year of acute cholera is known to return to normal, as shown by Gangarosa et al.(7) in Thai patients, the biopsies obtained now from the same 11 patients would serve as acceptable controls. As some technical problems were also encountered, such as pressure artefacts, poor orientation etc. biopsies taken from a few patients with acute cholera are also required. It is planned to use isotonic glutaraldehyde and osmium tetroxide as fixatives and both EPON and Araldite as embedding media. This, in essence, would complete the morphologic study.

We have also planned to study further the rabbit intestine from the loop preparations. In order to elucidate the role of water movement in producing epithelial cell separation we plan to study experimental situations of rapid absorption and

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secretion of fluid. For absorption studies, after dehydrating the rabbit for a period of time loops will be constructed and large amounts of saline infused thereinto. Biopsies will be taken from the loops at varying times after water infusion. For a secretory situation, a well hydrated rabbit will be used. Loops will be constructed and a hypertonic mannitol solution placed into the lumen, after which biopsies will be taken at varying time intervals. It is also planned to repeat the vibrio-inoculated loop experiments of the past year, using the same methods planned for the human biopsies in order to ensure that artefact has not been responsbile for the above mentioned finding. It is also planned to use the lanthanum hydroxide method of Revel and Karnovsky to test the intactness of the tight junctions.(8)

We feel that these studies are essential to the completion of the studies begun last season (1966-68) on cholera histology at the ultrastructural level. When the interesting preliminary finding noted above is better studied and confirmed, further studies to depict precisely the phenomenon in human patients are certainly in order.

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REFERENCES:

1. Dammin, G.J., Benenson, A.B., Feldman, D., Formal, S.B., Goldstein, H.B., Merril, T.G., Sprinz, H. - Clinical and histopathologic correlations in acute diarrheal disease. Proceedings of the Cholera Research Symposium, Honolulu, Hawaii, 1965, page 205.

2. Diamond, J.L., Tormey, J.McD.- Role of long extracellular channels in fluid transport across epithelia. Nature, 210: 817 (1966)-

3. Wheeler, H.O.- Mechanism of fluid and electrolyte transport in the mammalian gall-bladder epithelium. Symposium on Cholera. Palo Alto, California, 1967 page 29.

4. Taylor, J.O., Sachar, D.B., Kinzie, J. and Phillips, R.A. Enhancement of net sodium and water absorption in acute cholera in humans. Symposium on cholera, Palo Alto, California, 1967 page 36.

5. Gangarosa, E.J., Beisel, W.R., Chanyo, E., Sprinz, H. and Pivorat, P. - The nature of the gastrointestinal lesion in Asiatic cholera and its relationship to pathogenesis: A biopsy study. Am. J. Trop. Med. 9: 125 (1960).

.....cont'd

6. Kent, T.A., and Lindenbaum, J. - Correlation of jejunal function and morphology in patients with acute and chronic diarrhea in East Pakistan. Gastroenterology, 52: 972 (1967).

7. Gangarosa, E.J., Sribhibhadh, S., Sorinz, E., Benyajati, C., Kundel, M.M., Beisel, W.R. and Wolfstead, M.E. Intestinal Pathophysiology of Asiatic cholera. SEATO Conference on Cholera, Dacca, East Pakistan, 1960, page 76.

8. Revel, J.P., and Karnovsky, M.J.- Hexagonal array of sub units in intercellular functions of the mouse heart and liver. J. Cell. Biol. August 1967, page C7

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CI/P16

COMPARISON OF VIRUS ISOLATIONS IN CHOLERA AND OTHER BACTERIAL DIARRHEAS AND NORMALS

A. Abraham

The role of viruses in diarrhea is still controversial. Most of the reported studies on the association of viruses with diarrhea were done without proper controls and usually with techniques not permitting the recovery of the whole spectrum of human enteroviruses. Ramos-Alvarez (1) reported significantly higher virus isolation rates in infantile diarrhea in Mexico, while Melnick's Laboratory found no significant difference in the isolation rate in their studies of children from Houston, Texas (2,3) and Karachi, West Pakistan (4,5). A recent review of studies from North America and Western Europe (6) could report no significant differences in viral excretion rate between diarrheal infants and control subjects. We found (7), in a co-operative study with the NAMRU III Laboratory, in children with and without diarrhea in Cairo, Egypt, U.A.R. that a significantly higher virus isolation rate was in the control group (95%) than in the diarrheal group (75%). Enteropathogenic E. Coli and Shigella bacteria were isolated from the stool of the latter group in much higher frequency than from the control group.

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No relationship of viruses to cholera was found in the two studies of this problem (8,9). The negative result is significant in spite of the faults of experimental design and the small numbers studied. Cholera in children offers a test of the hypothesis that enteroviruses are actually less frequently found in children with diarrhea, where the cause of diarrhea is a bacterial pathogen, than in the normal control population. In addition, this study would give information on the enterovirus infection in East Pakistan.

This proposal is submitted for consideration for the 1968-69 cholera season, by which time the expected enlargement of the virus laboratory in the Peter Bent Brigham Hospital could take care of the specimens. The size of the study groups should be about 200 children in each group. Multiple stool samples - in the case of the cholera group, or rectal swabs - in the case of the control group should be kept and transported in the frozen state. The virus isolation and identification should follow the established procedures, using multiple cell cultures for virus isolation.

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REFERENCES:

1. Ramos-Avarez, M., Olarte, J.
Diarrheal diseases of children. A.M.A.J. Dis. Child, 107: 218, (1964)

2. Yow,M.D., Melnick, J.L., Blattner, R.J., Rasmussen,C.E.
Enteroviruses in infantile diarrhea. Amer.J.Hyg. 77: 283, (1963)

3. Yow, M.D., Melnick, J.L., Phillips, C.A., Lee, L.H., South, M.A., Blattner, R.J. An etiologic investigation of infantile diarrhea in Houston during 1962 and 1963.
Amer. J. Epid. 82: 255 (1966)

4. Ingram, V.G., Rights, F.L., Ali Khan, H., Hashimi, K., Ansari, K. Diarrhea in children of West Pakistan.
Amer. J. Trop. Med. Hyg. 15: 743, (1966)

5. Parks, W.P., Melnick, J.L., Queiroga, L.T., Ali Khan,H.
Studies of infantile diarrhea in Karachi, Pakistan. I Collection, virus isolation and typing of viruses.
Amer. J. Epid. 84:382, (1966)

6. Siewers,C.M.F. The etiology of gastro-enteritis in infants and children with emphasis on the occurrence of simultaneous mixed viral-bacterial infections.
Pediatrics 35: 885, (1965)

.....cont'd

7. Abraham, A., Cheever, F.S. Virus isolation studies of stool specimens obtained from patients with cholera. Proc. Soc. Exp. Biol. Med. 112: 981,(1963)

8. Johnston, P.B., Grayston, J.P. Studies of gastro intestinal viral flora of cholera patients. Ibid. 104; 293, (1960)

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PROTOCOL

STUDIES IN EXPERIMENTAL CHOLERA IN ANIMALS

David R. Nalin and Richard A.Cash

A. The effect of cholera-toxin on pancreatectomized dogs

Greenough[1] and Taylor[2] have speculated on the possible role of the pancreas in the pathogenesis of cholera. Both exocrine and endocrine mechanisms have been suggested. The purpose of testing Aziz-type cholera-toxin in pancreatectomized dogs is to examine that the response of Thiry-Vella intestinal loops to cholera-toxin may be mediated by an endocrine factor elaborated by the pancreas. The pancreas has already been implicated in the production of massive diarrhea associated with pancreatic islet cell adenomas[3]. If elaboration of a pancreatic factor is necessary for the diarrheal response to cholera-toxin in Thiry-Vella loops, then there should be no response to Aziz cholera-toxin in the pancreatectomized animals.

Thiry-Vella loops will be constructed in healthy female dogs and in monkeys. Following post-operative recovery, the loop response to Aziz-type cholera-toxin will be tested. An identical loop will be created and tested in a control animal. Following the determination of the response to Aziz cholera-toxin in the loop, a pancreatectomy and a control operation will be performed on the experimental animals. Daily insulin requirements will be administered.

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After recovery from pancreatectomy and a control operation the loops in the pancreatectomized and in the control animals will be rechallenged with Aziz-type cholera-toxin to determine whether pancreatectomy has any effect on the response to toxin. The response in both animals will be quantitated by the collection of the fluid output from the Thiry-Vella loop after administration of toxin. No.23 Foley Catheters placed in the loop stomata will be used to facilitate the collection of the loop fluid. In both animals control collections of loop fluid after administration of normal saline will be obtained prior to the administration of cholera-toxin.

B. <u>The effects of renin and epinephrine on the response of gut to cholera-toxin</u>.

The large quantities of fluid and electrolytes lost into the gut lumen in cholera are delivered to the gut from the intravascular fluid pool. Theoretically, pharmacologic agents capable of reducing blood flow to the gut should cause a decrease of the fluid and electrolyte losses into the gut in response to cholera-toxin. To test this hypothesis, Thiry-Vella loops will be constructed in dogs, monkeys and other experimental animals. The response of the individual loops to Aziz-type cholera-toxin will be measured. The response has been shown by Carpenter[4] and others to be reproducible for up to six weeks in dogs. Following quantitation of the loop response, the loop will be

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rechallenged with cholera-toxin during intravenous infusion of renin and, in other studies, during lavage of the loop with epinephrine. After an appropriate interval the animal will be rechallenged with Aziz-type cholera-toxin alone, to make certain that there has been no change in loop reactivity due to atrophy or to immunologic factors. The loop response to cholera-toxin with and without renin or epinephrine will be compared.

C. Relationship of Trauma to the response of the intestinal mucosal surface to cholera-toxin.

The fluid and electrlyte loss in cholera may depend primarily on secretory or on vascular mechanisms. One possibility is that cholera fluid and electrolyte losses occur directly through the vascular walls of the gut mucosa. Fluid may then pass through the basement membrane and inter-cellular spaces into the lumen.

If losses of fluid and electrolytes due to cholera-toxin are more dependent upon cellular secretory or active transport mechanisms, then a gut segment denuded of its epithelial cells should be refractory to cholera-toxin. The response of intestinal segments in experimental animals treated with Aziz-type cholera-toxin will be measured before and after denudation of the epithelium of the experimental gut segment. Deudation will be accomplised using standard enzymatic and abrasive preparations

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employed in cytological studies of the alimentary tract. The extent of denudation and associated Trauma will be determined by direct biopsy. Control loops will be challenged with cholera-toxin alone. If intracellular mechanisms are necessary for the response of the loops to cholera-toxin, then the denuded intestinal mucosal surface should react to cholera-toxin with production of only a small amount of fluid or none at all. In addition the composition of any fluid produced would be expected to differ from that produced by cellular secretion. The protein and electrolyte compositon of loop fluid samples will be measured.

The effect of other forms of Trauma will also be investigated, including irradiation and actinomycin application.

D. The effect of acetazolamide with or without glucose on the response of Thiry-Vella loops in experimental animals to Aziz type cholera-toxin.

Leitch et al[5] and Norris[6] have found that acetazolaminde alone can reduce by a small amount the response of the intestine to challenge with cholera-toxin. Norris also showed that addition of small amounts of glucose (20 m Mols/L) in addition to acetazolamide induced positive fluid balance in segments of rabbit intestine challenged with cholera-filtrate-toxin. The effect of this

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combined solution was greater than the effect of glucose alone.

The purpose of these experiments will be to attempt to confirm the observations of Leitch and Norris in monkeys, dogs and other suitable experimental animals. Thiry-Vella loops will be created in the experimental animals, and the response to Aziz cholera-toxin will be observed. Following initial challenge with Aziz cholera-toxin, toxin will be readministered to the lopp along with solution of acetazolamide in a dose of 50 to 100 mg per kg. administered in a solution containing 500 micro-grams per c.c. The effect on the response of the loop to cholera-toxin in the presence of acetazolamide will be determined. In some animals acetazolamide will also be administered before and after toxin challenge either into the loop alone or simultaneously with parenteral acetazolamide.

In another set of experiments the same design will be used but glucose solution (2-3%) will be infused directly into the acetazolamide - treated loops in the toxin-challenged animals. Following recovery from the experiment, animals will again be challenged with the Aziz type cholera-toxin alone to determine that no non-specific change in loop reactivity has occurred in the interval between challenges.

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E. The interaction of absorbing and chelating agents with Aziz type cholera-toxin.

The active principle contained in cholera-toxin can be completely removed by pretreatment with activated charcoal and other agents. However, this inactivation can only be accomplished if the cholera-toxin is mixed with the charcoal prior to administration to experimental animals. It has not been possible to demonstrate a beneficial effect of charcoal when it is administered to patients or to experimental Thiry-Vella loops following infection with cholera vibrios or the administration of cholera-toxin[3]. Among the studies planned in this area are (1) the testing of large quantities of absorbing agents administered by constant infusion to Thiry-Vella gut segments treated with cholera toxin to determine whether the effect of the toxin can be prevented; (2) the infusion of the charcoal plus toxin mixture into gut segments to determine whether the binding of the active toxic principles with charcoal can prevent the toxin from causing diarrhea; (3) the determination of the minimum period of contact between cholera toxin and the gut mucosa necessary for the response of the mucosa to the toxin. This will be determined by applying the cholera-toxin to the Thiry-Vella loops subsequently removing it by lavage at varying intervals. The response of the loop to the toxin will be measured and compared after different intervals;(4) determination of the effect of newer absorbing agents as well as chelating agents,

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renins and detergents on the response of the Thiry-Vella loops to the Aziz type cholera-toxin in experimental animals.

F. The effect of products of Vibrio metabolism on Thiry-Vella loops.

In vitro products of vibrio metabolism will be infused into Thiry-Vella loops in experimental animals to determine whether these products are capable of producing a diarrhea in the experimental loops. Among the substances to be studied are nitrites, indoles and ammonia. The effects on the quantity and composition of loop fluid after the administration of these substances will be measured.

Procedure for making crude cholera-toxin.

Vibrio cholerae Inaba 569B is inoculated in 100 ml culture medium containing 2% peptone and 1% sodium chloride, pH is adjusted to 7.5 by NaOH. A three-liter flask containing the inoculated medium is put in a shaking water bath at 30 °C, ± 1 °C, for 18 hours. By 150 alternate shakes per minute and with a lateral displacement of 35-36 mm., maximal aeration is achieved. The culture suspension is then centrifuged at 10,000 r.p.m. for 30 minutes and the supernatant is immediately passed through a 0.22 millipore filter. This sterilized filtrate is the crude cholera toxin and is stored between 0 °C and -25 °C

...cont'd

REFERENCES:

1. Greenough, W. Lancet 1:991, 1965

2. Taylor, J.O. Pakistan-SEATO Cholera Research Laboratory, unpublished experiments.

3. Matsumoto et al. Gastroenterology 50: No 2, pp.231-241, Feb. 1966.

4. Carpenter, C. Dept. of Infectious Diseases, Johns Hopkins University. unpublished observations.

5. Leitch et al. J. Infec. Dis. 116: 303-312, June 1966

6. Norris, T. Veterans Admin. Hospital, Seattle, Washington, unpublished observations.

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PROTOCOL

PART I: DETERMINATION OF DRUG EFFECTS ON INTESTINAL WATER AND SODIUM FLUX, VOLUME AND FLOW RATES USING Na^{22} AND A NON-ABSORBABLE MARKER.

David R. Nalin and Richard A. Cash

Summary

The initial study is designed to determine the effects of acetazolamide infusion into intestinal segments of actively purging cholera patients. The amount and type of fluid entering and leaving portions of the intestinal tract of patients with cholera will be measured using multiluminal tubes placed at various levels of the small intestine.

A marker dilution sampling technique will be used similar to that described by Fordtrand[1] and by Dillard[2] and Hare et al[3]. Intestinal segments will be perfused at a constant rate using a solution of known concentration containing a non-absorbable marker. Luminal fluid samples will be taken from separate distal tube orifices. Once a steady state is achieved the segment will be perfused with a total dose of 150 to 250 mg/kg. of acetazolamide as the soluble sodium salt. The effect on intestinal water and sodium flux, volume, flow rate and luminal fluid composition will be determined.

A. Introduction

The possibility of a useful oral maintenance solution for the cholera patient has long been a goal of cholera research. Although initially intravenous therapy would remain necessary to relieve shock, an oral follow-up solution would greatly facilitate cholera therapy during epidemics and in endemic, underdeveloped areas where necessary medical personnel and intravenous solutions are not available. An "ideal" solution would contain the sodium, potassium, chloride, bicarbonate and water needed to replace deficits still remaining

after the relief of shock. In addition, the therapeutic oral solution would contain substances capable of arresting the continued loss of fluid and electrolytes into the gut lumen during the post-shock period.

The effectiveness of glucose lavage in decreasing stooling rate and fluid and electrolyte losses in cholera was first studied in the Philippines by Dr. R. A. Phillips and co-workers.[4] This work has been continued at the Pakistan-SEATO Cholera Research Laboratory as well as at the Johns Hopkins Laboratory in Calcutta. It appears that glucose would have a role in an oral maintenance solution for use in cholera.

An additional ingredient of an oral maintenance solution would be tetracycline or one of the other antibacterial agents which reduce fluid and electrolyte losses by eliminating viable vibrios.

Recent observations on the action of acetazolamide on rabbit intestinal loop treated with diarrheogenic cholera toxins suggest that acetazolamide may have a place in cholera therapy. Leitch and his co-workers[5] have demonstrated that acetazolamide can reduce net water and ion plasma to lumen flux in rabbit intestinal loops. A decrease in bicarbonate secretion into the lumen (and chloride reabsorption) was also demonstrated. Acetazolamide (500 mg./L.) was given in the drinking water 24 hours before loop challenge. The total amount of acetazolamide imbibed was not mentioned in Leitch's article.

Norris[6] has reported unpublished observations of the effect of acetazolamide on rabbit intestinal loops treated with Finkelstein "choleragen". These loops were perfused with a solution containing 500 micrograms per cc of acetazolamide. The rabbits also received doses of 20 to 50 mg./kg. of intravenous acetazolamide. A moderate decrease in lumenal output resulted.

Similar results were obtained using a glucose lavage solution. Animals receiving intraluminal acetazolamide glucose enjoyed a total cessation of fluid and electrolyte losses into the gut lumen within 30 minutes of the beginning of the infusion. Intravenous acetazolamide alone had no effect on the fluid and electrolyte losses into the intestinal loops.

B. Materials and Methods:

1) Patients admitted to the study will be those patients weighing over 75 lbs. admitted to the ward of the Pakistan-SEATO Cholera Research Laboratory in shock* from acute choleraic diarrhea. Intubation of the jejunum with a triple lumen intestinal tube will be carried out concomitantly with initial intravenous therapy used to combat shock. All patients will receive the standard Cholera Research Laboratory 5:4:1** therapeutic solution for cholera. Tetracycline will not be given. Patient care will otherwise include all standard P-SCRL therapeutic measures until the patient is out of shock and is voiding urine.

The acetazolamide trial period will commence after the patient is out of shock and is voiding urine. During this phase the location of the intestinal tube will be determined radiographically. The gut segment containing the tube will be perfused at a constant rate with 1,000 cc of perfusing solution per hour. Gut luminal contents will be withdrawn from the tube collection ports. A Peristaltic pump will be used to maintain a constant perfusion rate. A non-absorbable marker (PEG) will be added to the perfusing solution.

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* Shock will be defined arbitrarily as the absence of pulse or measurable blood pressure on admission.

** Solution contains 5 gm. NaCl, 4 gm. $NaHCO_3$, 1 gm, KCl per liter.

Determinations will be made of the changes in fluid and electrolyte losses into the loop lumen.

After a steady state is achieved the segment will be perfused at a rate of one liter per hour with "Hypotonic Jejunal Solution"* containing acetazolamide as the soluble sodium salt. The acetazolamide perfusing solution will contain 500 mg. per liter of acetazolamide.

The total dose of acetazolamide will be 150-250 mg. per kg.** At regular intervals measurements will be made of changes in intestinal sodium and water flux, luminal fluid volume, flow and composition. Volume changes in the intestinal segment will be estimated by means of a "bolus" technique as described in the previously mentioned paper by Dillard et al[2]. The modifications of this technique described by Hare et al[3] will be employed. An intraluminal bolus of bromsulfalein containing Na^{22} will be infused rapidly via a triple lumen intestinal tube infusion port. Subsequent determinations will be made of BSP, PEG and Na^{22} collected at regular intervals from distal intraluminal collection ports. Patients will be carefully monitored for 48 hours from the beginning of the study. Recorded data will include intake and output, complete blood counts, electrolytes (potassium, chloride, sodium, bicarbonate, magnesium and calcium),*** pH **** of blood, urine, stool and lumenal contents, and plasma specific gravity

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* A solution which closely matches the composition of jujunal fluid in cholera patients. Na^+ = 120 mEq/L., K^+ = 6 mEq/L., CO_2 = 20 mEq/L., Cl^- = 106 mEq/L.

** Maximum amount previously used in man and found well tolerated.

*** Every hour to every 4 hours

**** Every two hours to every four hours

The characteristics of the gut segment after the end of the acetazolamide infusion will be observed for 12 to 48 hours to determine if acetazolamide has any effect on the choleraic gut segment and, if so, how long its effects last. Fluid intake sufficient to prevent acetazolamide crystalluria will be maintained. Urinalyses will be checked repeatedly for crystals. The effect of acetazolamide on stool volume, rate and composition will also be measured.

An additional series of patients will be studied using a perfusate containing a 2% glucose solution as well as acetazolamide in a dose of 150-250 mg. per kg. The effect of this solution on segments of choleraic intestine will be determined as described above. The response of the intestinal segment to glucose alone, to acetazolamide, and to the two combined will be measured in the same patient.

If no significant effect is noted of acetazolamide or of acetazolamide plus glucose (compared to glucose alone), then the same techniques will be used to study the effects of other drugs of potential interest in cholera on intestinal water and sodium flux. Atropine and adrenal corticosteroids are among the drugs being considered for this type of study.

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References

1. Fordtrand, et al: The kinetics of water absorption in the human intestine. Trans. of the Assoc. of Am. Physicians. 74: p.195, 1961.

2. Dillard, et al: Volume fluid relationship during the transport of fluid to the human small intestine. Gastroenterology 49: No.1, pp. 58-66, 1965.

3. Hare, Taylor, Kinzie and Phillips. (In preparation): A new method for measuring unidirectional fluxes of isotopically labelled compounds across the intestinal wall, using a bolus injection of isotope and a non-absorbable marker.

4. Phillips, R. : Fed. Proc. 23: No.3, pp. 705-712, part I, May-June 1964.

5. Leitch et al: Experimental cholera in the rabbit ligated ileal loop. Journal of Infectious Diseases. 116: 303-312, 1966

6. Private Communication - US - Japan Cholera Conference - July 1967

PROTOCOL

COMPARISON OF A COMMERCIALLY-AVAILABLE INTRAVENOUS ACETATE SOLUTION WITH STANDARD BICARBONATE REPLACEMENT SOLUTIONS IN THE RAPID CORRECTION OF THE ACIDOSIS OF ACUTE CHOLERA AND OTHER SEVERE DIARRHEAS.

Chief Investigator : Dr. A. Majid Molla

Other Investigators : P-SCRL Clinical Research Section and Ward Staff Personnel

Background & Purpose:

Intravenous bicarbonate has been proved to be an ideal substance for correcting the acidosis associated with the loss of large amounts of bicarbonate-rich stool in acute cholera. There are two major disadvantages of bicarbonate solutions however, that make it impossible to store such solutions for long periods of time. First is the fact that bicarbonate can be autoclaved only with great difficulty and therefore must be added to the solution immediately prior to use to avoid significant bacterial contamination, and second is that commercially-available intravenous bicarbonate solutions have a very short shelf life.

Intravenous lactate has been shown to be a satisfactory substitute for bicarbonate in the correction of the acidosis associated with cholera but lactate also has two major disadvantages. The first is that it is expensive and difficult to procure in bulk and secondly it supports bacterial growth and and therefore has a short shelf life. Lactate does have the advantage over bicarbonate of being stable during autoclaving.

Acetate has been considered as another possible substitute for bicarbonate in the correction of acidosis in acute cholera. Acetate is cheap, readily available, can be autoclaved and has a long shelf life as it does not support significant bacterial growth. Acetate solutions have been used in the correction of acidosis in normal clinical circumstances in the United States and acetate solution is commercially-available under the name of Isolyte-E (McGaw Laboratories Incorporated), a solution that contains 47 milliequivalents per liter of acetate and 8 milliequivalents per liter of citrate.

The fact that acetate is satisfactory in the correction of acidosis in ordinary clinical circumstances where rapid intravenous infusion of large volumes is not necessary, does not necessarily prove either its efficacy or safety in acute cholera when as much as 5 liters of this solution may be administered in an hour. The crucial factor here is time, i.e. how rapidly is acetate metabolized and thus how rapidly can acidosis be corrected in these circumstance? A subsidiary question is, would there be a dangerous build-up of ketone bodies if sufficiently rapid oxidation did not take place? At present the information available in the literature is insufficient to allow us to speak with certainty about these points. Mudge et al.(1), in experiments in dogs and humans comparing isotonic sodium acetate to sodium bicarbonate, gave 15 milliequivalents of alkali per kg. of body weight over short periods of time. They saw a

rapid rise of the CO_2 content at 15 minute intervals over 2 hours which appeared similar between acetate and bicarbonate. They did not see any detrimental effects of acetate. A pilot study was done at this Laboratory last year immediately following the cholera season during which 6 patients with acute gastroenteritis and mild acidosis were given a solution containing 50 milliequivalents per liter of acetate and the results were compared with 4 patients who received a similar solution containing 50 milliequivalents of lactate. All the patients who received acetate showed a rapid rise in CO_2 and a rapid rise in the pH which appeared to be of the same magnitude as the lactate. However, none of these patients had cholera and the most that any patient received during the first hour was $3\frac{1}{2}$ liters of this solution. In these circumstances acetate appeared to be as effective as bicarbonate but none of the patients had the severe dehydration and acidosis that are seen in severe acute cholera on admission.

With the recent spread of cholera El Tor across the Middle East there has been great concern on the part of the Middle East governments in stockpiling intravenous fluids in preparation for any sudden epidemic of cholera. For the reasons mentioned above, acetate intravenous replacement fluid would be ideal for this purpose because of its long shelf life and low cost. As a result of this interest in stockpiling of acetate solutions for cholera epidemics the NESA Emergency Cholera Program has

As soon as this initial blood is collected, an IV will be started immediately using either the acetate replacement solution or the 5-4-1 bicarbonate replacement solution, depending on the random selection of the patient. The rate of intravenous fluid administration will be based on the degree of dehydration and acidosis as determined by the clinical examination of the patient. The time of starting the solution and clinical examination will be recorded on the forms provided. The EKG will then be taken and labelled with the time. A full evaluation of the patient is repeated and recorded at 20 minute intervals and an EKG is taken and reviewed every 20 minutes. The doctor is to be with the patient at all times during the study. At the end of each liter of study solution another femoral arterial blood is taken and all of the above determinations are repeated. The study will continue till the end of 3 hours when a final blood will be taken and a final EKG done. All urine passed on the day of study, i.e. until 24 hours after the beginning of the study, will be collected and examined for acetone by the Acetest immediately after it is passed. A sample of each bottle of intravenous solution will be sent for electrolyte determinations. The study will be completed at the end of three hours.

Materials and Personnel Required:

The Acetate replacement solution will be the commercially-available Isolyte-E (McGaw) containing 140 milliequivalents per liter of sodium, 10 milliequivalents per liter of potassium, 103 milliequivalents per liter of chloride, 47 milliequivalents per liter of acetate and 8 milliequivalents per liter of citrate. The bicarbonate replacement solution will be standard 5-4-1 bicarbonate solution containing 133 milliequivalents of sodium, 13.5 milliequivalents per liter of potassium, 99 milliequivalents per liter of chloride and 48 milliequivalents per liter of bicarbonate. A clinical chemist familiar with the determination of pH and CO_2 in the blood will be available during the hours of the study and pH and CO_2 must be done immediately after collection. Determinations other than pH and CO_2 need not be done immediately, as long as the specimens are properly handled, i.e. the blood for electrolytes separated properly, etc.

References:

1) Mudge, G.H., J.A. Manning and A. Gilman. Sodium acetate as a source of fixed base. Proc. Soc. Exp. Biol. 71:136-138(1949).

2) Goodman, L. and A. Gilman. The Pharmacological Basis of Therapeutics. (The MacMillan Company, New York, 1965).

3) Sellards, A.W. Tolerance for alkalis in asiatic cholera. Philip. J. Sci. Section B. 363; (1910).

4) Schwartz, W.B. et al. Lactate versus bicarbonate. Am. J. Med. 32: 831-833 (1962).

5) Carpenter, C.C.J. et al. Physiologic studies during the treatment of acute cholera. Bull. Johns Hopkins Hospital. 118: 197-215 (1966).

Patient's Name: _____ CRL No._____Date:_____

Age:_____ Sex:_____ Weight on Admission:_____

Type of I.V. Fluid:_____Lactate/Bicarbonate:_____

Time	Vol. of I.V. Fluid in (cum)	Pulse Rate	Pulse Quality	Blood Pressure	Resp.Rate	Resp. Depth	Mental Status	Remarks

PROTOCOL

ORAL LAVAGE OF VIBRIOPHAGE IN ACUTE CHOLERA: EFFECTS ON VIBRIO COUNTS AND STOOLING RATE.

Dr. K. A. Monsur, Dr. Ataur Rahman, Dr. Z. Haque, Dr. R. Northrup and Dr. J.O. Taylor.

Background and Purpose:

Although there are various reports claiming success in using bacteriophage in the treatment of cholera, none of these reports stand up to critical analysis. Mortality rates are used as the criterion of success, intravenous fluid replacement has been grossly inadequate, insufficient clinical details are given and there are no proper controls.

This study of oral lavage of high doses of vibriophage was begun last year by Dr. K.A. Monsur and Dr.Hirschhorn in an attempt to demonstrate, once and for all, if bacteriophage is potentially effective in the treatment of acute cholera as measured by decrease in rate and duration of stooling. Results with several patients where the titer of phage in the stool was maintained at 10^{10} pfu/ml* or higher showed that for a period of time stooling rate did appear to decline more rapidly than would be predicted in an untreated patient. However, when the phage was stopped, the stooling rate increased again. Due to limitations on the amount of phage which could be produced it was never possible during the last cholera season to continue lavage of vibriophage until the cessation of diarrhea, thus a truly adequate trial of phage therapy was not possible. This year, with improvements in the technique of phage production developed by Dr. Monsur and his co-workers in the Institute of Public Health, and with a much

* pfu = plaque forming units

larger capacity for high speed centrifugation of large volumes of phage culture material, we will be able to produce adequate amounts of phage particles to continue the infusion for the total duration of diarrhea.

The principles of phage therapy are as follows:

1) Vibrios may be lysed within minutes if infected with a large number of particles at the same time ($>$ 100 particles/vibrio). This is known as "lysis from without" as distinguished from lysis from within in which a single phage particle enters and multiplies within the vibrio and eventually causes the vibrio to rupture. The average "burst time" from lysis from within is about 1 hour, which is twice the mean transit time from stomach to rectum in acute cholera. Obviously lysis from within will not be effective in reducing the vibrio count in the upper small bowel.

2) The average gut volume of an acute cholera patient is 1 to 2 liters.

3) Vibrio counts in the gut in acute cholera may reach 10^9 vibios/ml.

4) Therefore, the dose of phage must result in a concentration of 10^{11} pfu/ml in the gut to provide 100 particles per vibrio. The dose must also allow for dilution to 2 liters.

An initial dose of 100 ml of 5×10^{12} pfu/ml would result in a concentration of around 2.5×10^{11} pfu per ml. Assuming that the patient is purging 500 ml/hour an additional 25 ml of phage must be given hourly. In practice more will be given.

5) To protect against the possibility of a resistant mutant rendering phage therapy ineffective, a mixture of different phages will be used.

6) To provide more accurate counts of viable vibrios, these counts will be done in the presence of polyvalent antiphage sera from which bacterial antibodies have been absorbed. This technique has been developed by Dr. Monsur and his co-workers.

7) Some vibrios may be protected from attack by phage particles either by surrounding mucous, by being in inaccessible areas, crypts, or by other factors. Thus even with the large concentration of phage in the gut, it may be impossible to completely sterilize the gut for V. cholerae. This is probably why continuous phage therapy until diarrhea stops appears to be necessary.

8) This study will include administration of 30 ml of 10% oxoid peptone in 1% saline along with each dose of phage. It is felt that the peptone may help the lytic action of the phage and also encourage the growth of normal intestinal flora. Controls with peptone alone without phage will also be done.

Materials and Methods.

1) In a patient who qualifies as an active cholera patient (positive culture; more than 3.2 liters of stool in either of the first two eight-hour periods in the hospital), a double lumen tube with a mercury bag attached is passed. This tube

has a porthole that will be placed at or near the Ligament of Treitz and a proximal, independent porthole which will be at or just beyond the pylorus.

2) The tubes are flushed by drawing 10 cc of intestinal fluid and discarding.

3) Samples of rice water fluid are drawn from the lower port for vibrio and phage titration, and darkfield microscopy. A stool sample is taken for the same purpose, also for baseline electrolytes, via a rectal catheter (samples into new, sterile tubes).

4) Blood is drawn for baseline electrolytes, specific gravity, plasma protein, total and differential white count and hematocrit; also for initial phage titer and vibriocidal and agglutinating antibodies.

5) The duodenal tubes are flushed prior to any sampling by withdrawing 10 cc of intestinal fluid and discarding.

6) Via the proximal (pyloric) port 100 cc of 5×10^{12} titer phage is slowly given in ten minutes.

7) After a 15 minute wait, the distal tube is flushed again by withdrawing 10 cc of intestinal fluid and a sample is taken for vibrio and phage titration (into a new, sterile tube).

8) 10 cc of stool via a sterile rectal catheter is put into a brand new, sterile tube every 30 minutes from the start of the study until there is a detectable difference in the

darkfield. After one hour and 15 minutes, 50 cc of stool is taken for special washing to isolate vibrios.

9) After the first hour of phage and the 15 minute wait, the same concentrate (5×10^{12}) is given at a slower rate for the duration of diarrhea.

10) After a change in darkfield, stools are taken every four hours for phage and vibrio count. Stools are taken every six hours for electrolyte determinations.

11) Blood is taken for phage titer after the first 30 minutes of therapy and then twice daily, for phage titer and for white count and differential.

12) Antibody titers will be followed daily.

13) Stool output (by weight and measurement) and vital signs will be followed every four hours.

This study will be done in a room completely separate from the ϕ-SCRL ward and all equipment and specimens will be handled separately to avoid contamination of the ward with vibrio phage. Such contamination might interfere with immunologic studies.

Summary:

A study of oral therapy with high doses of vibrio phage (a mixture of phage types) will be done. The effectiveness of phage therapy will be determined by the reduction of stool output, duration of diarrhea and titer count of the vibrios in the stool.

The clinical aspects of the program will be managed by Dr. Z. Hoque, Dr. R. Northrup and Dr. J. Taylor. The preparation of phage and phage counting will be done by Dr. Monsur, Dr. Ataur Rahman and the Institute of Public Health Staff. Vibrio counts will be done by Dr. Monsur and his co-workers.

PROTOCOL

BILE SALTS AND BILE PIGMENTS IN RICE WATER STOOL

Drs. Irvin A. Rosenberg and Norbert Hirschhorn.

Introduction and Aims:

The effect of cholera on the liver and biliary system and their role in the pathogenesis of cholera has never been clearly defined. Clinicians have often commented on the appearance of green pigment in the stool of cholera patients as a herald of cessation of diarrhea, while the acholic appearance of rice water stoool has been variously attributed to spasm of the bile ducts or Spincter of Oddi.

It has also been suggested from this laboratory that the liver, by hypersecretion, can contribute to the copious rice water stool of cholera.

Bile salts are either conjugated (taurcholate, glycocholate) or unconjugated (cholate, deoxycholate). The unconjugated bile salts are created by action of lower gut flora on the conjugated salts. V.Cholerae is not known to deconjugate bile salts. Normally these deconjugated bile salts are reabsorbed in the ileum and reexcreted in the duodenum. In the malabsorption of bile salts, these salts would be present in the stool but not in duodenal drainage.

During acute cholera, in which the gut is largely populated with V.Cholerae, and where the rice water stool is clear and copious, the appearance of unconjugated bile salts in the stool, especially at the time of color change, may signal the return of activity of other microorganisms and diminution of diarrhea. Failure to find unconjugated bile salts in the duodenal drainage at the time

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of their presence in the stool would be another measure of malabsorption in cholera diarrhea. The presence or absence of conjugated bile salts and bile pigment in the duodenal aspirate, before and after stimulation by a fatty meal may give information on the relative contribution of the liver to the makeup of the rice water stool.

Method and Procedure:

A patient who is vigorously purging clear rice water stool, will swallow a double lumen tube with a mercury balloon, a distal sampling port in the duodenum and a proximal infusion port in the stomach. Daily, during diarrhea, a 100 cc sample of duodenal content will be aspirated for measure of bile pigment (by the diazo method of Malloy and Evelyn), for quantitative aerobic and anaerobic counts (vibrios and others), and then taken to dryness for measure of bile salts. The latter will be done by Dr. Rosenberg at the Thorndike Memorial Laboratory, Boston, by thin-layer and gas chromatography. A sample of stool will be taken at the same time and treated similarly. In some patients a fatty meal will be given into the stomach after the initial duodenum aspiration and a duodenal aspirate taken fifteen minutes later. Any changes in color will be noted.

Significance of Study:

We hope to secure further information on the possible role of the liver and biliary system in the pathogenesis of cholera, the absorptive processes in cholera diarrhea; in addition the historical question of the cause of acholic stools may be answered.

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Facilities Available:

All facilities of the Clinical Investigation and Bacteriology Sections are available for the study. One bacteriology and one chemistry technician will be needed.

Facilities Required:

No new or unusual facilities are required.

Work Already Done in This Area:

In this laboratory, BSP given intravenously has been recovered (about 60%) in the stool of patients with acute cholera. Studies with a Miller-Abbot tube have suggested that a considerable amount of fluid in cholera comes from above the Ligament of Trietz. Studies here have also shown widespread malabsorption in cholera and other diarrheas.

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PROTOCOL

THE PURIFICATION AND PROPERTIES OF CHOLERA TOXIN

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

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 relation to a permeability factor.

Pakistan-SEATO Cholera Research Laboratory, Dacca, is a unique place to work with cholera patients and rice water stool. The identification of the causative agent of the cholera syndrome, should obviously begin with the rice water stool.

The specific aims of this project are to purify the cholera toxin 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 luman flow, vibrio count and toxicity. The effect of vaccination, hyperimmune plasma, various antibiotics and enzyme poisons on the toxic product(s) should also be investigated. Ultimately this may lead to better treatment of cholera patients and the

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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 out rice water stool at a minimum rate of 300 ml 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 subbed and maintained in pure culture. This culture will be diluted in gel-borate buffer and injected intradermally into rabbit skin. 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/u millipore filter. If necessary prefiltration through microfiber glass will be done to facilitate quick filtration. The filtrate will be stored at -20°C. Three

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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 infant rabbits (11-12). The remaining 230 ml will again be stored at -20 ^{o}C for future reference. The dialysing will be carried out under vacuum in a special apparatus. An eight mm glass tube will be fitted in a rubber bung in the mouth of a conical sidearm flask. At the lower end of the glass tube an eight mm diameter dialysing tube, 1 meter long, will be attached. The lower end of the dialysing tube will be sealed off and the supernatant will be poured in the funnel. Suction will then be applied and dialysis carried out until one liter is reduced to about 10 ml. This viscous fluid will be tested in all the three animal models for toxicity. 5 ml of this fluid will be lyophilized and stored at -20 ^{o}C. The remainder of the fluid will be passed through DEAE-Cellullose chromatography column. The fractions will be collected in ten test tubes. Each of these will then be tested for toxicity in all the three animal models mentioned above. The active fractions will be pooled and dialysed to make 1-2 ml and molecular sieve

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filtration (13) will be done by sephadex G 100 or G 200 column chromatography to obtain further fractions. These fractions will again be pooled in 10 screw capped tubes and tested for toxicity by the three animal assay techniques. The active fraction(s) will be further purified by gel and paper 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. A microtechnique of immuno-electrophoresis as proposed by Darcy (14), may also be carried out. The ultraviolet absorption spectrum will be determined. The purified product will be concentrated by dialysis, lyophilised and stored at $-20^{\circ}C$.

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 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 (13-17). The molecular weight may also be determined by density gradient separation by ultracentrifugation and the results compared.

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After a 95% or more purification of the toxin is achieved some peptide mapping and structural work may be carried out in this laboratory. For this purpose a peptide mapping will be done after tryptic and other enzymatic digestion. The sequence of amino acids will be found out in each peptide and this amino acid sequence of the toxin molecule may be established.

Significance of This Research:

This research may lead to the discovery of the toxic componant(s) in the cholera stool that causes the cholera syndrome. If curative and prophylactic measures are to be improved we must know the 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. Some chemicals and equipments have already been procured. Some more equipment is on order. The laboratory is ideally located close to the Clinical Research ward. The animal house of the Pakistan-SEATO Cholera Research Laboratory is presently undergoing improvement to meet the standards that animal experimentation needs. About 1200 adult and 2400 infant rabbits will be needed. The following is a list of items of permanent equipment:-

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Equipment Available in the Laboratory:

1. International centrifuge.
2. Chromatography column.
3. Refrigerator.
4. 30 °C Incubator.
5. Vacuum pump.
6. Lyopholizing machine.
7. Beckman pH meter.
8. Millipore Filters
9. Spectrophotometer.
10. Model-L International Ultracentrifuge.

Equipment needed:

1. Fraction collector.
2. Deep freeze -20 °C
3. Micro-electrophorasis unit.
4. Paper chromotography apparatus.
5. Disc electrophoresis unit.
6. Pressurized Nitrogen gas.
7. Beckman Du Spectrophotometer.
8. Temperature controlled room(0-10 °C).
9. Temperature contolled room (30 °C).
10. Apparatus for structural work on Protein Molecules.

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REFERENCES:

1. Finkelstein, R.A. Immunological aspects of experimental Cholera. Proc. Cholera Res. Symp., 58-63, 1965.

2. Finkelstein, R.A. Observation on the nature and mode of action of the choleragenic products of cholera vibrios. Proc. Cholera Res. Symp., 264-270, 1965.

3. Finkelstein, R.A., Atthasampunna, P., Chulasamaya, M. and Charunmethee, P. Pathogenesis of experimental cholera. Biologic activities of purified procholeragen A.J. Immunol., 96:440-449, 1966.

4. Finkelstein, R.A., Norris, H.T., and Dutta, N.K. Pathogenesis of experimental cholera in infant rabbits. J. Infect. Dis., 114:203-216, 1964.

5. Feeley, J. Personal communication, Sept., 1967

6. Burrows, W. Personal communication October 1967

7. Craig, J.P. The effect of cholera stool and culture filtrates on the skin of guinea pigs and rabbits. Proc. Cholera Res. Symp., 153-158, 1965.

8. Craig, J.P. A permeability factor (Toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature, Vol. 207, No.4997, 614-616, 1965.

9. De, S.N. and Chatterjee, D.N. An experimental study of the mechanism of action of Vibrio cholerae on the intestinal mucous membrance. J. Path. Bact., 66:559-562, 1953.

....cont'd

10. Schafer, D.E.,and Lewis, G.W. 1965 - Studies on the properties of V.cholarae enterotoxin and the time and dose-dependence of its effects on the ligated intestinal loop. Proc. cholera Res. Symp., 160-162, 1965.

11. Dutta, N.K., and Habbu, M.K. - Experimental cholera in infant rabbits. A method for chemotherapeutic investigation. Brit. J. Pharmacol.,10:153-159, 1955.

12. Dutta, N.K., Panse, M.V., and Kulkarni, D.R. - Role of cholera Toxin in experimental cholera. J. Bact., 78:594-595, 1959.

13. Flodin, P. and Killander, J. - Fractionation of human serum proteins by gel filtration. Biochim. Biophys. Acta, 63:403-410, 1962.

14. Darcy, D.A. - Micromethod for the estimation of specific proteins in mixtures. Nature (London), 206/4986: 826-827, 1965.

15. Flodin, P. and Porath, J. - Molecular Sieve Process. In Chromatography, Ed. by Heftmann, E., Reinhold, N.Y., 328-343, 1961.

16. Fasold, H.,Gundlach,G., and Turba, F. - Chromatography of proteins: Molecular sieve filtration, in chromatography, Ed. by Heftmann, E., Reinhold, N.Y. 406-410, 1961.

....cont'd

17. Whitaker, J.H. - Determination of molecular weights of proteins by gel filtration on Sephadex. Anal. Chem., 35:1950-1953, 1963.

18. Blackwell, R.Q. - Personal communication November 1967.

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PROTOCOL

SPINAL FLUID IN ASIATIC CHOLERA

David R. Nalin, M.D.

A recent clinical study by Posner and Plum[1] provides evidence for a relationship between coma and CSF pH. Severely acidotic patients with normal CSF pH had normal mental states. Patients whose blood acidosis was corrected but whose CSF was still acidotic were comatose. A study comparing the characteristics of the blood and CSF in cholera can clarify the cause of coma in cholera and the complex relationship between mental and metabolic status. Therapeutic implications would include evaluation of the warning against over-rapid bicarbonate infusion in acidotic patients as given by Posner in the New England Journal article referred to above.* The possible relationship between CSF pH and respiratory pattern could be explored.[2,3,4] The effects of severe water sodium and potassium losses on CSF composition could be described.

Justification

Justification for this study includes (1) the need for understanding the cause of coma in acidotic patients, including those who have received therapy sufficient to correct plasma pH; (2) the need for dealing with the therapeutic implications of CSF acidosis or for adopting prophylactic measures to avoid inducing CSF acidosis therapeutically; (3) the need to

*Posner feels that over-rapid HCO_3 infusion worsens CSF acidosis by causing more rapid increase in blood HCO_3 than in CSF. Blood and CSF pCO_2 rise quickly, however, CO_2 is readily diffusable into the CSF, while HCO_3 rises only slowly in the CSF; therefore, relative CSF acidosis may be induced.

understand the relationship of CSF pH and respiration, and (4) exploration of possible relationship between CSF changes in cholera and neurologic or mental manifestations in the acute and convalescent phases of the disease.

Materials and Methods

All patients admitted to the P-SCRL in shock due to cholera will be candidates for admission to the study series of 5-10 patients. Treatment will not be altered in any way from the standard P-SCRL method. In addition to the abbreviated P-SCRL history and physical examination used during the cholera season, a neurologic examination will be done. Basic data describing the neurologic and general mental status will be recorded during the study period.

Once on admission and twice during the subsequent 24 hours, spinal fluid will be obtained by routine tap for laboratory studies. Simultaneous blood samples will be drawn. At each spinal tap 6.5 cc of spinal fluid will be taken for tests.

Routine intake and output with fluid and electrolyte balance data will be collected.

The following determinations will be made on all specimens: (in order of importance and priority for CSF).

Blood (10 cc total)		CSF (6½ cc total)
Urea nitrogen		Opening pressure
Acetone		(Closing pressure)
Phosphate	I.	Culture - bacteria
Glucose		- fungi
Specific gravity		Cell count and Differential
Total proteins		pH

Blood	CSF
Hematocrit	II. Total Protein
Sodium	Sodium
Potassium	Potassium
Chloride	Chloride
Bicarbonate	Bicarbonate
Arterial pH	Glucose
WBC-Differential	Osmolarity
Osmolarity	III. Specific gravity
Vibriocidal antibody titer	Ca
Ca	Mg
Mg	IV. Vibriocidal antibody titer

Routine P-SCRL history and physical forms and i & o sheets will be used.

Neurologic and mental data will include:

1) General Level of Mental State

a. Normal - conscious, alert, responds appropriately.

b. Comatose - No evidence of response to or awareness of external stimuli.

c. Obtunded - conscious but confused, with mild delirium - some appropriate behavior and responses to questions or commands. Varying orientation and varying level of ability to respond.

d. Delirium, severe - conscious but disoriented to time, place, person.

e. Chronic organic brain syndrome. Type to be specified.

2) Neurologic Review

a. Cranial nerves
b. Autonomic system
c. Sensory nerves
d. Reflexes
e. Motor and Coordination

} Results of examination will be reported.

Reference

1. New England Journal of Medicine. 277: No.12, 605-613, 1967.

2. Pappenheimer et al: American Journal of Physiol.208: 436-450, 1965.

3. Pappenheimer: Harvey Lect. 61: 71-94, 1967.

4. Fisher and Christianson: J. Applied Physics. 18: 712-716, 1963.

PROTOCOL

DETERMINATION OF PERMEABILITY OF CHOLERA-AFFECTED BOWEL TO MANNITOL 14C INJECTED INTRAVENOUSLY

J. Kinzie, J.O.Taylor, R.S. Gordon and R. Hare.

Purpose:

To determine the extent to which mannitol (labeled with 14C for easy and accurate measurement) injected into the circulation passes into the diarrheal fluid of cholera.

Significance:

A cholera patient taking nothing by mouth puts out enormous volumes of isotonic stool. Animal and human studies agree that this fluid enters the lumen of the G.I. tract primarily in the jejuno-ileal segment of the small intestine, but the basic nature of the process whereby this fluid mixture is separated from other blood constituents (such as cells and proteins) which are not lost, and transported into the lumen of the bowel is not known. There are two principal possibilities:

1) That a head of hydrostatic pressure, operating to force fluid through a membrane with pores small enough to retain proteins, provides the energy needed to accomplish the relatively small amount of thermodynamic work involved in the separation of macromolecular solutes and cells from the fluid which is to make up the stool. This process would be entirely analogous to the operation of the glomerular filter in the kidney.
2) That the active transport of some solute or solutes produces (perhaps in a very limited and inaccessible space) a gradient

of chemical potential which causes water and other solutes to migrate outward from blood to bowel lumen. Whereas the first possibility would indicate that the ultimate source of the energy producing the stool is the pumping action of the heart, the second would require that the energy come from the (perhaps misguided) metabolism of the cells of the intestinal epithelium.

The first possibility - that in cholera the intestinal epithelium filters fluid after the manner of the renal glomerulus - would require that mannitol in the extracellular fluid be cleared at the same rate as sodium, since it has virtually the same effective size in solution as the hydrated sodium ion. Since mannitol is not reabsorbed to any appreciable extent by the human gut, and since there is probably some sodium reabsorption even in the most severe cases of cholera, this hypothesis would require that, relative to its blood concentration, the stool contain more mannitol than sodium, or at least as much.

The second possibility would allow for very significant discrimination between sodium and mannitol in the formation of diarrheal fluid. As mannitol is not actively transported by any known mammalian tissue, it is unlikely that it would be specifically transported out in cholera. It does not penetrate cells in general, and it has been clearly shown that the normal gut is virtually impermeable to it; hence it would not be expected to enter the gut by diffusion down chemical gradients established by the active outward transport of any other solute. Therefore possibility number two would require that the newly formed stool contain much more

sodium than mannitol (again, relative to concentrations of these solutes in extracellular fluid).

The most elegant way to carry out this study will be to determine simultaneous outward fluxes of sodium and of mannitol in a defined study segment of small bowel, following the general outlines of protocols numbered CI/P1,2,3. Whereas the bolus technique with intraluminal administration of isotopic sodium should be entirely satisfactory for the determination of the flux of sodium ion from blood to gut, the mannitol would best be administered intravenously as a constant infusion, with measurement of efflux under steady state conditions. If, however, the efflux rate of mannitol is virtually zero, it may be possible to obtain a satisfactory negative answer by the simpler approach outlined here. This study, which can be undertaken with limited clinical facilities, should be considered as a preliminary approach, to be followed by more sophisticated measurements if necessary.

Procedure:

1) Select a male patient with severe cholera, at least 18 years of age. Establish the diagnosis by clinical and darkfield examination, and follow stool output for 6 to 24 hours to be certain that vigorous purging is to be anticipated. Net output should be 300 ml/hour or more.
2) Four hours before labeled mannitol is to be given, administer tetracycline and mannitol orally or by stomach tube. After a first dose of 1 gm, give 1/4 gm. tetracycline each hour through the study. Give mannitol at a rate of one half gm. per hour.

Tetracycline will kill the great majority of intestinal bacteria and prevent their attacking and degrading such mannitol 14C as enters the gut. Carrier mannitol given orally will also help preserve labeled mannitol from incidental loss. Aside from these materials, allow only water by mouth.

3) At time zero, give 5 ml. of labeled mannitol (2.2 uC per ml., total dose 11 uC) by vein. Sterile carrier mannitol should be added to the intravenous fluids used for maintenance therapy at this time or shortly before. The precise dose is unimportant, as this agent is also only a carrier, but it should be in the range of 0.2 to 0.5 gm. per hour.

4) Collect 10 ml. specimens of blood for separation of serum at 5, 15, 30, 45 and 60 minutes, and hourly thereafter until the sixth hour. Separate serum as soon as practicable, and refrigerate or freeze.

5) Collect each stool separately, measure volume, and save 2 30 ml. aliquots. Heat these to 100 degrees for 10 minutes in a water bath to kill all bacteria, add a drop of toluene, and save in the refrigerator.

6) Empty the bladder at time zero, then collect voided urine each hour, and treat as above. It is essential that no drop of urine be allowed to contaminate the stool specimens.

7) At the fourth hour, give one ampule of BSP orally. Terminate the collection of urine and stool specimens at six hours if BSP has been detected in the stools, or when the dye is found if its transit is delayed.

8) Determine radioactity in all specimens by liquid scintillation counting. Processing of data will be outlined separately.

APPENDIX 1.

RADIATION DOSIMETRY CALCULATIONS

Mannitol injected by vein is rapidly distributed throughout the extracellular fluid volume, and may be regarded as having whole body distribution. It is cleared at a rate determined by glomerular filtration rate. The rate constant for clearance = ECF volume / G.F.R.

The only tissue in which mannitol is concentrated is kidney and urinary system, neither of which is unusually radiosensitive. Exact dosimetry for urinary tract is impossible, as it will vary with rate of urine flow. However a reasonable approximation would be to assume tissue levels no higher than 100 times the initial E.C.F. levels, and periods of effective exposure not exceeding one hour.

Consider a 40 kg Bengali male subject. ECF volume is about 20% of body weight, or 8 liters. GFR would be about 100 ml (0.1l) per minute. Thus biologic half-time of mannitol would be determined by a rate constant for removal of 0.1/8.0, or 0.0125 per minute. This yields a half-time of 0.038 days (about one hour). An 11 μC dose of the labeled mannitol would result in an initial whole body concentration of the isotope of 11/40,000 or 2.8×10^{-4} μC /gr. Using standard formulae, the cumulative beta dosage is then found to be about 5×10^{-5} rads.

As a rough approximation to the anticipated extra dose to the tissues of the urinary tract resulting from concentration of mannitol in newly-formed urine, we may assume a transient concentration of 14C 100 times greater than that of whole body acting for 1 hr. This yields a value of 3×10^{-3} rads of extra irradiation of the urinary tissues.

APPENDIX 2.

Equipment required at or near bedside for study of one patient (aside from routine needs for therapy).

1. Mannitol:

One vial 14C mannitol.
One ampule sterile unlabeled mannitol for intravenous use.
3 - 5 gm. mannitol powder.

2. Syringes for blood samples, etc.

1 - 5 ml. glass syringe to inject mannitol.
1 0 10 ml. glass or disposable syringes for blood samples.
11 or more needles (20 gauge x 1 inch, or similar).

3. Sample Containers.

10 test tubes to receive blood specimens.
10 other test tubes or plastic tubes for separated serum.
About 30 glass bottles with stoppers or screw caps to receive urine and stool specimens. These will be heated, so should not be ordinary plastic.

4. Miscellaneous:

Refrigerator to store specimens.
Graduated cylinders to measure urines and stools.
Cylinder used for urines must not be used for stools, so several of each size will be needed.
Male urinal.
Centrifuge if possible.
Pipettes or droppers to transfer serum.
Labels.
BSP.
Alkali to demonstrate BSP qualitatively in stools.
Toluene.

TECHNICAL COMMITTEE MEETING 1967

EPIDEMIOLOGY RESEARCH PROTOCOLS

E/P1 Dacca Comparative Urban Cholera Study. N.O.

A. Martin, A. Momin Chowdhury, Md. Shahidullah & Nuzhat Jahangir.

E/P2 Naogaon-Nagda-Dogarpur Rural Cholera Study. N.O.

A. Martin & Md. Nuruzzaman.

E/P3 Antibiotic Treatment Study - Matlab Hospital 1967-68. N.O.

W.M. McCormack & A.S.M. Mizanur Rahman.

E/P4 Intradermal Cholera Vaccine.

W.M. McCormack & R.K. Barui.

E/P5 Purging of Family Contacts of Cholera Cases.

R. Barui and W.M. McCormack.

E/P6 An Assessment of the risk of Hospital and Laboratory Infections with V. Cholerae.

R. Barui and W.M. McCormack.

E/P7 Long-Term Serologic Response to Cholera Infection.

W.M. McCormack, W.H. Mosley & Ansaruddin Ahmad.

E/P8 Serologic Study of Bacteriologically Proven Cholera Cases at Various Intervals After Illness.

A. Martin & A. Momin Chowdhury.

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

A. Martin.

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

W. McCormack and W.H. Mosley.

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

W. M. McCormack and Md. Shafiqul Islam.

(Cont'd.....2,

EPIDEMIOLOGY RESEARCH PROTOCOLS

E/P12 The 1967 Cholera Vaccine Field Trial.

W.H. Mosley and W.McCormack.

E/P13 Program and Protocols for the Immunology Laboratory.

PROTOCOL E/P1

DACCA COMPARATIVE URBAN CHOLERA STUDY

A. Martin, A. Momin Chowdury, Md. Shahidullah and Nuzhat Jahangir

BACKGROUND

Numerous well-defined studies have been carried out to determine the pattern of cholera within the families and in the immediate surrounding environment of cholera patients. These have produced a good deal of information about the spread of the disease within a very circumscribed area such as a family or a bari with a common water source, but little information about the introduction or spread of cholera in the community as a whole. It has become apparent that cholera outbreaks are usually not limited to just a portion of the community, and that study must be expanded to a larger area in order to define the epidemiologic patterns. Such work has been begun in a number of rural areas in East Pakistan and preliminary data indicates that a larger percentage of the population may be infected each year than would be suspected. These infections can be detected by a rise in the serologic titer during the epidemic period in individuals who have had no outward manifestations of cholera. The data, in fact, suggests that many villages may have widespread outbreaks of cholera with all or most infections being asymptomatic.

Relatively little information is yet available on the means by which cholera is introduced into these communities and about the mechanism of spread. Particularly lacking is our knowledge of urban areas which are inherently more difficult to study and which differ significantly from the villages.

PURPOSE

The purpose of this study will be to compare the pattern of cholera in three well delineated urban communities which have different geographic characteristics. Attention will be focused on the means by which cholera is introduced into a community and the degree to which immunity and physical factors in the environment influence its pattern of spread. One of the areas chosen, Rayaraznaran Dhar Road, is situated adjacent to the river, a major source of water for the community. A second area, Goalnagar, is in the center of Old Dacca and depends primarily on municipal taps and dug wells for water. The third, Babupura Bosti, is near New Market and uses tube wells exclusively as water source. The first two areas have consistently been infected with cholera and the third has had a relatively small number of cases. A number of patterns will be analyzed including, in addition to water supply, occupation, travel, migrations, food sources, vaccination history and serologic titer.

METHODS

A. Census

A census, including name, age, sex, occupation, and relation of each family member will be obtained. A code letter will be assigned to each area and families within the area will be numbered consecutively starting with 1. Individuals within each family will be numbered consecutively also beginning with 1.

2. Occupation and location of worker or school will be determined for appropriate members of the family.

3. A history of cholera vaccination or prior infection with cholera will be obtained for each family member.

4. Water sources for drinking, bathing, cooking and washing clothes will be determined for each family and designated numerically according to a code which geographically locates the source.

5. Food sources for major food items (rice, fish, dhal, oil, vegetables) will be determined for each family.

6. Each area will be mapped so as to locate the families and water sources.

B. Routine Surveillance

1. Each family will be visited every day by one of two field teams and questioned about the onset of diarrhea in family members.

All cases of diarrhea will be recorded and the person involved will have a rectal swab examination taken for detection of Vibrio cholerae. Patients with severe diarrheal illness will be taken to PSCRL for treatment.

2. Daily information about births, deaths, and migrations in or out of the area will be obtained. This information will be used to update the census book weekly.

3. Water samples for bacteriologic examination will be taken daily from all major public water sources.

4. Information will be obtained each day about guests entering the households, and guests will be registered on a weekly log sheet with an indication as to the area from which they came. A guest will be defined as any individual, member of the household or not, who enters the household for at least an overnight stay after an absence of at least one night.

5. All bacteriologically positive cases of cholera or positive water samples will be reported immediately to the principal investigator and to the field staff by the bacteriology laboratory. These will be followed up immediately with a complete epidemiologic investigation directed at trying to locate the source of introduction and the means of spread of the disease.

C. Special Studies

1. Each person in the population will have a 0.05 ml. finger-tip blood sample taken in October and again after the cholera season. The date of the second bleeding will be decided later. The pairs will be run for vibriocidal titer after the second bleeding to detect a significant rise in vibriocidal titer during the cholera season.

2. Environmental sampling of rectal swab culture surveys may be done in localized areas within water useage groups where clinically apparent cases of cholera occur.

ANALYSIS OF DATA

Analysis will center around the bacteriologically and serologically positive cases. Given a reasonable number of cases, it will be possible to determine attack rates for apparent and inapparent infections in each area. A number of comparisons can then be made:

1. The ratio of cases detected by paired serology to those detected by case finding and rectal swabs will be calculated.
2. The attack rate for common users of water supply when one user is infected will be compared with the community attack rate.

3. An attempt will be made to determine if cholera infections can best be grouped by water useage groups, geographic areas, common food sources, or if they are distributed throughout the community.
4. It will be determined if a number of factors such as occupation, location of work, history of vaccination, educational status of the family have an effect on the incidence of cholera.
5. Within common exposure groups, the relationship between serologic titer and infection rate can be determined.

CONCLUSIONS

The thorough study of each community should provide valuable information about the factors important in the spread of cholera within that community. In addition, by comparing areas with different geographic and sociologic characteristics, the effect of these characteristics on the pattern of cholera should become apparent.

PROTOCOL E/P2

NAOGAON-MAGDA-DOGARPUR RURAL CHOLERA STUDY

A. Martin and Md. Nuruzzaman

PURPOSE

The purpose of this study is to try to define the patterns of introduction and spread of cholera in a well defined and controlled rural area. In three contiguous Muslim agricultural villages, an attempt has been made to define, as far as possible, the sociologic and geographic relationships which may be important in the spread of cholera. With this information as background, the population will be followed through the cholera season. Bacteriologic and serologic testing will be used to detect the presence of cholera infection, and a control prophylactic trial of the effectiveness of tetracycline in preventing the introduction of cholera will be performed.

METHODS

A. Census

1. Each village is divided into baris. The baris are defined by the villagers and are usually related families living in one geographical area. The baris are to be numbered consecutively in each village beginning with 1, and the families to be lettered consecutively in each bari beginning with the letter A. Each individual in the family is then numbered consecutively beginning with 1.

In this way, a census number (i.e., Naogaon 15-B-5) defines the family and bari relationship of each individual and allows him to be geographically located on a map by bari.

2. Each individual will be included in the census and listed by name, age, sex, occupation and relationship to head of the family.

3. Occupation and location of work or school will be determined for appropriate members of the family.

4. A history of cholera vaccination or prior infection with cholera will be obtained for each member of the family.

5. Water sources for drinking, bathing, cooking, and washing clothes will be determined for each family and designated numerically according to a code which geographically locates the source.

6. Sources of major food items (rice, fish, dhal, oil, vegetables) will be determined for each family.

7. Level of education will be obtained for each individual.

8. Each area will be mapped so as to locate the families and water sources.

B. Routine Surveillance

Each house is visited each day by one dai and every other day by the field assistant responsible for the dai. Households will be checked for diarrhea cases and a rectal swab culture will be taken from each case. The field assistant will also search for all births,

deaths, and migrations and record them on the birth-death-migration form and the family census card. Births, deaths and migration information will be recorded in the village census book kept in the village so that this book will be completely updated at the end of each month.

Families will be questioned about guests coming into the household. (A guest is defined as any person, resident or not, remaining overnight in the village after having been away for more than 24 hours.) These will be recorded on a guest list by bari number, date of entry and place from which they came.

Water samples for bacteriology will be collected daily at five points along the canal running from the river through Dogarpur to Naogaon.

All bacteriologically positive cases of cholera and positive water samples will be reported immediately to the sanitary inspector in charge of the area who will investigate the case along with the principal investigator.

C. Social Studies

1. Naogaon Antibiotic Study

Baris totalling 2,000 persons have been selected in village Naogaon for an antibiotic study. All guests (as defined above) entering these baris will be given a 1 gram dose of tetracycline

per day for three days or until they leave the bari if less than three days. The remaining baris including 1,000 persons will be treated similarly using a placebo (multi-vitamin) preparation instead.

Drugs have been assigned according to the areas covered by the dais. The area of four dais will be assigned tetracycline and the remaining two, placebo. The assignment has been adjusted so that the antibiotic and non-antibiotic areas have the same proportion of persons using canal water and so that no water supply except the canal is common to both populations. In order to ensure antibiotics being given on the day on which the guest arrives, rounds are to be made twice per day in Naogaon. The morning rounds will be routine diarrhea rounds. Evening rounds will be a brief search for guests who enter during the day. Villages Nagda and Bogarpur will not be given antibiotics or placebo, but will be considered part of the control group.

2. Serologic Studies

Each person in the population will have a 50 lambda sample of fingertip blood taken in October and again after the cholera season. The date of the second bleeding will be decided later.

A random sample (probably 25%) of the pairs will be run for vibriocidal titer after the second bleeding. All pairs should be run in baris which are shown to have bacteriologically or serologically-proven cases of cholera.

ANALYSIS

All data listed above will be punched on I.C.T. cards following collection of the second serologic sample. A code has been set up for individual data and a separate code for family data. Analysis will center around bacteriologically and serologically positive cases. The following analysis will be made:

1. The ratio of cases detected by paired serology to those detected by case finding and rectal swabs will be found.
2. The case rate will be defined for the three villages for various occupational groups and for persons of different age groups, sex and religion.
3. The relationship of case rate to educational status will be determined.
4. The attack rates for users of different types of water supplies can be determined and this can be further broken down by water useage (i.e., a comparison of those who use canal water for drinking as opposed to those who use tube well water for drinking.)

5. Attack rates can be determined for each water useage group (i.e., Pond 5 or Canal 2). This will help to determine if individuals living in the same geographic area or within the same bari have differing cholera attack rates depending on the useage of water.
6. An attempt will be made to determine if cholera infections can be grouped by geographic locality or by source of food, as well as by water useage pattern.
7. Within common exposure groups, the relationship between serologic titer and infection rate can be determined.
8. The effectiveness of tetracycline treatment in the prevention and introduction of cholera by migrants will be observed.

PROTOCOL

ANTIBIOTIC TREATMENT STUDY

MATLAB HOSPITAL

1967 - 68

J.M.McCormack and A.S.M.Mizanur Rahman

RESEARCH PLAN

A. Specific Objectives

The objectives of this study are:

1. To assess the possible effect of chloramphenicol treatment on the antibody response to infection with the cholera vibrio.

2. To test a simplified regimen for cholera treatment suggested by D. Craig Wallace on the basis of his experience in Calcutta.

3. To test in all age groups furoxone and a low-dose tetracycline regimen which has been previously demonstrated to be effective in children.

B. Method of Procedure

All admissions to the Matlab Hospital except pregnant women and children under age one year will be included in this study. They will be assigned in strict alternation to the six groups listed. For the purpose of this study, a child will be considered one who is under ten years of age, and an adult a person ten years of age or older. The specific treatment groups are:

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	10 Years of Age and Above	Under 10 Years of Age
1.	1A No treatment daily serologies	1C No drug treatment daily serologies
2.	2A Tetracycline capsules 500 mg (2 caps) every six hours for 5 days Daily serologies	2C Tetracycline syrup 10 cc (250 mg) every six hours for 5 days Daily serologies
3.	3A Chloramphenicol capsules 500 mg (2 caps) every 6 hours, for 5 days Daily serologies	3C Chloramphenicol capsules 250 mg every 6 hours for 5 days Daily serologies
4.	4A Tetracycline capsules 1 Gm (4 caps) on admission 1 Gm (4 caps) 24 hours after admission	4C Tetracycline syrup 20 cc on admission 20 cc 24 hours after admission
5.	5A "Special" syrup 10 cc every six hours for 5 days	5C "Special" syrup 5 cc every six hours for 5 days
6.	6A Furoxone 100 mg Tab one tablet every six hours for 5 days	6C Furoxone liquid 5 cc every six hours for 5 days

Those patients who are negative for Vibrio cholerae on both "direct" and "enriched" examination of the initial rectal swab stool specimen will be dropped from the study and discharged when their clinical course indicates.

Rectal swab stool cultures will be obtained daily from all study participants. All study patients will be kept in the hospital for 12 days or until both the "direct" and "enriched" stool samples have been negative for V. cholerae for three consecutive days, whichever is longer.

On the day prior to discharge, mannitol will be given in doses sufficient to induce purging (15 to 40 Gm, depending on age) and not less than three liquid stool specimens cultured.

Admission and discharge venous blood samples will be obtained from all study patients. In addition, daily fingertip samples for serology will be collected from all individuals in Groups 1, 2 and 3.

C. Significance of the Research

The first three schedules are designed to compare the effect of tetracycline, chloramphenicol and control on the serologic response to cholera infection. Chloramphenicol is known under certain circumstances to inhibit the formation of antibodies. Several of the patients found by Dr. Gangarosa in Iran to be convalescent cholera carriers had been previously treated with chloramphenicol. Also, chloramphenicol was shown to be less effective in the treatment of childhood cholera than tetracycline by Lindenbaum, et al. While it is more likely that this was due to the use of chloramphenicol palmitate which was not hydrolyzed in the intestine of

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the cholera patient, the possibility that there is some inhibitory effect on antibody formation by chloramphenicol remains.

Schedule four has been recommended by Dr. Wallace as one which provides excellent clinical results with no bacteriologic failures. This regimen offers considerable logistic advantages over the presently recommended PSCRL five day treatment schedule and, therefore, it should be tested in a larger group of patients.

Schedule five will test low-dose tetracycline in all age groups. This schedule, which is a five-fold reduction in the usual amount of tetracycline given over the period of five days, was proven to be as good as the ordinary tetracycline treatment schedule in children by Curlin and Karchmer during the last cholera season. If these results can be confirmed in all age groups, this regimen could then be generally recommended.

Regimen six will test furoxone which was also found to be quite effective in children by Curlin and Karchmer last year. Furoxone offers some advantages over tetracycline, particularly in its stability, and it would be a useful addition to the cholera armamentarium.

D. Facilities Available

We would anticipate that the Matlab Hospital during the coming cholera season will admit at least 200 patients with bacteriologically documented cholera. This should be enough for the assessment of the effect of all of the schedules in this protocol. The hospital is staffed by a well trained full-time senior PSCRL physician and a trained nursing staff. Facilities are available to provide food and lodging for the

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patients who will be kept in the hospital for about 12 days. All of the clinical information (stool volume, intravenous fluid volume, length of diarrhea, length of bacteriologic positivity) which will be used to compare the effectiveness of the various regimens are routinely recorded on all patients. Serologic specimens will be examined in the serology unit at PSCRL, Dacca, using the microtechnique developed by Dr. Benenson.

SUPPORTING DATA

A. Previous Work Done on This Project

Tetracycline and other antibiotics have been shown in this and other laboratories to dramatically modify the clinical course of cholera. Baseline information on the normal serologic response to cholera infection has been obtained which will serve as a baseline for comparison with the results obtained in this study.

B. Personal Publications

None.

C. Results Obtained From Others

Lindenbaum has shown that tetracycline in all age groups and chloroamphenicol in adults are highly effective in the treatment of cholera, dramatically modifying the clinical course. Wallace has shown in a relatively small group of patients in Calcutta that the administration of one gram of tetracycline at the time of admission and one gram of tetracycline 24 hours after admission provided excellent results. Curlin and Karchmer in a study confined to the pediatric age group demonstrated

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that tetracycline in one-fifth the usual dosage gave results comparable to those obtained with the usual dose of tetracycline. They also found furoxone to be effective.

D. Justification of the Budget

The major expense involved in this study will be the cost of the antibiotics and the additional cost of maintaining the patients in the hospital for approximately one week longer than would otherwise be required. No permanent equipment will be needed. The increased burden placed on the CRL staff may necessitate the payment of overtime or the hiring of extra nursing personnel.

BUDGET

A. Personnel

1. Allowance for overtime by nursing personnel during the period of this study	1,000
B. Permanent Equipment	
None.	
C. Expendable Equipment	
1. Antibiotics	Rs 2,000
2. Food for 200 patients for an additional 7 hospital days	Rs 4,200
3. Miscellaneous Expendable Equipment (approximately)	Rs 500
D. Travel and Transportation	
No known expenses	
Total	Rs 7,700

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PROTOCOL

INTRADERMAL CHOLERA VACCINE

W. N. McCormack and R. K. Barui

Research Plan

A. Specific Objectives - to compare the serologic response to cholera revaccination administered by the intradermal route both by syringe and needle and by jet injector with the response obtained with subcutaneous administration of vaccine.

B. Method of Procedure - 75 adults who are over age 20 and who have been previously vaccinated against cholera will be selected from the population of the Balisera medical department in South Sylhet. These 75 individulas will be assigned to the following three groups by strict alternation:

Group 1	0.1 cc of cholera vaccine intradermally by jet gun
Group 2	0.1 cc of cholera vaccine intradermally by needle and syringe
Group 3	1.0 cc of cholera vaccine subcutaneously by needle and syringe

An 0.05cc sample of capillary blood for serologic analysis will be obtained before the injection of cholera vaccine and on day 7, day 30, day 60, day 90, day 120 and day 180 following vaccination. The 0.05cc finger-tip blood sample will be immediately placed in 0.45 cc of normal saline.

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Specimens collected in the field will be stored on wet ice until they are transported to the Dacca laboratory. In Dacca, the plasma supernate will be separated and frozen at -20 degrees centigrade until analyzed. Serum vibriocidal antibodies will be estimated using the micro technique developed by Benenson.

C. Background of the Study- Initial studies by the PSCRL have suggested that the intradermal route may be a satisfactory one for cholera revaccination although not for primary cholera vaccination. These preliminary studies were done using cholera vaccine administered by syringe and needle. This study will allow us to confirm the efficacy of intradermal revaccination as well as to compare the relative efficacy of administration via jet injector and syringe and needle.

D. Significance of Research - Intradermal revaccination, if efficacious, would allow a 5 to 10-fold reduction in the amount of vaccine required for revaccination. This would be of economic advantage when large groups are to be vaccinated. In addition, it would probably reduce those systemic reactions to vaccination which are dose related. As intradermal vaccination by syringe and needle is a tedious process, the intradermal method cannot by recommended unless the results obtained with the jet injector apparatus are equally good.

E. Facilities Available - The Balisera Medical Department in South Sylhet offers a well defined population which has received cholera vaccine intermittently and would be quite satisfactory for a study of the effect of revaccination. The serology unit of the Epidemiology

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Section can process the specimens for the estimation of vibriocidal antibodies using the micro technique.

Supporting Data

A. Previous Work Done on This Project

In a study which was performed in the Balisera Medical Department in South Sylhet during 1966, it was shown that intradermal cholera vaccine administered by syringe and needle was not a satisfactory method of primary vaccination, whereas revaccination using intradermal cholera vaccine given by syringe and needle produced a mean vibriocidal titer as high as that produced by 2.0 cc of cholera vaccine given by the subcutaneous route.

B. Personal Publications - None

C. Results Obtained by Others - Noble (J.HYG.C.MB., '62:11,1964) has shown that the protection afforded by cholera vaccine administered by the intradermal route as demonstrated by active and passive mouse protection tests, active guinea pig protection tests and agglutination titers is equal to that given by subcutaneous innoculation. This confirmed the earlier work of Panje and Das (1947) and of Singer, Wei and Hoa (1943) who found better antibody formation after intradermal vaccination than after innoculations by the subcutaneous route in human volunteers. (Pollitzer, 1959)

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Budget

1.	Transportation and per diem for collection of the specimens	Rs 2,000
2.	Time of one serology unit technician for two weeks	Rs 300
3.	600 microcaps, 600 vials, cotton, spirit and other miscellaneous expenses.	Rs 700
		Rs 3,000

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PROTOCOL

PURGING OF FAMILY CONTACTS OF CHOLERA CASES

R. Barui and A.M.McCormack

Research Plan

A. Specific Objectives: To do a comparative study of multiple rectal swabs, whole stool examination and purging as techniques for the demonstration of V.cholerae in individuals with inapparant infection.

B. Method of Procedure : Soon after admission to the P-SCRL hospital, patients likely to be positive for V.cholerae will be selected on the basis of their clinical signs. A study team comprising one physician and one lady field assistant will visit the home of each patient and bring as many members as possible from the family to the P-SCRL hospital on the same day where a rectal swab will be taken from each member(definition of family is:- Related persons who eat and live together). The swab will be streaked directly on Gelatin Agar media and Monsur's Media (Na-Taurocholate & K-Tellurite + Gelatin) and then placed in Bile Peptone broth for subculture. These persons will then be made to purge by taking manitol in water by mouth (dose 15 g to 40 g in one single dose depending upon the age of the patient). Subsequent stools (upto 4 samples) will be collected in sterile bottles and sent for V.cholerae culture. These contacts will be kept in the hospital for 7 additional days. On the 5th hospital day they will be made to purge for the second time by using manitol in the same manner.

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A daily rectal swab for culture will be collected during their stay in the hospital. In addition, a formed stool will be examined for V.cholerae daily.

Background of the Study

Initial studies of a group of families of cholera patients admitted to the P-SCRL hospital in Dacca during the last few years have revealed some interesting features concerning subsequent occurrence of V.cholerae infection. 20% of family contacts were found to be infected. The rate is higher than in community contacts but whether this is due to common source exposure or person to person spread within the family is uncertain.

Significance of Research:

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

Facilities Available :

During the coming cholera season, we expect a sufficient number of cholera patients in P.SCRL hospital whose families can be studied. Bacteriology Section will process the routine culture for V.cholerae.

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Accommodation for the family contacts can be arranged in the convalescent ward.

Supporting Data:

A. Previous Work Done on This Project:

During the last cholera season 46 contacts from 12 families of 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 became positive after purging, 5 persons were found to be negative on both initial rectal swab and on purging but become positive on subsequent daily rectal swabs.

B. Personal Publication - None

C. Results Obtained by Others - Gangarosa et al (1966) reported that eight (21%) of thirty-eight patients purged 1-2 weeks after their last routine positive stool culture were vibrio positive. Wallace et al (1967) reported that 2 of their 23 cases were found to be vibrio positive after purging and duodenal intubation.

BUDGET :

Cost of Material	Rs	650.00
Fooding expenses for family contacts	Rs	1750.00
Salary of field team ...	Rs	3000.00
	Rs	5400.00

(Rupees five thousand four hundred only (approx.))

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PROTOCOL
AN ASSESSMENT OF THE RISK OF HOSPITAL AND LABORATORY INFECTIONS WITH V.CHOLERAE

R. Barui and W.M.McCormack

Research Plan

A. Specific Objective:

To determine the risk of inapparent infection of cholera among persons working in the hospital and in the laboratory.

B. Method of Procedure:

For the purpose of the study all the volunteers in the laboratory will be divided in 3 groups:

1. Those who are exposed directly to cholera patients
2. Those who are exposed indirectly (in laboratory).
3. Those who are not exposed.

50 lambda of finger blood in 0.45 cc of N/ saline will be collected from volunteers in each group in July, 1967 and then monthly during the coming cholera season from November until March, 1968. The blood samples will be sent to serology unit where serum will be separated and frozen until processed to measure the amount of vibriocidal antibody by the microtiter method devised by Dr. Benenson. A history of recent cholera vaccination will be taken from each person.

A 4-fold rise of titer not associated with cholera vaccination will be considered as an evidence of infection with V.cholerae.

C. Significance of This Survey:

It has been said that there is no danger of person to person spread of cholera in the hospital ward. However, it has been observed that when

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there is a cholera case in a family, other members of that family are uniquely at risk. Family contacts yield positive stool culture for V.cholerae with or without symptoms. A 4-fold rise in vibriocidal antibody titer is seen in both symptomatic and asymptomatic cases. This survey will allow us to assess accurately the risk of becoming infected with V.cholerae in the hospital and laboratory situation.

D. Facilities Available:

It is expected that a good number of volunteers in the laboratory will be available to cooperate in this survey. Serology unit will process the blood samples for vibriocidal antibody and the data will be analyzed in the statistics unit of the Epidemiology Section.

Supporting Data

A. Previous Work Done on This Project: None

B. Personal Publication: None

C. Results Obtained by Others: To our knowledge a study of this type has not previously been conducted.

D. Justification of The Budget:

We expect that 120 people will be involved in this survey for 6 months. This will include 7 monthly blood specimens from each person for estimation of vibriocidal antibody. Four laboratory

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technicians and one physician will be required to work for this survey.

BUDGET

Cost of material to be used	Rs 410.00
Salaries of persons involved	640.00
Total	Rs 1,050.00

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PROTOCOL

LONG-TERM SEROLOGIC RESPONSE TO CHOLERA INFECTION

W.M.McCormack, W.H.Mosley, Ansaruddin Ahmad

Research Plan

A. Specific Objective:

To determine the duration of vibriocidal antibody response following infection with Vibrio cholerae and to relate this response to vaccine status, age, pre-existing antibody titer, and antibiotic treatment.

B. Method of Procedure

During the 1966-67 cholera season, every patient admitted to the Matlab Hospital with bacteriologically documented cholera will be included. A report of each included patient will be forwarded by the Matlab Hospital Ward Staff to the VTS office. Serologic specimens will be collected in the field one month, two months, three months, six months and nince months following admission. An additional sample will be collected on day 10 following admission for those patients who were discharged from the hospital before the 7th hospital day.

Serologic specimens will be collected from fingertip blood with a 50 lamda capillary pipet. They will be immediately discharged into 0.45 cc of sterile saline. Specimens will be kept on ice in the field and during transportation to the P-SCRL laboratory in Dacca where the plasma supernate will be decanted and frozen at -20 degrees centigrade until analyzed. Vibriocidal titers will be determined by the microtechnique developed by Dr. Benenson.

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In the statistics unit of the Epidemiology Section, a punch card will be prepared for each participant and information will be recorded regarding age, sex, vaccine status and antibiotic therapy. These factors will be correlated with the vibriocidal titers.

C. Significance of the Research

Evidence is accumulating that the vibriocidal antibody titer is related to (although perhaps not responsible for) protection from cholera. If this is the case, then there is considerable benefit in determining the length of immunity as measured by the vibriocidal titer following natural infection with the cholera vibrio.

D. Facilities Available;

It is anticipated that over 100 patients with documented cholera will be admitted to the Matlab Bazar Hospital from the villages of the vaccine trial area. Patients so located in the vaccine trial area are readily available for follow-up collections. No difficulty is anticipated in obtaining fingertip blood on repeated occasions from cholera patients who have been treated in the hospital. Therserology unit of the Epidemiology Section P-SCRL Dacca, can process the specimens. The statistics unit will be able to analyze the data using the I.C.T. equipment available.

Supporting Data

A. Previous Work Done on This Project:

A preliminary analysis of 37 sera collected at various intervals up to three months after cholera injection has suggested that the vibriocidal titers in cholera patients are not at all long-lasting.

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B. Personal Publications : None

C. Results Obtained by Others:

Benenson and Sack have analyzed small groups of patients from whom sera was obtained upwards of three months following cholera infections. No long-term stydies have been done.

D. Justification of the Budget:

The major expense will be time of one sanitary inspector in Matlab who will devote about half of his time to the collection of these chronic serum samples in the field. Also 1,000 vibriocidal antibody titer determinations will have to be performed.

Budget

A. Personnel

1. One sanitary inspector, half-time, for one year	Rs 2,400
2. One serology technician for approximately one month	500
B. Permanent Equipment: None	
C. Expendable Equipment: Miscellaneous supplies required for the collection of and performance of 1,000 vibriocidal antibody titers	Rs 1,000
Total	Rs 3,900

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PROTOCOL E/P8

SEROLOGIC STUDY OF BACTERIOLOGICALLY PROVEN CHOLERA CASES AT VARIOUS INTERVALS AFTER ILLNESS

A. Martin and A. Momin Chowdury

PURPOSE

To determine the duration of immunity, as measured by vibriocidal micro titer technique, in a group of proven cholera cases at various intervals after illness. This group would include cases with mild or asymptomatic infection as well as with hospitalized illness.

Materials, Methods and Personnel

1. Vibriocidal micro titer technique
2. Equipment for collecting fingertip blood
3. Transportation - one car and driver for use in Dacca area 5 days/week
4. Records from 3 previous family studies and Rayer Bazar studies
5. Male field worker - supervisor and one or two female field assistants.

PROCEDURE

1. Bacteriologic proven cases, either hospitalized or field cases, will be selected from records of previous family studies (Dr. Mosley 1965-1966; Dr. McCormack 1966-1967; Dr. Martin 1966-1967) and from cases detected in the Rayer Bazar area.

2.

a) when cases can be located a single finger tip blood will be collected for serology.

b) where possible, a single finger tip blood will be collected from other members of the patient's family.

3. History of cholera infection since the time of illness will be elicited.

Analysis of Data:

1. Comparison of antibody profile of this population with bacteriologically proven cholera with serologic survey of East Pakistan population (Matlab).
2. Comparison of hospitalized vs mild or asymptomatic cases in regard to maintainace of antibody titer.
3. Comparison of antibody profile of bacteriologically proven cholera cases vs uninvolved family members.
4. Comparison of cases occurring in 1965-66 season vs those occuring in 1966-67 season.
5. In those cases here serologic data was available at time of illness, comparison of acute vs. convalescent vs. long term titer.

Conclusions:

This study should provide data as to what infection with cholera does in terms of producing sustained immunity in various age groups and determine whether severity of infection is a factor in determining sustained immunity.

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DETECTION OF REINFECTION WITH V. CHOLERAE IN THE MATLAB VACCINE TRIAL AREA

E/P9

By A. Martin

The duration of immunity resulting from one cholera infection is not known. Work performed at the PSCRL during the past cholera season indicates that it is not lifelong and in fact may be quite short. Serum samples were taken from persons with cholera at various times after infection. It was shown that most individuals developed a significant rise in titer after infection, but that after four to six months, more than 50% no longer had a vibriocidal antibody titer against cholera. Since the vibriocidal titer has been shown to correlate well with immunity to cholera, it is very possible that these individuals are again susceptible to cholera infection.

Further evidence that multiple infections occur comes from the fact that 80% of adults eventually develop a sustained vibriocidal titer against cholera. If the chance of developing a sustained titer is less than 50% for one infection, then it seems reasonable to assume that multiple infections are necessary for such a high level of vibriocidal antibody to be present in adults.

As the attack rate for clinically apparent cases of Vibrio cholerae is quite low (1 to 2 per 10,000 in Dacca city, and 2 to 3 per 1,000 in Matlab VTS), the chance of detecting a second clinical

case of cholera in one individual is very small. However, inapparent cases, detected serologically, are much more common (probably greater than 5% per year in Matlab VTS). Therefore, paired serology provides a practical means of looking for reinfection. If paired sera are taken from 300 to 500 persons who have been previously documented as having cholera infection, and if at least 50% are again susceptible to cholera, we can easily expect to establish the occurrence of re-infection.

Of equal importance is whether or not the vibriocidal titer has the same relationship to susceptibility in these individuals as in the general population. From other serologic studies which will be carried out during the season, it will be possible to determine the expected number of cases among persons in the general population with a given titer. Applying these figures to this study population will indicate whether the same relationship of titer to susceptibility exists.

METHODS

The name, age, sex, date of infection, census number and case status (hospitalized or field case) of all bacteriologically confirmed cholera cases reported from Matlab Vaccine Trial area during the past four seasons will be obtained from the records.

These individuals will be listed by census number, with a separate sheet for members of each village. Columns will be provided on the sheet to designate vaccine given in 1966 or 1967, during the present vaccine trial.

Individuals who will have paired serology performed next season for other studies (Meharan, Vaccine Trial, Naogaon-Nagda-Dogarpur Study, Chronic Serology) will not be bled again, but the date and results of their test will be recorded for use in this study. All remaining persons will have 0.05 ml fingertip blood collected for vibriocidal titer in October, 1967 and again in January or February, 1968 (date to be determined). Data will be processed to detect individuals with a four-fold or greater rise in vibriocidal titer between the two collections. Individuals will also be grouped by titer present at the first reading. Attack rates when determined for each titer group will be compared with other serologic surveys performed in the VTS area.

CONCLUSIONS

Given the usual rate of inapparent cases in the Matlab area, one would expect a minimum of 25 cholera infections among 500 individuals. Even if only half of these individuals are susceptible to cholera (because of previous known infection or vaccination), one would still expect to see 10-15 infections in this group. It,

therefore seems likely that by doing paired vibriocidal titers on 500 former cholera cases, one should be able to document reinfection if, in fact, these people are susceptible as indicated by their titer. If sufficient cases occur, it will also be possible to determine whether the relationship of vibriocidal titer protection is the same among persons known to have had cholera infection and in the general population.

These studies should contribute significantly to our understanding of the development of immunity in the population and clarify the relationship between serologic titer and protection.

E/P10

PROTOCOL

1966 CHOLERA VACCINE FIELD TRIAL
1967-68 SEROLOGIC SURVEYS

W. McCormack and W. H. Mosley

RESEARCH PLAN

A. Specific Objectives

1. To determine the mean vibriocidal antibody titers of the various vaccine trial study populations before and after the administration of this year's dose of cholera vaccine.

2. To relate the mean antibody titers present in each of the vaccine groups to the cholera case rates in these groups.

3. To estimate the frequency of inapparent cholera infections occurring in the control group.

B. Method of Procedure

During September 1967, before the administration of this year's dose of cholera vaccine, a serologic survey will be conducted. All children under age 15 in every 30th household will be included. A 0.05 ml sample of fingertip blood will be collected from each subject. This blood will be discharged immediately into 0.45 ml of saline in a sterile screw cap vial. Specimens will be kept on ice in the field until transported to the serology laboratory in Dacca where the plasma supernate will be separated and frozen at -20° C. until analyzed.

Following vaccine administration in October, a second serologic sample will be collected in the same manner from the same children. A third and perhaps a fourth sample will be collected from these same children during the cholera season as indicated by the occurrence of cholera. Vibriocidal titers on matched specimens will be determined by the microtechnique developed by Dr. Benenson.

C. Significance of the Research

Further information concerning the relationship of antibody titer as measured by the vibriocidal test to susceptibility to cholera will be obtained. In addition, the availability of serum samples from a large number of individuals in an endemic cholera area will enable us to estimate the number of inapparent serologic conversions that occur.

D. Facilities Available

In the vaccine trial area where an accurate census is available, there will be no difficulty in locating each of the approximately 1300 individuals included in this survey. Rapport is such that obtaining several samples of fingertip blood from the same individuals will not be difficult. Transport of the samples from Matlab to Dacca will present no difficulty. The serology unit of the epidemiology section will be able to process the specimens.

SUPPORTING DATA

A. Previous Work Done on This Project

During the first portion of the 1966 cholera vaccine field trial, unpaired serologic samples were obtained before vaccination and twice during the cholera season. The comparability of the groups prior to vaccination was demonstrated. The differences in mean antibody titers at various points during the vaccine trial were related to the cholera case rates.

In addition, a comparison of the titers in the control population in January, i.e., at the height of the cholera season, with those in the control population before the cholera season suggested that there were a large number of serologic rises among the control population. Although the samples were not paired, the sample size was so large as to suggest that this was a valid observation. The possibility that this might have been due to the inappropriate administration of cholera vaccine to those persons who were scheduled to receive the control preparation was considered, but would appear to be unlikely. In any event, the collection of the samples this year after the administration of cholera vaccine but before the beginning of the cholera season will enable us to eliminate this objection.

B. Personal Publications

The results of last year's cholera vaccine field trials are being prepared for publication.

C. Results Obtained from Others

There is little else in the literature concerning the relationship of vibriocidal antibody titer to susceptibility to cholera. As far as we know, the serologic approach has not been previously utilized by others to determine the relative frequency of inapparent cholera infections.

D. Justification of the Budget

Each serologic collection will require four teams of two men each who will have to be paid a food allowance for a period of approximately three weeks. Materials for process of approximately 5000 to 7000 serologic specimens will be required.

BUDGET

A. Personnel

1. Serologic collection teams	Rs 3,000
2. One serologic technician for approximately two months	1,000

B. Permanent Equipment

None.

C. Expendable Equipment

Miscellaneous supplies required for the collection and performance of approximately 6000 vibriocidal antibody titers	6,000
TOTAL	₦10,000

PROTOCOL E/P11

A COMMUNITY STUDY OF INAPPARENT CHOLERA INFECTIONS

1967-68

W. M. McCormack and Md. Shafiqul Islam

Research Plan

A. Specific Objectives:

To further study in an endemic area community the number of inapparent infections with the cholera vibrio which occur during an outbreak of cholera, i.e.,to estimate the infection: case ratio.

B. Method of Procedure:

Village Meharan, population 1608, will be studied again this year. This is a Hindu fishing village in Matlab, Comilla District, just outside of our vaccine trial area. A 0.05 ml sample of fingertip blood will again be collected from each available resident of the village before the cholera season. Intensive surveillance will be maintained for diarrheal disease by four female field workers who are residents of the village under the direct supervision of a health assistant. A rectal swab stool culture for V.cholerae will be obtained from each individual with diarrhea of acute onset. Patients with severe diarrhea will be referred to the PSCRL Hospital at Matlab Bazar. At the end of the cholera season, a second serologic sample will be collected from each available resident. Those individuals who have a two tube(four fold) or greater rise in antibody titer between the two samples will be presumed to have become infected

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with vibrio cholerae in the interim. We will have the number of actual cases of cholera from the daily surveillance and will thus be able to estimate the infections: case ratio.

In addition, about 100 children under age 10 will be selected by taking the name of every fifth child in the census book. From each of these children, a rectal swab for cholera culture will be obtained daily during the cholera season. A note will be made each day as to the presence or absence of diarrhea on that day, but specimens will be collected from each subject daily regardless of symptoms.

All specimens collected in the field will be collected with a tellurite impregnated rectal swab and placed directly into bile peptone transport medium. Specimens will be transported daily to the PSCRL Laboratory in Matlab Bazar. After overnight incubation, they will be subcultured onto TTGA and gelatin agar and examined for V.cholerae.

C. Significance of the Research

This continuation of the study in Meharan with the addition of daily rectal swabs on a number of the children should enable us to better define the relative frequency of inapparent cholera infections. Bacteriologic confirmation of some of the inapparent infections detected by examination of the paired serum specimens will provide an indication of the specificity of a rise in vibriocidal titer.

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D. Facilities Available

With the exeeption of the addition of daily rectal swabs on some of the children, the study will be exactly the same as that which was performed in Meharran during the 1966-1967 cholera season. Rapport with the villagers is excellent. We anticipate no difficulty in carryihg out this continuation of the study program. Serologic samples can be processed by the serology unit, Epidemiology Section, PSCRL.

Supporting Data:

A. Previous Work Done on This Project:

During the 1966-67 cholera season, we were able to show that there were five mild(non-hospitalized) cases of bacteriologically confirmed cholera in village Meharan. Analysis of the serum pairs obtained from 954 individuals before and after the cholera season revealed an additional 30 individuals who had a four-fold or greater rise in vibriocidal titer. These individuals were clustered geographically around the patients with bacteriologically confirmed cholera, and a number of them also had rises in toxin neutralizing antibody titer. This observation of a relatively large number of inapparent cholera infections in a village which had no cholera serious enough to require hospitalization, leads us to believe that inapparent infection with the cholera vibrio is more common than previously recognized.

B. Personal Publications:

The results of the first year's study are being prepared for publication.

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C. Results Obtained by Others

Studies in the Philippinos by Mosley et al in which community contacts of cholera patients were examined for the presence of vibrio cholerae indicated that the percentage of individuals with inapparent infection was relatively low (about 2%).

D. Justification of the Budget:

An additional two female field workers will be required because of the addition of daily rectal swab collection from some of the children. The present field team consisting of one field assistant and two female field workders will continue to work. A serologic survey team will have to work in the village for approximately six days to collect the samples. The serology laboratory will be required to process approximately 3000 sera.

BUDGET:

A. Personnel :

Serologic survey teams, 8 people, for one week	Rs 500
Health assistants, 1 for 4 months	4,000
Female field workers, 4 for 4 months	1,000

B. Permanent Equipment - None

C. Expendable Equipment

Miscellaneous equipment necessary for the collection of serologic specimens and the performance of the vibriocidal test approximately	2,000

D. Travel and Transportation

No additional cost

TOTAL	Rs 7,500

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PROTOCOL

THE 1967 CHOLERA VACCINE FIELD TRIAL

W.H. Mosley and W. McCormack

Introduction and Specific Aims:

The 1967 vaccine field trial will be a follow-up of the studies initiated in 1966 with the specific objectives of determining the duration of protection produced by a two-dose schedule of cholera vaccine through a second cholera season and determining the effect of a booster dose of cholera vaccine given prior to the 1967 cholera season. Serological surveys will be continued as a part of this trial to further relate the protection produced by the vaccine to the vibriocidal antibody response to the vaccine.

Methods

The 1966 field trial included three vaccine groups. Group 00 included approximately 10,000 children who received two dose of diphtheria-tetanus toxoid. Group X0 included 10,000 children who received one dose of standard cholera vaccine followed by an injection of diphtheria-tetanus toxoid. Group XX received two doses of cholera vaccine at an interval of approximately 30 days. Group XX was twice as large as the other two vaccine groups, consisting of approximately 20,000 children. In the 1967 trial a booster injection will be given to the study population. There will be four vaccine groups. Group 000 - this is the control group 00 of the 1966 trial which will be given a booster dose of diphtheria-tetanus toxoid. Group X0X - this is the one dose group which will receive a booster dose of cholera

vaccine prior to the 1967 cholera season. Group XXO - this group consists of one-half of the children in Group XX in the 1966 trial, who will receive a booster dose of diphtheria-tetanus toxoid. Group XXX - this represents the other half of Group XX which will receive a booster dose of cholera vaccine.

Standard commercial cholera vaccine will again be used in this year's study in a dosage of 1/2 ml. to all trial participants. The dosage of diphtheria-tetanus toxoid will also be 1/2 ml. for those persons in the control groups.

The vaccine campaign for the 1967 trial was initiated on September 20, 1967 and was completed by October 30th. Preliminary tabulations indicate that 97.4% of the participants in the 1966 field trial were given a booster injection and will thus be included in the 1967 field trial. A serological survey was conducted in September prior to the booster injection and additional surveys will be carried out during November, January and May.

The methods of surveillance for cases in the hospital and in the field have been described in detail in the original field trial protocol.

Significance of This Research

The 1966 field trial demonstrated that a single injection of cholera vaccine lowered the case rate by 47%, while two injections

had a protective efficacy of 66%. The enhanced protection in the two-dose schedule was primarily due to its effect on children under the age of five years. Although the number of cases was small, the data from the 1966 field trial suggested that protection in both the one and two-dose schedules had disappeared almost completely within four to six months after injection. The 1967 field trial will involve a follow-up of 10,000 persons who received two doses in 1966 to determine if there is any residual protection left in this population one year after immunization. Booster doses will be given to the group that received only a single injection of cholera vaccine in 1966, as well as one-half the group that received two doses. By this means, we will be able to evaluate the effectiveness of an annual booster prior to the cholera season. In addition, it will be possible to determine whether or not there is a difference in the booster response in individuals who have been primed with only a single dose of cholera vaccine as compared to those who have been primed with two doses of cholera vaccine a year previously.

Facilities Available and Budget

This has been outlined in detail in the original protocol for the 1966 vaccine field trial.

PROGRAM AND PROTOCOLS FOR THE IMMUNOLOGY LABORATORY

INTRODUCTION:

The Immunology Laboratory of the Epidemiology Section has two major objectives. The first is to provide the laboratory support for the epidemiological field studies including the development and refinement of new serological tests which can be used in the field. Studies over the past year have clearly demonstrated the value of serological tests, particularly the vibriocidal antibody titration test in identifying inapparent infections, and in estimating the protection afforded by cholera vaccine. Field studies set up this year with particular emphasis on searching for inapparent infections will require vibriocidal antibody titrations on approximately 20,000 specimens.

Recent observations have suggested that the vascular permeability factor (toxin) neutralization tests as devised by Dr. Craig, may prove to be a valuable tool in the field in defining patterns of spread of cholera. Thus, the Immunology Laboratory is working toward the standardization of this test so that it can be run on a fingertip blood specimen. More recently, there has been data suggesting that opsonizing antibody may be important in protection against Vibrio Cholerae and, more specifically, may be active in intestinal secretions. Thus, the Laboratory is involved in setting up and standardizing opsonizing antibody tests which could be utilized in support of field studies of immunity in cholera.

The second main objective of the Immunology Laboratory is to define the mechanisms of immunity that are active in protecting an individual against cholera. This involves characterization of the immunoglobulins involved in the antibody response to cholera, defining the antibody activity associated with these immunoglobulins, and attempting to define which antibodies and antibody activities are important in protecting man from intestinal infection with Vibrio cholerae. The following procedures have been set up and are being standardized in the Immunology Laboratory.

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1. Routine electrophoresis and immunoelectrophoresis of serum proteins.
2. Ouchterlony double diffusion techniques.
3. Single and double immunodiffusion techniques for quantitating of serum proteins.
4. Chromatography of serum proteins on Sephadex G 200.
5. Density gradient separations of serum proteins.
6. Reduction of macroglobulins with 2-mercaptoethancl.
7. Measurements of antibody activity by the vibriocidal agglutination and opsonization test.
8. Titration of vascular permeability factor (toxin) neutralizing antibody.

The following are specific studies which are being carried out during the 1967-68 fiscal year.

1. Quantitation of Total Protein and Immunoglobulins in Acute Cholera Stool - Dr. Ahmed and Mrs. Molla :

Preliminary observations in this laboratory have already demonstrated that gamma-A globulin is the major immunoglobulin in acute cholera stool. This will be a systematic follow-up of that observation in a series of 10 acute cholera patients. Measurements will be made of the total stool volume in these patients in the first 24 hours, the total protein content, and the amounts of immunoglobulins in the stool, to prepare a report on the protein and immunoglobulin loss by acute cholera patients in the first 24 hours of hospitalization.

2. Further Studies on the Characteristics of Vibriocidal, Agglutination and Vascular Permeability Factor Neutralizing Antibody in the Serum of Acute Cholera Patients -

Dr. Bhattachargee, Dr. Ahmed, Dr. Mahmoud :

This is a continuation of the studies of fractionation of serum antibodies in cholera that have been going on for the past year. Specific studies will be : first, the separation and characterization of the antibodies in cholera by density gradient ultracentrifugation, and a comparison of these results with results of sephadex fractination..

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Second will be a study of the heat lability of gamma-M and gamma-G globulin antibodies, and a comparison of the results of heat(65°c. 1/2 hour) with treatment with 2-mercaptoethanol. These studies will be carried out on whole serum as well as fractions from sephadex chromatography and density gradient untracentrifugation.

Observations in the literature suggest that heat lability may be a more specific (and simpler) method than 2-mercaptoethanol for distinguishing between gamma-M and gamma-G antibody. If this is the case, then this simple procedure could prove a valuable epidemiologic tool in the study of serum specimens from serologic surveys since natural (non-specific) antibody against many bacteria is characteristically gamma-M globulin whereas specific antibody is found in the gamma-G as well as the gamma-M globulin fraction.

3. Studies on Opsonizing Antibody in Cholera Serum and Stool -

Miss Diana Bowen and Dr. Ahmed

A report by Dr. Derrick Rowley has suggested that immunity in the rabbit loop could be associated with the presence of opsonizing antibody demonstrable in the intestinal secretions. We plan to extend these observations in this laboratory. Methods for demonstrating opsonizing antibody are being established. An in vivo model that is presently being set up is the clearance of intraperitoneally injected vibrios in mice. In vitro techniques that are being established will involve the direct demonstration of phagocytosis by macrophage preparations from mouse or rabbit peritoneum. Once the model has been established, the development of opsonizing antibody will be studied in acute and convalescent serum from cholera patients as well as from individuals receiving cholera vaccine. The serum will be fractionated by column chromatography and density gradient ultracentrifugation to study the immunoglobulin characteristics of the opsonizing antibody. In addition, acute and convalescent stool specimens will be taken from cholera patients to see if opsonizing activity is demonstrable in these specimens. Again, if activity is demonstrable in these specimens,

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then the stool proteins will be fractionated to determine which immunoglobulins are active.

4. Cytological Studies of Cholera Stool and Intestinal Secretions with Particular Reference to Phagocytic Cell Population - Dr.Ahmed

With the observation by Dr. Rowley that opsonization may be important in intestinal immunity, it is important to study the cell population in the intestinal secretions to determine if adequate phagocytic cells are present. Stools and intestinal secretions from acute as well as convalescent cholera patients will be collected and processed to determine the numbers of phagocytic cells and also to observe the activity of the phagocytic cells in terms of ingested vibrios.

5. A Survey of Vascular Permeability Factor Antibody in Various Population Groups - Dr. Mahmoud and Dr. Ahmed

Recent studies by the Epidemiology Section suggest that while vascular permeability factor antibody cannot be associated with protection against cholera, it may prove to be a valuable epidemiologic tool in identifying infection with Vibrio cholerae. The PF neutralizing test has been re-standardized in our laboratory so that precise titrations of PF antibody can be determined from fingertip blood specimens. This study will involve the titration of PF antibody in the sera of various population groups that we have in our serum file including specimens from Czechoslovakia, United States, Philippines, West Pakistan, as well as several districts in East Pakistan. Based on these studeis, it will be possible to determine the epidemiologic significance of PF neutralizing antibody in terms of exposure to Vibrio cholerae. These titrations will also be performed on the serum specimens from the serological surveys in the vaccine field trial as well as in the field studies set up this year.

The epidemiologic evidence to date indicates that the PF neutralizing antibody titrations in conjunction with vibriocidal antibody titrations from fingertip bloods may prove to be a highly specific tool for identification of inapparent infections with Vibrio cholerae, particularly

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since PF neutralizing antibody is not influenced by the present-day cholera vaccines.

6. Study of "Diarrhea Toxin" Neutralization in Human Sera and the Relationship to Vibriocidal and Vascular Permeability Factor Neutralizing Antibody - Dr. Ahmed, Dr. Mahmoud, Dr. Bhattarcharjee, in collaboration with Dr. Cash and Dr. Nalin

Studies by both Dr. Carpenter and Dr. Finkelstein have suggested that animals immunized with cholera culture supernatants can be protected against challenge both by the diarrhea toxin as well as by living vibrios. Epidemiologic studies in man have suggested that protection from cholera may be due entirely to anti-bacterial antibody, particularly since vaccines which apparently do not contain the toxin antigen are protective. It has been shown that man produces antibody against vascular permeability factor during the course of cholera, however, epidemiological studies have demonstrated that this antibody cannot be associated with protection from cholera. Therefore, it is of great interest to determine whether or not cholera patients develop an antibody which neutralizes the factor producing diarrhea in the various animal models.

This study will initially utilize the rabbit loop model. A standardized vibrio challenge and a standardized toxin challenge will be established to produce uniformly positive loops. The vibrios as well as the toxin preparation will then be treated with convalescent cholera serum prior to challenge of the rabbit loops. If there is protection, the protective serum would be fractionated by sephadex chromatography and the gamma-M and the gamma-G fractions would be studied separately. The protective capacity of the serum against both vibrio and toxin challenge would be related to the vibriocidal and PF neutralizing antibody titers. If convalescent cholera serum protects against both vibrio challenge and toxin challenge, these studies would be extended to examine serum from vaccinated Americans who have not had intestinal infection with Vibrio cholerae and who do not have PF neutralizing antibody.

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Based on the observations of other investigators, it is reasonable to expect in this study that any serum which has high anti-bacterial antibody as measured by the vibriocidal titer will protect the rabbit loop against vibrio challenge. This antibody may be induced either by natural infection or by vaccination. The exact mechanism of protection has not been defined. Whether or not the convalescent cholera serum will neutralize the diarrhea toxin is unknown. Presumably, if the toxin is antigenic, it should. If there is neutralization, it will be of great interest to study serum from individuals never naturally infected with Vibrio cholerae but immunized with a proven potent cholera vaccine. The high potency field trial vaccine will be used. This vaccine is known not to produce antibody against vascular PF.

By these studies, it may be possible to determine whether or not there are two mechanisms whereby antibody protects against cholera, the first being anti-bacterial immunity which is produced both by natural infection and by vaccination, and the second possibly an anti-toxic immunity which may or may not be produced during the course of natural infection and with the vaccines now in use. If two mechanisms of immunity are defined, the relationship between anti-bacterial and anti-toxic immunity can be studied and more particularly the relationship between antibody against PF and against diarrhea toxin can be defined.

7. Chemical Characterization of the Antigenic Groups of Vibrio cholerae - Dr. Bhattacharjee.

This study will involve progressive fractionation of the antigens of Vibrio cholerae by chemical techniques. The assay system to be used to follow the fractionation procedure will be the vibriocidal inhibition test as devised by Dr. Finkelstein. This test which depends on the binding of

antigen by specific antibody has been shown by Dr. Verwey to be a useful method of quantitating antigen even when it is available as a haptene and no longer antigenic. It is hoped that by this procedure of successive breakdown of the antigen the smallest chemical unit that will specifically bind antibody can be defined and characterized. It may be possible to determine if there are differences in the chemical characteristics of Ogawa and Inaba antigen by this procedure. Likewise if the haptene is a simple molecule such as has been found in similar studies with Salmonella typhimurium, it may be possible to synthesize this molecule and artificially couple it to a large carrier molecule and prepare an artificial antigen which could be useful as a vaccine. Again, such a procedure has been carried out with Salmonella typhimurium antigen in mice.

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TECHNICAL COMMITTEE MEETING
FY67

VISITORS TO PSCRL

I: VISITORS ATTENDING PSCRL MEETINGS.

1. Clinical Investigation Committee, November 1966.

Professor Douglas A.K. Black	Manchester University, England.
Dr. Carl V. Moore	Washington University, St. Louis.
Lt. Colonel S. M. Bokhari	National Health Laboratories, Islamabad.

2. Technical Committee, February, 1967.

Dr. Colin MacLeod	Vice President for Medical Affairs, Commonwealth Fund, New York.
Dr. J. W. Howie	Public Health Lab. Service, London.
Dr. Ali Nawab Khan	Deputy Director General of Health, Govt. of Pakistan.

3. Directing Council, March, 1967.

Colonel S. A. Mallick	Director of Health Services, Govt. of East Pakistan.
Dr. Willard Boynton	Senior Public Health Officer, USAID/Pak, Lahore.
Dr. Robert Cruickshank	Dept. of Social and Prev.Med. University of the West Indies, Jamaica.
Mr. David A. Wraight	Deputy Secretary General, SEATO, Bangkok.
Dr. Robert S. Gordon	Clinical Director, NIAMD, NIH, Bethesda.

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II. CONSULTANTS:

July 1966:	Dr. William Greenough, National Heart Institute, NIH. Setting up of laboratory procedures.
July 1966:	Dr. Randolph Eichner, Communicable Disease Center, Atlanta. Work with John Craig on skin tests.
July/ August 1966:	Mr. Charles Myers, Management Consultant, NIAID, NIH. TDY, Executive Officer.
August 1966:	Dr. Abram S. Benenson, (former Director, PSCRL) Department of Preventive Medicine, Jefferson Medical College, Philadelphia. Review of Clinical Studies.
August 1966:	Mr. Billie Myers, Chief, Electrical Engineering Division, NIH. Review of electrical requirements.
August 1966:	Dr. John P. Craig, State University, N.Y. Skin test program in Epidemiology Sec.
August 1966:	Dr. John Feeley, Division of Biologics Standards, NIH. Review of bacteriological and serological procedures.
August 1966:	Dr. Michael Clark, NAMRU-2, Taipei, Taiwan. Virus isolations.
October 1966:	Dr. George Curlin, Communicable Disease Center, Atlanta. Epidemiology Studies.
October 1966:	Dr. Adolph Karchmer, Communicable Disease Center, Atlanta. Epidemiology Studies.
November 1966:	Dr. George Whalen, Marquette University, U.S. Fluid and electrolyte transport studies
November 1966:	Dr. Andrew Abraham, Department of Pathology, Harvard Medical School. Fluorescein-tagged microscopy.

II. CONSULTANTS

November/December	1966:	Mr.Wallace de Witt; Communicable Disease Center,Atlanta. Setting up field bacteriology laboratory at Matlab.
December	1966:	Dr.Howard Goodman & Dr.Elvin Kabat. (W.H.O.sponsored visit) Review of Immunology
January	1967:	Dr. Larry Griffith, NAMRU -2, Taipei. Pediatric cholera studies.
January	1967:	Dr. C. Campbell, School of Tropical Medicine, University of Sydney. Study of treatment of cholera.
January	1967:	Dr. Nathaniel Pierce, Johns Hopkins Center, Calcutta Review of current clinical studies
January	1967:	Dr. John Banwell, Johns Hopkins Center, Calcutta. Review of current clinical studies
January	1967:	Dr. Paul Crabbe', Louvain University, Belgium Immunology study.
January	1967:	Dr. Calvin Linneman, Communicable Disease Center,Atlanta Epidemiological studies
January	1967:	Dr. Thomas Vernon, Communicable Disease Center,Atlanta Epidemiological studies.
February	1967:	Dr. Kenneth Goodner, Professor of Microbiology, Jefferson Medical College,Philadelphia Consultant in bacteriology at Technical Committee meeting.

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II. CONSULTANTS: (continued)

February	1967:	Dr. Alexander Langmuir, Chief of Epidemiology Branch, Communicable Disease Center, Atlanta. Consultant in Epidemiology at Technical Committee meeting.
March	1967:	Dr. Poul Astrup, Professor of Clinical Chemistry, Rigshospitalet, Copenhagen. Acid base balance studies.
April	1967:	Dr. Carl E. Miller, Chief, Animal Development Section, Laboratory Aids Branch, NIH. Survey of animal facilities.
April	1967:	Dr. Charles C.J. Carpenter, Chairman, Division of Allergy and Infectious Disease, Baltimore. Visit Vaccine Trial project at Matlab

III. TRAINING VISITS:

July	1966:	Dr. Anthony C. H. Chan, Infectious Diseases Hospital, Hong Kong. W.H.O. sponsored study tour in infectious diseases.
December	1966:	Twenty W.H.O. doctors for regional training course in epidemiology and treatment of cholera, sponsored by W.H.O.
January/ February/ March	1967:	Twelve Turkish and eight Iranian doctors on CENTO sponsored training course in the epidemiology and treatment of cholera.

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IV. INSTITUTIONAL REVIEW VISITS:

July	1966:	Dr. P.W. Dill-Russell; Deputy Medical Advisor, Ministry of Overseas Development, U.K.
September	1966:	Dr. Dorland Davis, Director, NIAID, NIH.
October	1966:	Dr. James Mason; Communicable Disease Center, Atlanta. General Laboratory Advisor. Follow-up on Hornig Committee visit.
January	1967:	Mr. Herbert Spielman, SEATO Affairs, American Embassy, Bangkok.
January	1967:	Mr. Anthony Curnow; Public Relations Department, SEATO Secretariate, Bangkok. To review PSCRL activities for SEATO publications.
February	1967:	Dr. Howard Minners, Special Assistant to Chief, Office of OIR, NIH. Discussion with OIR staff.
February	1967:	Jean Fitzpatrick, Editor, PHS World.
April	1967:	Professor J.A.R. Miles, Department of Microbiology, University of Otago Medical School Dunedin, New Zealand.
June	1967:	Colonel Stefano Vivona, Director, U.S. Army Medical Component, SEATO, Bangkok.

APPENDIX II

PAPERS READ AT SCIENTIFIC MEETINGS

FY 1966-67

EPIDEMIOLOGY SECTION:

A. Pacific Science Congress - Tokyo - August 1966

1. A Serological Survey for Cholera Antibodies in the Cholera Vaccine Field Trial Populations in Rural East Pakistan

   W. H. Mosley

2. Field Studies in East Pakistan on Cholera Vaccines

   A. S. Benenson

B. Jinnah Postgraduate Medical Symposium - Karachi - August 1966

3. Salmonella Surveillance

   W. M. McCormack

4. Present Trends of Birth and Death in Rural East Pakistan

   K. M. A. Aziz

5. Serological Studies in Cholera with Special Reference to IgG and IgM antibodies as Determined by 2-mercapto-ethanol Fractionation.

   A. Ahmed

6. Field Trials of Cholera Vaccine in Rural East Pakistan

   M. Fahimuddin

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C. East Pakistan Medical Services Association - Seminar - Dacca - August 1966

7. Present Trends of Birth and Death in Rural East Pakistan

K.M.A. Aziz

D. Annual Conference of Pakistan Medical Association and British Medical Association - Karachi - December 1966

8. Pattern of Cholera Endemicity in East Pakistan

M. Fahimuddin

9. Fertility Pattern in Rural East Pakistan

M. Fahimuddin

10. Fractionation of Immunoglobulins of the Serum of Patients with Cholera

Samin Ahmed

E. National Research Institute of Family Planning - Seminar - Karachi - October 1966

11. The Prosent Trends of Birth and Death in Rural East Pakistan

K. M. A. Aziz

F. East Pakistan Medical Conference - Dacca - December 1966

12. The Role of Boatmen in the Transmission of Cholera

Moslemuddin Khan

G. East Pakistan Christian Medical Association - Rajshahi - February 1967

13. Epidemiology of Cholera

R. Barui

H. Pakistan Medical Association Cholera Conference - Dacca - 1967

14. Epidemiology of Cholera Based on the CRL Experience

Moslemuddin Khan

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I. Pakistan Medical Association Annual Seminar - Khulna - March 1967

15. Endemicity Characteristics of Cholera in East Pakistan

M. Fahimuddin

J. Trained Nurses Association of Pakistan Conference - Karachi - March 1967

16. The Nurses Responsibility Toward Her Profession

Binipani San

K. Dacca Medical College Reunion Symposium - Dacca - May 1967

17. Epidemiological Patterns of Cholera and Non-Cholera Diarrheas

Moslemuddin Khan

L. E.I.S. Conference - Atlanta - April 1967

18. Endemic Cholera in Rural East Pakistan

W. M. McCormack

19. Cholera on a Tea Estate

A. W. Karchmer

M. Medical Education for National Defence Symposium - Atlanta - April 1967

20. Epidemiology of Cholera and Evaluation of Vaccines

W. M. McCormack

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N. All Pakistan Sociological Conference - Abotabad - August 1967

21. Socioanthropological Observations on the Workers of a Tea Garden

A. Momen Chowdhury

O. Jinnah Postgraduate Medical Symposium - Karachi - August 1967

22. Epidemiologic Pattern of Diarrhea and Cholera in a Semiurban Community

Moslemuddin Khan

P. U.S. - Japan Cooperative Medical Science Program Cholera Symposium - San Francisco - July 1967

23. The Vibriocidal Antibody Titer as a Measure of Immunity to Cholera in Man

W. H. Mosley

24. A Community Study of Inapparent Cholera Infections

W. M. McCormack

Q. E.I.S. Training Course - Atlanta - July 1967

25. Epidemiological Patterns of Cholera as a Model for the Study of Infectious Diseases

W. H. Mosley

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CLINICAL INVESTIGATION SECTION

A. Pacific Science Congress - Tokyo - August 1966

1. Some consequences of acidosis in cholera.

   W. B. Greenough

B. Jinnah Postgraduate Medical Symposium - Karachi - Augst 1966

2. Antibiotic therapy of cholera

   Md. Rafiqul Islam

C. W.H.O. Training Course on Cholera - Dacca - December 1966

3. The pathogenesis of cholera diarrhea

   Norbert Hirschhorn

4. The pathogenesis of the typical symptoms and signs of cholera and secondary features.

   Norbert Hirschhorn

D. Pakistan Medical Association: East Zone Annual Conference - January 1967

5. Approaches to therapy of cholera - Oral Gastric Lavage

   Hirschhorn, J. Taylor, D. Sachar, R. Northrup, J. Kinzie and R.A.Phillips.

6. Search for persistent vibrio carriers among convalescent cholera patients.

   A. Majid Molla

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E. Federation of American Societies for Experimental Biology - Chicago - April 1967

7. Transmural Electrical potentials and their response to sugars in the intestine of normal subjects and acute cholera patients.

   D.B. Sachar, J.R. Saha, K.W. Hare

8. Enhancement by intestinal glucose lavage of net sodium and water absorption in acute cholera patients.

   J.O. Taylor, N. Hirschhorn, R.A. Phillips

F. U.S. - Japan Cooperative Medical Science Program: Cholera Symposium. San Francisco - July 1967

9. Transmural electrical potentials and their response to sugars in the intestine of acute cholera patients and normal subjects.

   D.B. Sachar

10. Enhancement of net sodium and water absorption in active cholera in humans by intestinal glucose lavage.

    J.O. Taylor, D.B. Sachar, J. Kinzie, R.A. Phillips

11. Reduction of stool output in cholera by glucose and electrolyte lavage.

    N. Hirschhorn, J. Kinzie, D.B. Sachar, J.O. Taylor, R.S. Northrup, R.A. Phillips.

G. Jinnah Postgraduate Medical Symposium - Karachi - August 1967

12. An analysis of 41 consecutive fatal diarrhea cases in children admitted to the Pakistan SEATO Cholera Research Laboratory - Dacca.

    M. M. Rahaman

13. Some studies on cholera patients in relation to cholera toxin.

    K.M.S. Aziz, A.K.M. Mohsin

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PSCRL PUBLICATIONS FY67

Published:

1.	Hypoglycemia in Children with Acute Diarrhea.	Hirschhorn, N., M.D. Lindenbaum, J., M.D. Greenough, W.B.III, M.D. Mahmood Alam, S. Lancet 2: 128-132, 1966.
2.	Phage Resistance in Vibrio Cholerae.	Rizvi, S.S.H., Ph. D. Benenson, A.S., M.D. Bull. of W.H.O. 1966, 35, 675-80.
3.	Role of Carriers in the Intrafamilial Spread of Cholera.	Khan, A.Q., MBBS. The Lancet, Feb.4, 1967. pp.245-46.
4.	The Role of Boatmen in the Transmission of Cholera.	Khan, M., MBBS Mosley, W.H., M.D. East Pakistan Medical Journal April 1967, pp.61-65.
5.	Present Trends in Birth and Death in Rural East Pakistan.	Aziz, K.M.A., Mosley, W.H., M.D. Fahimuddin, M., MBBS McCormack, W.M., M.D. & Islam, M.S. Pakistan Journal of Family Planning Vol.1, No.1. pp.35-40 (1967).
6.	Cholera in the Perspective of 1966.	Phillips, R.A., M.D. Annals of Internal Medicine, Vol.65, No.5, Nov.1966.
7.	Malabsorption and Jejunitis in American Peace Corps Volunteers in Pakistan.	Lindenbaum, J., M.D. Kent, Thomas H., M.D. Sprinz, Helmuth, M.D. Annals of Internal Medicine, Vol.65, No.6, Dec.1966.

Accepted for Publication.

1.	1963 and 1964 Field Trials of Cholera Vaccine in Rural East Pakistan, Final Report. I. Result of Field Trials.	Benenson, A.S., M.D. Mosley, W.H., M.D. Fahimuddin, M., MBBS & Oseasohn, R.O., M.D. Bull. of W.H.O.

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Accepted for Publication.

2.	1963 and 1964 Field Trials of Cholera Vaccine in Rural East Pakistan, Final Report. II. Preliminary Studies, Reaction and Antigenicity Data.	Benenson, A.S., M.D. Joseph, P.R. & Oseasohn, R.O., M.D. Bull. of W.H.O.
3.	Serological Studies in Cholera. I. Vibrio Agglutinin Response of Cholera Patients Determined by Microtechnique.	Benenson, A.S., M.D. Saad, A., & Mosley, W.H., M.D. Bull. of W.H.O.
4.	Serological Studies in Cholera. II. The Vibriocidal Antibody Response of Cholera Patients Determined by Microtechnique.	Benenson, A. ., M.D. Saad, A., & Mosley, W.H., M.D. Bull. of W.H.O.
5.	Serological Studies in Cholera. III. Serum Toxin Neutralization-Rise in Titer in Response to Infection with Vibrio cholerae and the level in the "Normal" Population of East Pakistan.	Benenson, A.S., M.D. Saad, A., Mosley, W.H., MD. & Ahmad, A. Bull. of W.H.O.
6.	A Serological Survey for Cholera Antibodies in the Cholera Vaccine Field Trial Population in Rural East Pakistan. I. The Distribution of Antibody in the Control Population and the Relationship of Antibody to the Pattern of Endemic Cholera.	Mosley, W.H., M.D. Benenson, A.S., M.D. Barui, R.K., MBBS. Bull. of W.H.O.
7.	A Serological Survey for Cholera Antibodies in the Cholera Vaccine Field Trial Population in Rural East Pakistan. II. A Comparison of Antibody Titers in the Immunized and Control Population, and the Relationship of Antibody Titer to Cholera Case Rate.	Mosley, W.H., M.D. Benenson, A.S., M.D. & Barui, R., MBBS. Bull. of W.H.O.
8.	The Relationship of Vibriocidal Antibody Titer to Susceptibility to Cholera in Family Contacts of Cholera Patients.	Mosley, W.H., M.D. Ahmed, S., M.Sc., Benenson, A.S., M.D. & Ahmad, A.,MBBS. Bull. of W.H.O.

Accepted for Publication:

9 .	Asiatic Cholera (with emphasis on pathophysiological effects of the disease).	Phillips, R.A., M.D. Annual Review of Medicine Vol.19.
10.	Antibiotic Therapy of Cholera.	Lindenbaum, J., M.D. Greenough, W.B.III, M.D. & Islam, R., MBBS. Bull. of W.H.O.
11.	Antibiotic Therapy of Cholera in Children.	Lindenbaum, J., M.D. Greenough, W.B., III, M.D. & Islam, R., MBBS. Bull. of W.H.O.
